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Meet the editors

Dr. Hemanth KN. Vasanthaiah graduated from University of Agricultural Sciences, Bangalore, India. He is presently working as a Research Scientist at Plant Biotechnology and Biochemistry Laboratory, Florida A & M University, USA. The overall goal of his investigation is to determine biochemical and molecular factors involved in disease tolerance, drought, nutraceutical and enological characteristics of grapes, raspberry and peanuts. The distinction of his research is association with Muscadine and Florida Hybrid Bunch grapes, which are unique to southeastern United States where the cultivation of European grapes is limited due to the prevalence of pest and diseases. Dr. Vasanthaiah has accomplished significant progress and achievements to address some of the issues in plant science and the outcomes have been published in leading scientific journals. He has also received meritorious awards and quite a few grants as Co-PI and collaborator. Dr. Vasanthaiah also has a computer science degree to compliment with this research work.

Dr. Devaiah Kambiranda expertise is in plant biotechnology and biochemistry. He obtained PhD in biotechnology at Bangalore University, India, where he worked on identification of genes involved in drought tolerance in peanut plants. He did his Postdoctoral fellowship at Gyeongsang National University, South Korea, where he gained expertise in protein expression and purification. He has also worked in the Indian Institute of Ayurvedic medicine where he developed molecular markers for several medicinal plants. Currently he is working as a Research Associate in Florida A&M University where he is working on identification of genes and proteins involved in carbohydrate metabolism, disease tolerance and berry ripening in Florida hybrid and Muscadine grapes. He has authored about thirteen publications in peer reviewed journals and has presented in several national and International conferences.

Contents

Preface

The purpose of this book is to discuss intricate problems associated with plant performance under hostile conditions. Plants are sessile organisms and are frequently exposed to extreme stresses. Therefore, they have to be considerably more adaptable to stressful environments and must acquire greater tolerance to multiple stresses. Individual plant performance varies with their magnitude of tolerance. Among the stresses, abiotic stress is known to repeatedly limit the growth and productivity of plants, which negatively affects the yield, quality and other characteristics of the crops. As plants do not posses immune system, they have evolved a complex and dynamic defense system for their adaptation in response to stress. Drought and temperature are the major abiotic stresses that affect the plants, along with light, salinity and minerals. Prolonged abiotic stress may cause irreversible damage to plant function or development.

The possibilities for increasing tolerance to abiotic stresses are enormous. Progress has been made worldwide to utilize advanced tools and techniques from all branches of science in order to understand how plants respond to abiotic stresses with the aim of helping to manipulate plant performance that will be better suited to withstand these stresses. Though several projects have been developed to tackle effect of stress, many questions still remain unanswered. Therefore, an effort is made through this publication to show some of the current research and critical roles of genetics, physiology, biochemistry, biotechnology, and other related science in crop improvement for the benefit of fellow researchers. It is necessary to constantly get acquainted with the past and present occurrences in order to learn lessons that could help in the acquisition of new knowledge or the further development of appropriate technology ensuing from it. Among the high-priority research areas identified in plant sciences, developing crops with high yielding characteristics under adverse conditions is gaining attention in view of the future predictable earth's environment and also for possible establishments of plants on other terrestrial bodies in our universe.

In this book an attempt has been made to unfold some of the new findings and achievements that will enhance ongoing research and help solve some of the pressing issues in plant science.

This book presents the holistic view of the affect of various abiotic stresses on plants and their response to it. Chapters offer a critical discussion on the available literature and major problems , as well as understanding of plant stress responses and mechanisms of their tolerance in detail. I honestly trust that this book will be of great help to students, researchers and professionals in various fields of science, both in academic and industrial sectors.

I would like to express my deep sense of gratitude and indebtedness to all the authors for their valuable contributions and also to the researchers who actually performed experiments and reported their findings. I must confess that it had been a rare privilege for me to be associated with InTech publishers. Thanks is the least word to offer to Ms. Viktorija Zgela, Ms. Natalia Reinic and Ms. Dragana Manestar, Intech Publishing Process Managers, yet I shall avail this opportunity to extend my sincere gratitude for their help and co-operation at various phases of book publication. I would like to express my sincere thanks to Devaiah Kambiranda for helping me in editing some of the chapters. Last but not least I express my sincere thanks and affection to my wife Roopashri Puttaramu and my daughter Sahitya H. Setty for their sensible co-operation and cheerful encouragement. I hope the information available in this book will have a greater impact on the scientific world working towards crop improvement, which will pay off one day in the survival of life form on this beautiful planet, the Earth.

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Enhancing Phytoremediation Efficiency in Response to Environmental Pollution Stress

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1. Introduction

Environmental pollution, particularly contamination of soil and water resources, has been accelerated as a result of global industrialization and so is considered as a major risk for human communities throughout the world. Due to the adverse effects of organic and inorganic pollutants on human health and environmental safety, it is necessary to be removed in order to minimize the entry of these potentially toxics into the food chain. There are several methods to remove the soil pollutants which are categorized into 3 main parts including chemical, physical and biological methods. While conventional methods of soil clean-up including solidification, vitrification, electrokinetic, excavation, soil washing and flushing, oxidation and reduction etc. have shown to be effective in small areas, they need special equipments and are labor intensive. However, due to the side effects and highly costs of physical and chemical techniques, the biological methods especially phytoremediation, seem to be promising remedial strategies and so are highlighted as alternative techniques to traditional methodologies. Although phytoremediation as a "green technology" has shown many encouraging results, there have also been numerous inconclusive and unsuccessful attempts, especially in the field conditions, mostly because of biotic and abiotic stresses. "Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment" (http://en.wikipedia.org). Abiotic stressors as the plant stress factors including high concentration of organic and inorganic pollutants, salinity, drought, flooding etc**.** could be considered as the main general themes adversely affect phytoremediation efficiency. Therefore, decrease of abiotic stresses is considered as a promising approach to introduce phytoremediation technique more applicable even though in the present of environmental stressors which significantly affect plant growth and development (Dimkpa et al., 2009; Gerhardt et al., 2009; Weyens et al., 2009).
In this chapter we have discussed phytoremediation and its various types, as well as the plant response to abiotic stresses and the mechanisms which could be efficient to enhance phytoremediation efficiency regarding to abiotic stresses, especially considering environmental pollutants.

2. Phytoremediation

Phytoremediation is defined as an environmental friendly, cheap and large scale method which uses plants and their associated microorganisms to degrade, stabilize, reduce and/or

remove organic and inorganic pollutants from the environment (Pilon-Smits, 2005). It can be achieved in several ways including phytoextraction, phytostabilization, phytodegradation, phytovolatilization, phytorestoration, phytomining, rhizosphere-enhanced degradation and rhizofiltration (Vara Prasad & Freitas 2003; Pilon-Smits, 2005).

2.1 Phytoextraction and phytomining

Phytoextraction is the removal of heavy metals and metalloids by plant roots with subsequent transport to shoots (Vara Prasad & Freitas 2003). Generally, plants which can grow in heavy metal contaminated soils and waters are categorized to "tolerant", "indicators" and "hyperaccumulators" (Bert et al., 2003). A tolerant species can grow in contaminated soils while other plants have not this ability. For indicator species, there is a linear correlation between metal concentration in growth media and plant tissues. While both indicator and hyperaccumulator species are also tolerant, however since tolerant species could prevent entering metals to roots, they are not necessarily hyperaccumulators or indicators (Bert et al., 2003). Hyperaccumulators have a high potential to uptake and accumulate heavy metals or metalloids which could be more than 100 fold in comparison with common plants. A hyperaccumulator species has i) the ability to accumulate more than 100 μg g^{-1} dry weight Cd, 1000 μg g^{-1} dry weight Ni, Cu, Co, Pb, Se, As and 10000 μg g^{-1} dry weight Zn and Mn, and ii) the bioconcentration factor (i.e. the ratio of metal concentration in plant to soil) and translocation factor (i.e. the ratio of metal concentration in shoots to roots) greater than 1.0 (Sun et al, 2008). "The technique of phytomining involves growing a hyperaccumulator plant species, harvesting the biomass and burning it to produce a bio-ore" (Anderson et al., 1999).

2.2 Rhizofiltration

Rhizofiltration is a promising technique for removal of heavy metals from aquatic environments using suitable plants which could accumulate metals in their roots and shoots (Vara Prasad & Freitas, 2003).

2.3 Phytostabilization

Phytostabilization, where plants are used to stabilize rather than clean organic and inorganic pollutants in contaminated soils to prevent their movement to surface and groundwater and/or to prevent translocation of pollutants from plant roots to shoots. The latter would be important for prevention of pollutant's transport to the upper levels of food chain (Pilon-Smits, 2005; Vara Prasad & Freitas, 2003). Additionally, in phytostabilization plants accumulate pollutants in their roots or immobilize, precipitate and reduce soil contaminants. Phytostabilization could also be important for reduction of wind and water erosion (Vara Prasad & Freitas, 2003).

2.4 Phytodegradation and rhizosphere-enhanced degradation

Degradation of organic pollutants which are easily entered into the plant tissues or in the rhizosphere through the plant enzymes called phytodegradation. If this phenomenon occurs in plant rhizosphere by enhancing the activity of degrading microorganisms through the release of root exudates, named rhizosphere-enhanced degradation, which in fact is achieved by microbial enzymes rather than plant enzymes (Vara Prasad & Freitas, 2003; Pilon-Smits, 2005).

2.5 Phytovolatization

Plants can also remove toxic substances from soil through phytovolatization. In this process, the soluble contaminants are taken up by the roots, transported to the leaves, and volatized into the atmosphere through the stomata (Vara Prasad & Freitas, 2003; Pilon-Smits, 2005). Some heavy metals such as Hg, As and Se are inactivated when they are translocated from the soil into the atmosphere by bonding to free radicals in the air (Pilon-Smits, 2005).

2.6 Phytorestoration

Phytorestoration involves the complete remediation of contaminated soils to fully functioning soils which is an attempt to return the land to its natural state (Bradshaw, 1997).

3. Plant response to abiotic stresses

Plants react to environmental stresses on various levels including biochemical, cellular and morphological scales depending on type of species or population (Mulder & Breure, 2003). These mechanisms include production of reactive oxygen species by autoxidation and Fenton reaction (for Fe and Cu), blocking of essential functional groups in biomolecules (for Cd and Hg), and displacement of essential metal ions from biomolecules for different kind of heavy metals (Schützendübe & Polle, 2002). Malondialdehyde is a major cytotoxic product of lipid peroxidation and acts as an indicator of free radicals which its production together with chlorophyll, caretonoids, as stress markers, increases in response to metal stress (Ben Ghnaya et al., 2009). Basically, reaction of plants to abiotic stresses depends on type of plant species having fundamental differences in development and anatomy as well as environmental limiting factors (i.e. stressors) (Tester & Bacic, 2005). For example, while a flood may kill most plants in a certain area, but rice would be thrived there.

When plants are faced to metal stress (such as Cd) the abundance of stress-related proteins, like heat shock proteins, proteinases and pathogenesis-related proteins could be changed in leaves and roots. Since roots are not photosynthetical tissues, whereas metal stress could adversely affect CO_2 uptake, electron transport in chloroplasts by damaging photosystem I and II in leaves, proteomic changes in plant tissues upon an abiotic stress exposure would be different (Kieffer et al. 2009). Hence, plant exposed to high level of heavy metals causes reduction in photosynthesis, water and nutrient uptake, growth inhibition and finally death (Yadav, 2010; Soleimani et al., 2010a; Kieffer et al. 2009). The biosynthesis of ethylene as a gaseous plant hormone could be induced in response to environmental stressors which affect germination, growth and development of plant species as well as defence and resistance (Glick, 2004; Kang et al., 2010).

Although, one abiotic stress can usually decrease the ability of plant to resist a second stress (Tester & Bacic, 2005), interaction of various environmental stresses might decrease or increase plant tolerance to the growth limiting factors. For example; though soil salinity usually increases Cd bioavailability in heavy metal polluted soils and subsequently induces their toxicity, chloride salinity increased tolerance of an halophyte species (*Atriplex halimus* L.) to Cd toxicity both by decreasing the absorption of heavy metal and by improving plant tolerance through an increase in the synthesis of osmoprotective compounds in its tissues (Lefevre et al., 2009). The opposite trends were reported in the case of wheat which salinity increased Cd absorption and translocation by plants exposed to the metal in a nutrient solution (Mühling & Läuchli, 2003 In Lefevre et al., 2009; Liu et al., 2007) and for *Elodea canadensis* (Michx.) and *Potamogeton natans* (L.) in the presence of Cd, Cu and Zn (Fritioff et

al., 2005). It could also be considered that NaCl might increase the occurance of $CdCl^+$ which may be absorbed by the roots and translocated to the shoots (Lefevre et al., 2009).
Abiotic stresses such as salinity and organic and inorganic pollutants could adversely affect seed germination of plants (Soleimani et al., 2010b; Besalatpour et al., 2008). However, some plants such as *Frankenia* species have been reported to germinate successfully even though in response to abiotic stresses which demonstrate their uses in remediation and revegetation projects in areas affected by salinity (Easton & Kleindorfer, 2009).
Another response of plants upon exposure to heavy metals is oxidative stress which leads to cellular damage. In addition, metal accumulation by plant tissues disturbs cellular ionic homeostasis (Yadav, 2010). Salts and heavy metals could induce oxidative stress in plant which generate active oxygen species and consequently damage plant photosynthetic apparatus resulting in a loss of chlorophyll content and decline in photosynthetic rate and biomass production as well (Qureshi et al., 2005). Total antioxidant activity may increase with increasing environmental pollutants suggesting the capacity of plant to enhance antioxidant defense in response to pollutant stress. Antioxidant enzymes (e.g. dehydroascorbate reductase, glutathione peroxidase, glutathione-S-transferase and superoxide dismutases) may play an important role in plant cell against environmental abiotic stressors (Babar Ali et al., 2005). Reduced forms of phytophenolics act as antioxidant in plant facing to heavy metal stress, while oxidized form (i.e. phenoxyl radicals) can exhibit prooxidant activities under conditions that prolong the radical life time (Dimkpa et al., 2009; Sakihama et al., 2002). Hence, Johnstone et al. (2005) suggested that the test of total antioxidant activity could be mentioned as a new approach to identify putative algal phytoremediator as well as to monitor the effects of water quality on the biological components of polluted aquatic ecosystems.
Generally, the main mechanisms of higher plants in the presence of a metal stress include: stimulation of antioxidant systems in plants, complexation or co-precipitation, immobilization of toxic metal ions in growth media, uptake processes and compartmentation of metal ions within plants (Pilon-Smits, 2005; Liang et al., 2007; Jahangir et al., 2008). To minimize the detrimental effects of heavy metal stress, plants use detoxification mechanisms which are mainly based on chelation and subcellular compartmentalization (Mejáre & Bülow, 2001; Yadav, 2010). A principal class of heavy metal chelator known in plants is phytochelatins (PCs), a family of Cys-rich peptides. PCs are synthesized non-translationally from reduced glutathione in a transpeptidation reaction catalyzed by the enzyme phytochelatin synthase. Therefore, availability of glutathione is very essential for PCs synthesis in plants at least during their exposure to heavy metals (Yadav, 2010). One strategy of plants against xenobiotic stress such as phytotoxic chlorophenols is increasing of extracellular peroxidases enzymes capable of catalyzing their oxidative dechlorination which could be a protection approach of some aquatic plants (e.g. *Spirodela punctata*) against pollution stress (Jansen et al., 2004).
In the case of hyperaccumulators which are extensively used to remediate soil contaminated with heavy metals, the major involved processes in response to excess amounts of metals are i) bioactivation of metals in the rhizosphere through root–microbe interaction, ii) enhanced uptake by metal transporters in the plasma membranes, iii) detoxification of metals by chelation with phytochelatins, metallothioneins, metal-binding proteins in the cytoplasm and/or cell wall, and iv) sequestration of metals into the vacuole by tonoplast-located transporter proteins (Yang et al., 2005).

Understanding the plant response to abiotic stresses, mainly due to excess environmental pollutants, can be important in selecting a suitable approach to prevent decreasing phytoremediation efficiency.

4. Enhancing phytoremediation efficiency

Due to limitations of phytoremediation such as low biomass of hyperaccumulator species, plant sensitivity to high concentrations of environmental pollutants as well as other abiotic stresses and less efficiency of ions and compounds which have low bioavailability to uptake by plants, several approaches have been mentioned in recent decays to boost the efficiency of this technology. Although there are some chemicals (e.g. surfactants and ligands) which may increase phytoextraction, phytodegradation or phytostimulation of pollutants through the enhancement of bioavailability of organic and in-organic compounds in media, nature-based methods like using plant-microorganisms symbiosis not only seem to be more acceptable due to having less side-effects by protection of food chain but could also be efficient in remediation process by increasing plant biomass (Weyens et al., 2009). In the following, we mainly discuss several approaches including plant symbiosis with fungi and bacteria as well as plant genetic engineering which have revealed improvement of phytoremediation efficiency of various environmental pollutants consequently.

4.1 Plant-bacteria symbiosis

Generally there are several bacterial species in the rhizosphere called rhizobacteria. Root zone bacteria which have shown beneficial effects on various plants are named plant growth-promoting rhizobacteria (PGPR) and categorized into 2 main parts; extracellular and intracellular PGPR (Dimkpa et al., 2009). The latter group includes bacteria which are capable of entering the plant as endophytic bacteria and are able to create nodules, whereas extracellular PGPR are found in the rhizosphere, rhizoplane or within the apoplast of the root cortex, but not inside the cells (Dimkpa et al., 2009; Rajkumar et al., 2009). Since endophytic bacteria live within the plant, they could be better protected from biotic and abiotic stresses in comparison to rhizospheric bacteria (Rajkumar et al., 2009).

Plant-associated bacteria can promote plant growth as well as reduce and/or control of environmental stresses which together affect phytoremediation efficiency through several approaches directly and indirectly, within the plant and/or in the rhizosphere (Dimkpa et al., 2009; Glick, 2004, 2010; Kang et al., 2010; Rajkumar et al., 2009; Weyens et al., 2009; Yang et al., 2009). Furthermore, in the case of organic pollutants, there are a number of soil microorganisms that are capable of degrading xenobiotic compounds and consequently reduce their related stress to plants in contaminated soils (Glick, 2010).

Regarding to plant-bacteria symbiosis, there are several mechanisms which induce abiotic stress tolerance within the plant or in the rhizosphere which are mentioned in the following.

4.1.1 Mechanisms underlying abiotic stress tolerance within the plant

They are as follows:

1. Production of phytohormones (e.g. auxins, cytokinins, gibberellins) which can change root morphology is an adaptation mechanism of plant species exposed to environmental stresses (Dimkpa et al., 2009; Weyens et al., 2009). Indole acetic acid as a sub-group of auxins together with nitric oxide are produced in plant shoot transported

to root tips and consequently enhance cell elongation, root growth, root surface area and development of lateral roots (Dimkpa et al., 2009).

2. Inoculation with non-pathogenic rhizobacteria can induce signaling cascades and plant systemic resistance, alter the selectivity for Na, K and Ca ions resulting in higher K/Na ratios and change in membrane phospholipid content as well as the saturation pattern of lipids (Dimkpa et al., 2009).
3. Bacteria may produce osmolytes, such as glycine betaine, act synergistically with plant osmolytes, accelerating osmotic adjustment (Dimkpa et al., 2009).
4. PGPR containing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity reduces ethylene level within the plant and consequently facilitates plant growth under stress conditions (Dimkpa et al., 2009; Glick, 2004; Kang et al., 2010). The possible mechanisms described by Kang et al. (2010) accordingly. "ACC synthesized in plant tissues by ACC synthase is thought be exuded from plant roots and be taken up by neighboring bacteria. Subsequently, the bacteria hydrolyze ACC to ammonia and 2-oxobutanoate. This ACC hydrolysis maintains ACC concentrations low in bacteria and permits continuous ACC transfer from plant roots to bacteria. Otherwise, ethylene can be produced from ACC and then cause stress responses including growth inhibition." (Kang et al., 2010).

4.1.2 Mechanisms underlying abiotic stress tolerance in the rhizosphere

They are as follows:

1. Rhizobacterial with the capability of nitrogen fixation can positively influence on host plant growth by increasing nitrogen availability (Dimkpa et al., 2009; Kang et al., 2010; Rajkumar et al., 2009). Therefore they can act as a biofertilizer which affect plant growth (Gerhardt et al., 2009).
2. The mobility of heavy metals in contaminated soils can be significantly reduced through root zone bacteria which finally cause precipitation of metals as insoluble compounds in soil and sorption to cell components or intracellular sequestration (Dimkpa et al., 2009).
3. Bacterial migration from the rhizoplane to the rhizosphere plays a role in reducing plant uptake of some metals (e.g. Cd) in biologically unavailable complex forms (Dimkpa et al., 2009).
4. Iron–chelating siderophores complexes can be taken up by the host plant, resulting in a higher fitness (Dimkpa et al., 2009). They can also form complexes with other non-soluble metals (e.g. Pb) and enhancing their ability to uptake by hyperaccumulators such as *Brassica napus* (Rajkumar et al., 2009).
5. Bacterial exopolysaccharides lead to the development of soil sheaths around the plant root, which reduces the flow of sodium into the stele (Dimkpa et al., 2009).
6. Root zone bacteria can influence pH and redox potential in the rhizosphere, for instance, through the release of organic acids. This can have positive effects on the availability of nutrients (e.g. phosphorous) for the plant (Dimkpa et al., 2009; Weyens et al., 2009; Rajkumar et al., 2009).
7. Under abiotic stress such as high concentration of environmental pollutants causing plant less tolerant in response to biotic stress (e.g. disease, pathogens), PGPR can act as biocontrol agents which mitigate the effect of pathogenic organisms (Gerhardt et al., 2009)

4.2 Plant-fungi symbiosis

One of the approaches to enhance phytoremediation efficiency is using of plant-fungi association. In this regard using of arbuscular mycorrhizal fungi (AMF) that are naturally present in the roots of most plant species where they form a mutualistic association, as well as endophytic fungi which live systemically within the aerial portion of many grass species, can improve plant tolerance to biotic and abiotic stresses (Hildebrandt et al., 2007; Kuldau & Bacon 2008; Lingua et al., 2008; Soleimani et al., 2010a). The role of these groups of fungi on reducing abiotic stresses is mentioned in the following.

4.2.1 AMF and plant abiotic stress

Environmental stresses such as organic and inorganic pollutants trigger oxidative stress commonly showed by increasing content of malondialdehyde in plant. Mycorrhizal fungi are important factors which have the ability to regulate oxidative stress (i.e. reducing the amount of malondialdehyde), as a general strategy, to protect plants from abiotic and biotic stresses (Bressano et al., 2010). Several researches have revealed using AMF as a useful method showing more beneficial effects of phytoremediation, especially in metal contaminated soils (Bressano et al., 2010; Jiang et al., 2008; Lingua et al., 2008; Schützendübe & Polle, 2002). The enhancement of phytoextraction efficiency of *Brassica juncea* L. inoculated with Acacia-associated fungi reported by Jiang et al. (2008). It has been confirmed that mycorrhizal fungi in association with poplars are suitable for phytoremediation purposes underscore the importance of appropriate combinations of plant genotypes and fungal symbionts (Lingua et al., 2008). Furthermore, some fungi have the potential to degrade organic pollutants via extracellular or intracellular oxidation using various enzymes such as laccase, peroxidase, nitroreductase and transferases (Harm et al., 2011), and thereafter reduce stress of organic compounds in soil.

AMF can reduce metal stress in host plants or improve plant growth and development via several ways. Production and excretion of organic acids (e.g. citrate and oxalate) may increase dissolution of primary minerals containing phosphate which is one of the main nutrients for plant (Harm et al., 2011). Furthermore, release of siderophores can enhance iron uptake by plant and boost the growth. In the other hand, increasing the metal solubility or metal-complexing through acidification of the mycosphere could enhance metal uptake by plants which is important in phytoextraction. Extra-hyphal immobilization may occur through the complexation of metals by glomalin (i.e. metal-sorbing glycoproteins excreted by AMF) and biosorption to cell wall constituents such as chitin and chitosan (Harm et al., 2011). This process could be important in phytostabilization of heavy metals in contaminated soils considering that fungal mycelia and glomain could only increase soil aggregate stability against wind and water erosion (Harm et al., 2011). Methalothionin is another protein excreted by some mycorrhizal fungi which can also be important to reduce heavy metal stress in plants (Schutzendubel & Polle, 2002). It would be possible for heavy metals to storage in vacuoles or complex by cytoplasmic metallothioneins in fungi cells or volatilize via metal transformation (Harm et al., 2011). To alleviate heavy metal stress in plants associated with AMF, several genes encoding proteins (e.g. metallothionein, 90 kD heat shock protein, Glutathione-S-transferase) potentially involved in metal tolerance are expressed which are varied in their response to different heavy metals (Hildebrandt et al., 2007). However, improvement of plant mineral nutrition and health and also detoxification of metals in plants associated with AMF could be important to use them in phytoremediation of soil and water contaminated with heavy metals and/or organic pollutants (Lingua et al., 2008).

4.2.2 Endophytic fungi and plant abiotic stress

Endophytic fungi are a group of fungi that live their entire life cycle within the aerial portion of many grass species, forming nonpathogenic, systemic and usually intercellular associations (Soleimani et al., 2010a). Endophytes induce mechanisms of drought avoidance (morphological adaptations), drought tolerance (physiological and biochemical adaptations), and drought recovery in infected grasses (Malinowski and Belesky, 2000). In response to phosphorous deficiency, root morphology of host plant is altered or exudation of phenolic-like compounds may modify the rhizosphere conditions (Malinowski and Belesky, 2000). Aluminium toxicity mainly in acidic soils can be reduced on root surface of endophyte-infected plants through Al sequestration which appears to be related to exudation of phenolic-like compounds with Al-chelating activity (Malinowski and Belesky, 2000). Besides, drought and light stress as well as salt stress could be reduced in endophyte-infected plants via release of some proteins (e.g. dehydrins) and phenolic-like compounds in the rhizosphere (Kuldau & Bacon, 2008; Malinowski and Belesky, 2000). Several researches have also demonstrated the positive effect of endophytic fungi on phytoremediation of heavy metals as well as organic pollutants such as petroleum hydrocarbons (Soleimani et al., 2010a, 2010b). However, there is a lack of information regarding the effect of endophytic fungi on plant tolerance in response to stress of pollutants, especially organic pollutants, in both laboratory and field conditions.

4.3 Transgenic plants

Plants absorb toxic elements by the same pathways they take up essential elements. There should be a vast investigation on the processes involved in metal uptake, transport and storage by hyperaccumulating plants. Improving the number of absorption sites, changing specificity of uptake system to decrease competition by unwanted cations and enhancing intracellular binding sites should be considered to improve heavy metal accumulation in plants (Eapen & D'Souza, 2005). Modification of plant characteristics through the genetic engineering to enhance metal uptake, transport and accumulation as well as plant tolerance to abiotic stresses is a new approach for phytoremediation (Karenlampi,et al., 2000).

The first transgenic plants for phytoremediation were developed to enhance heavy metal tolerance including tobacco plants (*Nicotiana tabacum*) with a yeast metallothionein gene that gives tolerance to cadmium, and *Arabidopsis thaliana* that overexpressed a mercuric ion reductase gene for higher tolerance to mercury. Since each heavy metal may have a specific mechanism for uptake, therefore it is important to design suitable strategies for developing transgenic plants specific for each metal (Eapen & D'Souza, 2005).

There are different possible areas for genetic manipulation to create a suitable transgenic plant for phytoremediation. Generally, genes can be transferred from any living source to develop efficient transgenic plants for phytoremediation. Storage and detoxification of metals in some metal accumulating plants is due to metal storage in epidermal cells. Hence, genes can be inserted and/or overexpressed to produce metallothioneins, phytochelatins and metal chelators to improve plant tolerance and metal accumulation, thus play a role in detoxification of metals in plants (Eapen & D'Souza, 2005; Hassan et al., 2011). Genetic manipulation of metal transporters can be effective in modification of metal tolerance/accumulation in plants. One of the key essential features of metal hyperaccumulators is root-to-shoot translocation of ions. A strong metal sink in the shoots and improved xylem loading and repressed metal sequestration in root vacuoles are possible ways to enhance the root-to-shoot translocation (Hassan et al., 2011). Different metabolic pathways from various organisms can be presented into plants for

hyperaccumulation or phytovolatization resulting in plants being more tolerant to heavy metals. Alteration of enzymes which are involved in oxidative stress may also produce an altered metal tolerance in plants. Since having highly branched root systems with large surface area is important for efficient uptake of toxic metals, introduction of genes affecting root biomass can improve rhizofiltration of heavy metals in some hyperaccumulator plants (Eapen & D'Souza, 2005). Besides, the phytoremediation potential of most hyperaccumulating plants is limited because of their low biomass and slow growth and close association with a special habitant. Therefore, biomass of hyperaccumulator plants can be changed by introduction of genes affecting phytohormone synthesis resulting in enhanced biomass (Eapen & D'Souza, 2005; Kotrba et al., 2009). Transgenic plants expressing bacterial ACC deaminase genes can decrease ethylene level which is a major problem that reduces phytoremediation efficiency in plants exposed to abiotic stresses (Kawahigashi, 2009).

Genetically engineered plants can offer new characteristics that may not be met in normal plants (Table 1). Transgenic plants for phytoremediation presenting new or improved characteristics are engineered by the introduction and/or overexpression of genes taken from other organisms, such as bacteria or mammals. Bacteria and mammals are heterotrophs and have the enzymes necessary for achieving complete mineralization of organic pollutants; therefore bacterial and mammalian degradative enzymes can complete the metabolic efficiencies of plants (Van Aken, 2008).

Tolerance to toxic elements is a key factor in bioremediation. Plants which are more persistent in a harsh environment tend to maintain a high biomass and fast growth rate in regions unfavorable for growth and have more time for accumulating metals from the soil. (Eapen & D'Souza, 2005). Metal tolerance can significantly be increased by over-expression of proteins involved in intracellular metal sequestration but may not be utilized for metal accumulation. Tolerance and accumulation are highly independent traits, therefore they should both be manipulated to obtain a suitable plant for phytoremediation. (Eapen & D'Souza, 2005; Karenlampi et al., 2000).

Increasing the plant's ability to convert a toxic element into a less toxic form can improve its tolerance to excess amount of that toxic trace element. Typically, such a plant could be able to accumulate higher amounts of the detoxified form. In order to create a model system for phytoremediation of heavy metals a MerP protein was expressed in transgenic *Arabidopsis*. The transgenic *Arabidpsis* showed higher tolerance and accumulation capacity for mercury, cadium and lead when compared with the control plant (Hsieh et al., 2009). Organomercury can be converted to metallic Hg volatized from the leaf surface in plants with capability to produce bacterial mercuric reductase and organomercurial lyase (Kotrba, et al., 2009). Besides, volatiziation of selenium compounds could be promoted via overexpressing genes encoding enzymes involved in production of gas methylselenide species (Kotrba, et al., 2009). Some of the genetically engineered plants and sources of the genes involved in the process are mentioned in Table 1.

Ferredoxin, a stress-sensitive protein, was replaced in tobacco chloroplasts by an isofunctional protein, a cyanobacterial flavodoxin that usually exist in photosynthetic microorganisms such as algae and bacteria and is missing in plants. The resulting transgenic plants showed tolerance to some abiotic stresses such as drought, chilling, oxidants, heat and iron starvation (Zurbriggen et al., 2008).

There are specific genes called "pollutant-responsive elements" (PRE) that can be induced by the presence of particular toxic chemicals in the environment. They should be identified

and characterized so that they can be fused with reporter genes and be introduced into plants. For example the promoter of the barley gene HvhsplT which is expressed in the presence of some heavy metals had been fused to the reporter gene. This new gene combination was used to make a transformed tobacco plant which could be used as a bioindicator for monitoring heavy metal pollution (Mociardini, et al., 1998).
A combined use of transgenic plants and bacteria in the rhizosphere could improve phytoremediation of contaminated environments and may overcome the current limitations of phytoremediation such as low detoxification and absorption efficiency. The combination of plants for removing or degrading toxic pollutants and rhizospheric microorganisms for improving the availability of hydrophobic compounds can be effective in breaking down

Gene	Target plant	Effect
gshI	*Brassica juncea*	Cd tolerance , Cd, Zn, Cu and Pb accumulation
gshI and gshII	*Arabidopsis thaliana and Brassica juncea*	As and Cd tolerance and accumulation
GSH1 and AsPCS1	*Arabidopsis thaliana*	As and Cd tolerance and accumulation
OAS-TL	*N. tabacum*	Cd and Ni tolerance
APS1	*Brassica juncea*	Se tolerance and accumulation ,Cd accumulation
SMT	*Brassica juncea*	Se accumulation
SMT and APS1	*Brassica juncea*	Se accumulation
merP	*Arabidopsis thaliana*	Hg accumulation and tolerance
ADC	*Oryza sativa*	Heavy metal tolerance
CUP1	*Brassica oleracea Nicotiana tabacum*	Cd tolerance , Cu accumulation
TaPCS1	*Nicotiana. glauca*	Pb and Cd tolerance Pb, Cd, Zn, Cu and Ni accumulation
FRE1 and FRE2	*Arabidopsis thaliana Nicotiana tabacum*	Fe accumulation
merApe9 and merA18	*Arabidopsis thaliana L.tulipifera*	Hg and Au resistance
HisCUP1	*Nicotiana tabacum*	Cd accumulation , Cd tolerance
Ferritin	*Oryza sativa*	Fe accumulation
NtCBP4	*Arabidopsis thaliana*	Ni tolerance, Pb and Ni accumulation
MT-I and MT-II	*Nicotiana tabacum*	Cd tolerance
ZAT	*Arabidopsis thaliana*	Zn tolerance
AtPCS1	*Nicotiana tabacum Brassica juncea*	Cd accumulation, Cd and As tolerance
SOS1	*Oryza sativa*	Heavy metal tolerance
SOD	*Nicotiana tabacum and Zea mays*	Heavy metal tolerance
SAMDC	*Oryza sativa*	Heavy metal tolerance

Table 1. Genes involved in plant genetic engineering for phytoremediation of heavy metals (Hsieh et al., 2009; Karenlampi et al., 2000; Kotrba et al., 2009; Kolodyazhnaya et al., 2009).

many types of toxic chemicals (Kawahigashi, 2009). Special bacterial genes which encode enzymes involved in the breakdown of explosives, such as cytochrome P450 and nitroreductase have been also used in manipulating higher plants to enhance plant tolerance, uptake, and detoxification of contaminated environments (Van Aken, 2009).
Therefore, using transgenic plants or combined use of them with microorganisms in the rhizosphere could be mentioned as a promising technique to reduce abiotic stresses in plants which are used in phytoremediation. Future researches, especially in the field conditions, can distinguish the efficiency of this approach.

5. Conclusion

Abiotic stresses, especially due to high level of organic and inorganic pollutants, are major limiting factors which could adversely affect phytoremediation. To reduce the effects of these stresses in plants which are used in phytoremediation, using bacteria- or fungi symbiosis as well as plant genetic engineering could be a promising way to enhance remediation efficiency. In practical point of view, it should be considered that combined enhanced-phytoremediation approaches are possible to use too. Finally, understanding the involved mechanisms in the mentioned enhancing methods would be a useful tool to extend use of phytoremediation based on these approaches. Since most of researches have been carried out in laboratory conditions, field trials are needed to perform in contaminated sites.

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2

Morphophysiological Investigations in Some Dominant Alien Invasive Weeds

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1. Introduction

Allelopathy generally refers to any direct or indirect, harmful or beneficial effect of one plant on other plants, animals including microorganisms, through the production of chemical compounds that are released into the environment (Rice 1984). These donor plants affect the germination, growth and development of the recipient plant species (Einhellig 1987). The science of allelopathy has a very crucial role in maintaining the phytodiversity / biodiversity of a particular region. In fact, the phenomenon of biodiversity is the reflection of allelopathic interactions in that area. The losses in phytodiversity which are taking place at an alarming rate throughout the world is mainly ascribed to introduction of invasive / alien species which substitute the native ones. Invaded plant species and their success as well as secret have always threatened the world's biodiversity.

"The invasive plants are also known as alien, exotic or introduced ones, which are new to a specific area, become dominant, replacing / substituting the native plant species". These wide-spreading, non-indigenous species adversely affect the habitats they are invading in. The most important aspect of the alien plant is their rapid growth, establishment over new and large areas. Introduced species often find no natural enemies in their new habitat and therefore spread easily and quickly, especially in open disturbed areas. Invasive plants reproduce fast, either vegetatively or by seed. Their phenomenal growth allows them to overwhelm and displace existing vegetation and form dense Monothickets.

1.1 Ecological impacts of invasive plants on the environment

The general ecological impacts of invasive plants on natives and their surrounding environment are given in nutshell:-

- Competition with (and/or replacement of) native plants along with rare and endangered once.
- Loss of habitat and food sources of native insects, birds, wildlife and plants including microorganisms.
- Disruption of native plant-animal associations
- Elimination of native plant communities
- Prevention of establishment of native plants
- Acute competition for space, water, sunlight and nutrients due to its reduction
- Change of the soil structure and chemistry

- Morphophysiological interactions with native flora and fauna through release of allelochemicals / ecochemicals

1.2 Scope of allelopathy

At present it is well established that allelopathic phenomenon exist in different ecosystems including forest ecosystem and it can be exploited for increasing the productivity of crops as well as forest plant species in sustainable manner. According to Reigosa et al. (1999), allelopathy can affect distribution pattern of plants and biodiversity. They further explained that in a climax forest, germination and growth of understorey species must cope with allelochemicals released by the dominant trees. Those trees could release different chemicals, producing differences in the species composition. Similarly, Carballeira and Reigosa (1999) also indicated that monocultures (pure stands) allow the accumulation of particular allelochemicals affecting species composition. The occurrence of some weeds growing better than others within a monoculture could be a result of accumulation of allelochemicals. The allelopathic effects can affect small-scale vegetation patterns, by strengthening the associations between plants or not allowing them to grow in their vicinity. The research in allelopathy has increased greatly from 1960 onwards (Putnam 1985).

Inderjit *et al.* (2005a, b) has discussed in detail the challenges, achievements and opportunities in allelopathy research. They further highlighted that the novel research findings of allelopathy relevant to enzymes and genes involved in production of putative allelochemicals, allelochemical persistence in the rhizosphere, the molecular target sites of allelochemicals in sensitive plant species and the influence of allelochemicals upon other organisms will lead to enhanced utilization of natural products for pest management or as pharmaceuticals and nutraceuticals. The research and development in allelopathy is of extreme urgency for improvement of agriculture, forestry and global environment (Reigosa and Pedrol, 2002), because it deals majorly with invasive and native plant species.

2. Allelobiogenesis - concept and mechanism

Allelobiogenesis is nothing but the stress created by allelopathic effects of donor plants on recipient plants. In other words it is biotic stress and at the same time it is abiotic stress also. Allelobiogenesis is a typical stress combination of biotic and abiotic factors. Plant – plant, plant – animal and plant – micro-organism interactions can be considered as biotic stress.

The influence / stress of one plant on the other plant is mainly through the phytochemicals / ecochemicals / allelochemicals released by these donor plants. Hence the stress is obviously of abiotic nature. These allelochemicals are mainly secondary metabolites like alkaloids, glycosides, tannins, flavonoids, phenols etc. and the stress created by such allelochemicals is abiotic stress. The stress created by such allelopathic interaction or allelochemicals is allelobiogenesis. A weed exhausting nutrients from the soil voraciously and producing nutrient stress on associated crop as it shows dominance on associated crop by its faster growth and encroachment over crop species is biotic allelobiogenesis, e.g. *Parthenium* the invasive weed growing in association with *Sorghum.*

The exotic weed *Parthenium* is releasing large no. of allelochemicals through root exudation, leaching and volatilization in the surroundings and these allelochemicals cause very adverse effects on seed germination and growth and all the metabolic processes such as photosynthesis, respiration, absorption of water and minerals. This stress can be well explained as “abiotic allelobiogenesis”.

Weeds have many ill / negative characters, which cannot be neglected at all. Many of the weeds cause damages to agroecosystems and also disturb/ reduce natural phytodiversity. Weeds cause great harm to the crops in various ways as they cause 30 – 40% yield losses, increase the expenditure of various cultural practices, reduce the efficiency of agricultural implements. Perennial weeds reduce quality of fertile lands, cause obstacles for water flowing in canals. Weeds reduce crop yield and its quality as they compete with crops for resources like soil, water, nutrients and light. Weeds are alternative hosts for many pests and pathogens. Many weeds like *Prosopis, Calotropis* etc. reduce the germination capacity of crops' seeds due to the phytotoxins/ allelochemicals/ ecochemicals, many a times which are the secondary metabolites, secreted by them in the soil.

Aquatic weeds like *Eichhornia* and different types of algae produce toxins, which are harmful to aquatic flora and fauna. Weeds harbour organisms like mosquitoes, which cause or transmit diseases. Some weeds are poisonous to humans and produce pollens, which cause allergies. These studies will be more helpful, if emphasis on interactions among the plants is highly focused by the researchers. Studies on allelopathic potential and the biochemical characterization of native and invasive weeds has become the top priority to get rid of the ill effects of native and invasive weeds.

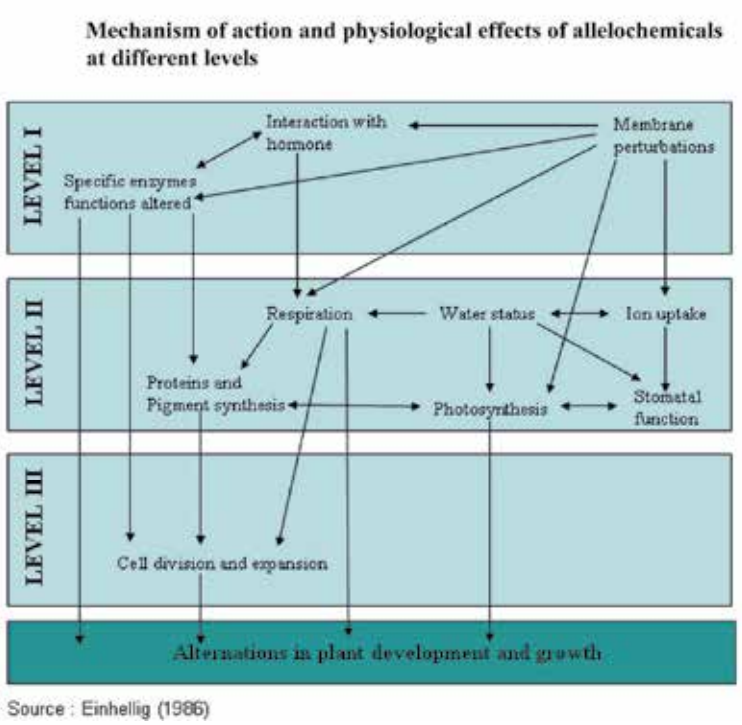


Diagram 1. Mechanism of action and physiological effects

2.1 Allelopathic interactions between plants

Allelopathic interactions are primarily based on the synthesis and release of secondary metabolites by higher plants that initiate a wide array of biochemical reactions, which induce several biological changes, however, many of these are yet to be understood. In

nature, many plant species grow together and interact with each other by inhibiting or stimulating the growth and development through allelopathic interactions. In any ecosystem, the dominant plants growing within it are exhibited in the form of pure stands or monothickets. Such ecosystems always show the zones of inhibition around them (Nilsen 2002, Chase and Leibold 2003). The ecosystems infested by dominant weeds show drastic alterations in their structure and function.

All the weed species, which are the part of dynamic ecosystems, originate in natural environment and become hurdle to the crops (Aldrich 1984). These weeds have some diagnostic features, such as short seed dormancy period, high rate of seed germination, rapid seedling growth, high reproductive ability, life cycle of a short span, very high environmental plasticity, self-compatibility, effective and efficient methods of dispersal of propagules, production of different types of novel ecochemicals or allelochemicals and tolerance to biotic and abiotic stresses (Baker 1965), which enable them to grow and survive in varied habitats and inhospitable ecological conditions.

As a result of this these weeds are becoming dominant throughout the world (Colautti and MacIsaac 2004, Lee and Klasing 2004, Jeschke and Strayer, 2005). As stated by Li et al. (2009), the invasion of exotic weeds is mainly due to their easier establishment and faster growth under diverse environmental conditions. Lonsdale (1999) claimed that the propagules' pressure, adaptive characters and susceptible environment favour the invasibility to which Carlton (2001) called biological invasion.

2.2 Plant invasions and encroachments

The whole biosphere is facing the problem of invasion of different weed species, hence studies on plant invasions and allelopathy will help in understanding the mechanism of invasions, and consequences of them on global biodiversity and ecosystem functioning. These invasions pose many ecological, economic and social problems. A team approach to solve these complicated problems is necessary.

According to MacDougall and Turkington (2005), the alien species highly out compete the native species or escape from adverse environmental conditions and dominate the community. According to vacant niche hypothesis (Elton 1958) the empty places such as barren lands, roadsides, open grounds etc. are generally invaded by such weeds. There are different hypotheses explaining the invasion mechanisms (Inderjit et al. 2005a, b). The diversity of these weeds is governed by population, ecosystem dynamics, disturbances, nutrient supply and climatic factors. The biotic restrictions on them, force to skip from their previous habitat and start surviving in new habitats, helping in the process of invasion. The enemy release hypothesis advocated by Mack et al. (2000) also supports the above view. If the invader is resistant enough and tolerant to herbivory, then its competitive ability increases and it becomes very aggressive due to production of some defensive chemicals (Carpenter and Cappuccino 2005).

The disturbances by some plant species, grazing pressure, fluctuation in resource availability (Davis et al. 2000), soil moisture, available light (Meekins and Mc Carthy 2001), phenotypic plasticity and hybridization (Daehler 2003) results in to successful invasion. The novel weapon hypothesis (Callaway and Ridenour 2004), biotic resistance hypothesis (Maron and Vilà 2001), and the genetic shift hypothesis (DeWalt et al. 2004) also explain the mechanism of invasion. To understand the distribution of invasive weeds and their associates in a natural community, the eco-distribution mapping is of paramount importance.

Biological species invasions alter ecological systems in a multitude of ways. Worldwide an estimated 80% of endangered species could suffer losses due to competition with or predation by invasive species.

3. Compilation of updated work

To have the information about the previous work done on allelopathy in general, its role in different fields of agriculture and botany, different types of interactions such as weed – weed, weed – crop, the impact of leachates, extracts and residues on recipient plants, allelochemicals existing in different donor plants, their chemical structures, mode of release of these ecochemicals in the environment, their accumulation, mechanism of action, their effect on seed germination, seedling growth, mineral nutrition, microbial activity in the soil etc. a review of literature is given in nutshell.

Studies on allelopathy were made thousands of years before the term was coined by Molisch (1937). The term allelopathy is derived from two Latin words *Allelon* means each other and *pathos* means to suffer. He, for the first time studied the effect of numerous plant species and their plant parts viz.- roots, shoots, leaves, flowers, fruits, leachates, extracts and residues on seed germination, seedling growth and maturity of crops. Later on many scientists at different corners of the world, contributed to this field by carrying out the research on various aspects of allelopathy. At present the research on allelopathy is being carried out in more than 85 countries. In India, the research in this field took a great speed after 1950.

Vilai-Santisopasri (2003) studied the allelopathic effects of *Eupatorium adenophorum* Spreng. on growth of some crops and weeds. Hierro and Callaway (2003) had investigated in detail the invasion of exotic plants and their role in allelopathy. Many workers like Rice (1979), Gill and Sandhu (1996), Pawar and Chavan (1999), Chou (1999), Wang et al. (2001), Cheema et al. (2002) had great contribution in allelopathy through their basic research. Recently, many researchers like Narwal et al. (2003a, b), Podolska et al. (2003), Navaz et al. (2003), Batish et al. (2002), Singh and Singh (2003) and Azania et al. (2003) have introduced multidisciplinary approach in allelopathy.

According to Fujii et al. (2002) allelopathy now refers to any process involving secondary metabolites produced by plants, microorganisms, viruses and fungi, that influence the growth and development of agricultural and biological systems. The allelopathy workers like Bhatt and Chauhan (2000), Singh and NarsingRao (2003) and Leather and Einhellig (2005) also claimed that secondary metabolites produced by donor plants, when released into environment, play a key role in ecology and physiology of recipient plants. They further advocated that the released allelochemicals as well as the phytochemicals present in the leachates / extracts have stimulatory or inhibitory influence on seed germination, seedling growth and yield of recipient plants.

The allelopathic impact of invasive weeds on seed germination, seedling growth, growth parameters like plant height, number of leaves per plant, leaf area, yield contributing parameters like number of flowers and fruits per plant, weight of fruit and grains etc in different crops had been studied in detail by Rice (1979), Patil and Hegde (1988), Devi et al. (1997), Kulvinder et al. (1999), Bhalerao et al. (2000a, b), Wang et al. (2001), Kong and Hu (2001), Lin et al. (2002), Bhalerao (2003), Jadhav (2006), Hase (2008) and Vaidya (2009). Presently the allelopathy research work is mainly focused on identification of allelochemicals, their mode of action and ecological significance.

According to many researchers allelopathy now refers to any process involving secondary metabolites produced by plants, microorganisms, viruses and fungi that influence the

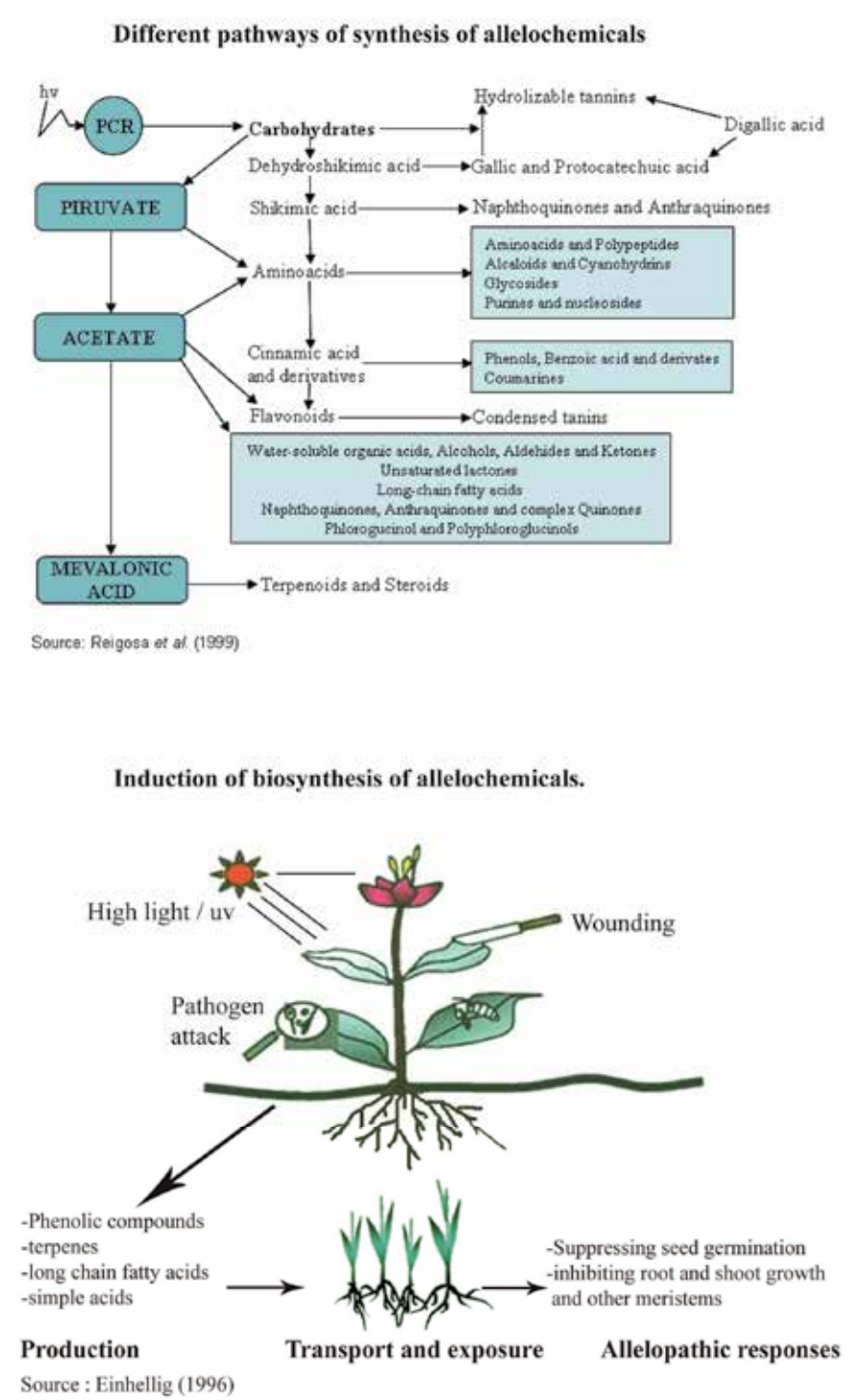


Diagram 2. Different pathways of synthesis of allelochemicals

growth and development of agricultural and biological systems. The allelopathy workers also claimed that secondary metabolites produced by donor plants, when released into environment, play a key role in ecology and physiology of recipient plants. They further advocated that the released allelochemicals as well as the phytochemicals present in the leachates / extracts/ residues have stimulatory or inhibitory influence on seed germination, seedling growth and yield of recipient plants.
Today this subject has come into lime-light because of its multidisciplinary nature, which covers agriculture, biological sciences, biochemistry, physiology, biotechnology and even genetic engineering.

3.1 Invasion success of weeds

The light has been thrown on the success of invasive alien weeds outside their native boundary and probable causes of this. The biological processes and specific characteristics of invasive weeds are important factors in their introduction, spread, and establishment that threatens the ecosystems, habitats, or species with economic/ environmental harm. Therefore the detailed investigations on the ecological, physiological and molecular aspects of invasive weeds' allelopathy should be conducted in order to understand community structure and declining phytodiversity.

3.2 Allelochemicals in invasive and native weed species

Isolation, identification and characterization of allelochemicals present in roots, stems, leaves, flowers, fruits, seeds, bark, residues, litter, dried leaves (trash) and their leachates, extracts and residues have a pivotal role in allelopathy research, without which any predictions, possibilities, hypothesis and explanations are not possible. Asteraceae plants with their leachates, extracts and residues of different plant parts are well known for their allelopathic activity because of their allelochemicals like phenolic acids and terpenoids (Chon et al. 2003).
Many researchers like Ghayal et al. (2007a, b, c) and Li et al. (2009) have given prime importance for identification of allelochemicals, ecochemicals, novel bioactive compounds which are the secondary metabolites existing in their leachates, extracts and residues. They have characterized diverse groups of allelochemicals like terpenoids, flavonoids, phenolic compounds and essential oils existing in the invasive and native weeds.

4. About the study area

The big campus of University of Pune established in 1949, at Ganeshkhind occupies an area of 164.8 hectares, which is situated about seven km north-west of Pune city proper and lies between $18^0$34′ North latitude and $73^0$53′ East longitude at an elevation of about 1880 m. At present 1/4 th area is occupied by roads, buildings and gardens.
Ganeshkhind stands on pediment surface of amygdaloidal basalt. These rocks are traversed by many veins and veinlets of silica and chalcedony. The poor soil of study area is reddish brown on higher grounds and deeper dark brown (black cotton soil) on flat areas. The soils are alkaline and are of pedocal type (Varadpande 1972). The average rainfall, climate and other environmental conditions of the campus are more or less similar to that of Pune city. The railfall is restricted to couple of months in monsoon and the maximum annual rainfall is 31.78cm. The temperature during hot season goes up to 40 – 42^0C but normally it is cool as compared to Pune city.

4.1 Weed floristic of study area

The phytosociological studies were conducted at all the four selected sites in Pune University campus. The different plant species were recorded with respect to their distribution, frequency, density and abundance. All four study sites (I to IV) showed the vegetation composition of various types of herbs, shrubs, trees, climbers and twinners.

The results on phytosociological studies at site I reported in Table 1 (a) indicated that this site was having highest weed diversity. The most abundant weeds at this site with maximum frequencies were *Cassia uniflora* (93.33%), *Achyranthes* (90.00%), *Synedrella nodiflora* (90.00%) and *Oplismenus* (83.33%). These were followed by *Acalypha*, *Bidens, Boerhaavia* and *Euphorbia geniculata,* all these were at par having frequency (80.00%). *Alternanthera tenella* was also showing better population with frequency 76.67%. Remaining native plants like *Cynotis* and *Calotropis* and invasive plants have shown less frequency, density and abundance.

The results of site II recorded Table 1(b) revealed that at this site, 43 different weed genera were recorded including about 19 genera of invasive weeds. The most abundant weeds with maximum frequencies were *Synedrella nodiflora* (96.67%), *Cassia uniflora* (93.33%), and *Cassia absus* (86.67%). These were succeeded by *Acalypha*, *Bidens* and *Euphorbia geniculata* which were at par having frequency (83.33%). Remaining plants showed discrete occurrence. At sites I and II *Cassia uniflora* was showing very thick population density, as these sites were exposed to sunlight for longer period.

The results on phytosociological studies of site III reported in Table 1 (c) illustrated that at this site, only 24 genera were reported including about 12 genera of invasive weeds. The plants recorded at this site in order of highest frequency were *Synedrella nodiflora* (93.33%), *Cassia uniflora* (80.00%) and *Rauwolfia* (73.33%). As opposite to sites I and II, at site III, there was complete dominance of *Synedrella nodiflora* only, which was virtually forming monothickets / pure stands. *Synedrella nodiflora* being shade lover was showing luxuriant growth and very high dominance at this site. The shady conditions along with high soil moisture favoured the luxuriant growth of *Synedrella nodiflora* and *Rauwolfia.*

The results listed in Table 1 (d) regarding phytosociological studies carried out at site IV revealed that, there were about 32 genera of weed species including 14 genera of invasive weeds. The weeds with highest frequency were *Cassia uniflora* (96.67%), *Synedrella nodiflora* (93.33%) and *Euphorbia geniculata* (76.67%), which were followed by *Acalypha, Achyranthes* and *Alternanthera*. At this site there was highest human interference as compared to the remaining three sites. The fast growing invasive weed *Lantana camara* showed successful invasion only at this site with frequency 63.33%.

No.	Dominant species	Associated species	Nature of association
1	*Cassia uniflora*	*Achyranthes aspera*	Very common
2	*Cassia uniflora*	*Blainvillea acmella*	Occasional
3	*Acalypha ciliata*	*Cassia uniflora*	Rare
4	*Alternanthera tenella*	*Cassia uniflora*	Common
5	*Synedrella nodiflora*	*Cassia uniflora*	Common
6	*Oplismenus compositus*	---	Very common
7	*Euphorbia geniculata*	*Cassia uniflora*	Common

Table 1. (a) Weed-weed interactions at site I in Pune university campus

No.	Dominant species	Associated species	Nature of association
1	*Bidens pilosa*	*Cassia uniflora*	Very common
2	*Cassia uniflora*	*Achyranthes aspera*	Very common
3	*Triumfetta rhomboidea*	*Cassia obtusifolia*	Common
4	*Alternanthera tenella*	*Boerhaavia erecta*	Common
5	*Cassia absus*	*Achyranthes aspera*	Common
6	*Cassia uniflora*	*Synedrella nodiflora*	Occasional
7	*Cassia uniflora*	*Parthenium hysterophorus*	Rare

Table 1. (b) Weed-weed interactions at site II in Pune university campus

No.	Dominant species	Associated species	Nature of association
1	*Synedrella nodiflora*	*Cassia uniflora*	Very common
2	*Synedrella nodiflora*	*Achyranthes aspera*	Common
3	*Cassia uniflora*	*Tithonia tagetiflora*	Common
4	*Cassia uniflora*	*Achyranthes aspera*	Common
5	*Rauwolfia tetraphylla*	*Euphorbia geniculata*	Common

Table 1. (c) Weed-weed interactions at site III in Pune university campus

No.	Dominant species	Associated species	Nature of association
1	*Cassia uniflora*	*Achyranthes aspera*	Common
2	*Cassia uniflora*	*Alternanthera tenella*	Common
3	*Lantana camara*	*Cassia uniflora*	Occasional
4	*Acalypha ciliata*	*Cassia uniflora*	Common

Table 1. (d) Weed-weed interactions at site IV in Pune university campus

Mishra et al. (1997), Chapin et al. (2000), Kumar et al. (2004), Jadhav (2006), Saswade (2007) and Thakur and Khare (2009) have also carried out the phytosociological studies on various invasive and native weeds. The phytosociological studies on *Potentilla recta* and other species, the invasive, noxious weed from Eurasia were carried out by Werner and Soule (1976). Zouhar (2003), Endress and Parks (2004) had also conducted phytosociological studies on these invasive weeds from U.S. The dominance of *Cassia uniflora* and *Synedrella* in the study area may be attributed to their aggressive nature, allelolpathic potential, adaptations in morphological and reproductive features along with specific type of physiological, biochemical and enzymological mechanisms allowing their faster growth and tolerance to biotic and abiotic stress conditions.

4.2 Invasion components

Along with the two dominant weed species like Cassia uniflora and Synedrella nodiflora at all the four sites, the major co-occuring species recorded were Acalypha ciliata, Boerhaavia erecta, Cassia obtusifolia, Lagasca mollis, Peristrophe bicalyculata, Parthenium hysterophorus and Triumfetta rhomboidea. The fact worth to mention was establishment of

monothickets (Bhakat et al. 2006) of Cassia uniflora at sites I, II and IV and that of Synedrella at site III.

The results of GPS mapping of weeds in Pune University campus strongly support the phytosociological observations recorded through quadrat studies. The GPS mapping had given the exact latitude, longitude and altitude of each plant species. More than 200 waypoints were recorded to confirm the dominance of selected weed species like *Cassia uniflora, Synedrella nodiflora, Alternanthera tenella, Bidens pilosa, Blainvillea acmella, Acalypha ciliata, Euphorbia geniculata, Triumfetta rhomboidea, Cassia obtusifolia* etc. It has also indicated the dominance of *Cassia uniflora* and *Synedrella nodiflora* at all the four selected sites. These selected weeds were located at $18^{0}55'$ north latitude and $73^{0}82'$ east longitude and an altitude of 568.63m and 571.48m respectively.

The Millenium Ecosystem Assessment (2005) have claimed that invasive species are the most important drivers of ecosystem change, which can very well alter the vegetational set up of a particular area. Such phytosociological studies on various weeds occurring in crop ecosystems have been carried out by researchers like Bartariya et al. (2005) and Seal et al. (2009) have also reported the dominance of invasive weeds in different ecosystems.

4.3 Secrets of invasion / encroachment and aggressiveness

The invasive weeds can exploit many niches left available and keep changing the phytodiversity of these niches or ecosystems. Unless the phytosociological studies of such areas are carried out, it is difficult to know the extent of encroachment over natives and invasion by the invasive plants. Phytosociology will help to understand the growth characteristics, dominance, distribution and adaptations which enable these plants to sustain the changes in the environment. These studies help to determine the distribution, prevalence, competing ability, behaviour and survival of weeds (Rao, 2000). The results recorded in Tables 1(a, b, c and d) clearly showed the higher dominance of *Cassia uniflora* and *Synedrella nodiflora*, at different sites in the university campus amongst the co-occuring species. These two invasive weed species have caused the reduction in the native phytodiversity of Pune University campus. As suggested by Rizvi and Rizvi (1992), allelopathic interactions of these weeds might be playing a crucial role in existing vegetation pattern of Pune University campus.

The dominance of *Cassia uniflora* and *Synedrella* in the study area may be attributed to their aggressive nature, allelolpathic potential, adaptations in morphological and reproductive features along with specific type of physiological, biochemical and enzymological mechanisms allowing their faster growth and tolerance to biotic and abiotic stress conditions.

Plants are chemically well defended in their environments, because their exposure to any stress leads to the qualitative and quantitative changes in the plant biochemicals and enzymes as a part of defense mechanism. These defensive chemicals are nothing but allelochemicals only, which act as feeding deterrents or alter the physiology and development of the attacking organisms (Pathipati UshaRani 2008). Even the different organic compounds often have role in ecological development which mediates interactions between the donor plants and the recipient organisms. The defensive allelochemicals and organic compounds have crucial role in the weed–weed associations formed in the campus of Pune University. The allelopathic potential of invasive weeds like *Cassia* and *Synedrella* can be ascribed to the above mentioned factors.

Allelochemicals always affect many aspects of plants' ecology e.g. distribution, growth, succession, structure of communities, dominance, diversity and productivity (Takeuchi et al. 2001). The population of *Cassia uniflora* had always shown its shifting nature, i.e. area with monothickets during first year will show very less population next year on the same spot. It may be due to the resource exhaust, autotoxicity and heavy accumulation of allelochemicals making the area inhospitable for its own growth. However, it requires further confirmation and experimentation in details.

4.4 Morphological specifications of invasive and native weeds

An attempt was made to study the morphological and reproductive features of invasive and native weeds from Pune university campus (Table 2). The maximum plant height was recorded for *Cassia uniflora,* (104.66cm), which was followed by *Triumfetta* (103.66cm) and *Achyranthes* (96.33cm).

The root length indicates easy and proper establishment of the plants. The maximum root length was recorded for *Alternanthera* followed by *Rauwolfia* and *Achyranthes*.

The highest number of branches per plant was observed in *Achyranthes* and followed by *Triumfetta,* while the weed species like *Acalypha* and *Oplismenus* were without any branch. The number of branches in remaining weeds was next to the above weed species.

Cassia obtusifolia had maximum third leaf area and it was followed by *Rauwolfia* and *Triumfetta.* The third leaf area in remaining weeds was very less as compared to them.

The results on fresh biomass per plant indicated that *Cassia obtusifolia* was having highest biomass and *Triumfetta* was next to it. The weight of fresh biomass in the remaining weeds was comparatively very less.

The dry biomass per plant showed wide variations ranging from 0.89g to 17.43g. The *Cassia* species e.g. *C. obtusifolia* and *C. uniflora* were having highest dry biomass 17.43g and 12.4g per plant respectively.

The highest fresh biomass per m^2 area was recorded in *Triumfetta, Cassia uniflora* and *Blainvillea.* The remaining weeds recorded comparatively less biomass per m^2 area in the campus of Pune University.

Many research workers like Sen (1977), Weaver and McWilliams (1980), Wilson (1988) had given due importance to morphological studies of native and invasive weeds. Ehrenfeld (2003) proposed that the invasive plants share many physical characteristics and tend to alter habitats. They have very high productivity and above ground biomass. They grow earlier in the season and show faster growth rate than native species. All such features might be applicable to *Cassia* and *Synedrella,* because of which they are dominant in the campus, showing luxuriant growth.

Unless we know the morphological features of weeds, the attempts for their effective management are difficult. With this view, Sutherland (2004) has described all the morphological details of different terrestrial and aquatic weeds of India. Similarly Monaco et al. (2002) had also investigated the various morphological characteristics of different weeds from USA. The life cycle of a plant can be understood well by its morphological structures, developmental processes and whole plant activities that occur during each phase of its life cycle. He further stated that selected phenotypes dominate their neighbours, because the timing of their life history optimizes their relative fitness and minimizes mortality. Same explanation may be true for the dominance of different invasive weeds including *Cassia* and *Synedrella* over the natives of Pune University campus.

*Weed spp.	Plt. Ht. cm.	Root length cm.	No. of Branches/plt.	No. of leaves / plt.	III Leaf area cm^2	Fresh biomass / plt. g	Dry biomass / plt. g	Fresh biomass / m^2 g
Cul	104.66 a± 4.18	17.66 c ± 0.70	7 d ± 0.28	18 e ± 0.4	20.41 d ± 0.81	36.3 d ± 1.45	12.4± 0.49	2316.2 b ± 92.64
Snl	63.66 e ± 3.18	17 c ± 0.85	8 c ± 0.4	31a ±1.5	14.9 e ± 0.74	15.68 g ± 0.78	4. 1± 0.20	282.42 f ± 14.12
Alt	91 b ± 6.37	22.66 a ± 1.58	9 b ± 0.63	28 b ± 1.96	12.8 ef ± 0.89	21.5 f ± 1.50	8.3± 0.58	356.84 e ± 24.98
Eug	73.66 c ± 2.20	13.3 d ± 0.39	3 h ± 0.09	10 g ± 0.3	18.41 d ± 0.55	19.06 f ± 0.57	4.6± 0.13	114.36 g ± 3.43
Bod	46.33 g ± 2.77	8.16 g ± 0.48	4 g ± 0.24	15 f ± 0.9	11.8 fg ± 0.70	31.13 e ± 1.86	10.7± 0.64	108.39 g ± 6.50
Ach	96.33 b ± 5.77	20 b ± 1.2	11 a ± 0.66	24 d ± 1.44	12.2 fg ± 0.73	34.72 d ± 2.08	10.2± 0.61	347.2 e ± 20.83
Bln	66 de ± 2.64	5.5 h ± 0.22	6.33 e ± 0.25	7.33 h ± 0.29	13 ef ± 0.52	12.33 hi ± 0.49	1.43± 0.05	579.51 c ± 23.18
Aca	49.66 f ± 3.47	4.5 hi ± 0.31	0 i ± 0	26 c ± 1.82	10.5 g ± 0.73	9.33 i ± 0.65	1.75± 0.12	171.67 g ± 12.01
Tum	103.66 a± 3.10	9.66 f ± 0.28	11 a ± 0.33	25 cd ± 0.75	33.66 c ± 1.00	88.33 b ± 2.64	3.1± 0.09	2473.24 a ± 74.19
Cab	52.26 fg± 2.61	11.4 e ± 0.57	8.33 c ± 0.41	7.33 h ± 0.36	18.5 d ± 0.92	11.66 hi ± 0.58	3.02± 0.15	139.92 g ± 6.99
Cfl	71.83 cd± 2.15	13.16 d ± 0.39	6.66 de ± 0.19	14.66 f ± 0.43	45 a ± 1.35	91.66 a ± 2.74	17.43± 0.52	366.64 e ± 10.99
Bdn	55.23 f ± 3.31	7.96 g ± 0.47	8.33 c ± 0.49	9.66 g ± 0.57	33.1 c ± 1.98	12.9 gh ± 0.77	2.35± 0.14	472.14 d ± 28.32
Raw	63 e ± 4.41	20.76 b ± 1.45	5.33 f ±0.37	20.33 e ±1.42	42.66 b ± 2.98	56.66 c ±3.96	9.5± 0.66	396.62 e ±27.76
Opl	23.12 h ± 0.92	3.2.i ± 0.12	0 i ± 0	10 g ± 0.4	3.5 h ± 0.14	5.35 j ± 0.21	0.89 ± 0.03	112.35 g ± 4.49
p=	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Data are the pooled means of three estimates each over two years ±standard deviation. 'p-value' denotes the significance of difference between the means by one way ANOVA statistics. a The values followed by different letters differ significantly by Duncan's multiple range test at p=0.05.
* **Cul**: *Cassia uniflora* Mill.non Spreng ; **Snl**: *Synedrella nodiflora*(L) Gaertn; **Alt**: *Alternanthera tenella* Colla; **Eug**: *Euphorbia geniculata* Orteg.; **Ach**: *Achyranthes aspera* L.; **Bod**: *Boerhaavia erecta* L.; **Bln**: *Blainvillea acmella* L.; **Aca**: *Acalypha ciliata* Forsk.; **Tum:** *Triumfetta rhomboidea* Jacq.; **Cab**: *Cassia absus* L.; **Cfl:** *Cassia obtusifolia* L.; **Bdn**: *Bidens biternata* Lour.; **Raw:** *Rauwolfia tetraphylla* L.; **Opl:** *Oplismenus compositus* P.Beauv.

Table 2. Morphological features of invasive and native weeds

4.5 Reproductive capabilities of invasive and native weeds

The invasive and native weeds showed considerable variations in reproductive characters (Table 3 a). The type of inflorescence in majority of the weeds studied was mostly head,

spike or cyme. Only in *Euphorbia,* it was cyathium, and in *Oplismenus* it was panicle. In almost all the weeds the inflorescence was axillary, but only in *Achyranthes* and *Cassia absus*, it was terminal.

For the number of inflorescences per plant *Triumfetta,* was at first rank, which was followed sequentially by *Alternanthera, Synedrella* and *Cassia uniflora.* The weed species like *Blainvillea*, *Acalypha* and *Achyranthes* were at medium position, while the least number was noted in *Oplismenus.*

The number of flowers, florets and flowerbuds per inflorescence was also a diagnostic feature for all the weeds. *Acalypha* was having highest number of male and female flowers together, whilst *Achyranthes* was following it (Table 3a). *Oplismenus* was in third position and it was followed by *Synedrella*. Remaining weeds had very few numbers of flowers**/** florets**/** flowerbuds and the lowest number of flowers was in *Triumfetta.*

The number of fruits per inflorescence is very important reproductive character which was in the order of –

Snl > Ach > Bdn > Alt > Tum > Bln > Aca > Cul > Cfl > Cab > Opl > Eug > Raw (Table 3b).

The number of seeds per plant was not showing the same order as that of number of fruits (Table 3b). This was due to the number of seeds per fruit, which is again a specific and variable character for a particular plant. The descending sequence for number of seeds per plant was:

Cfl > Snl > Ach > Cul > Aca > Bdn > Tum > Cab > Alt > Bod > Bln > Eug > Opl > Raw.

Weed species	Type of Inflorescence	No. of Inflo. / plant	No. of flowers, florets or floral buds per inflo.
Cul	Axillary raceme/ subsessile pairs, Crowded upwards	38.0 d ± 1.52	8.0 fgh ± 0.32
Snl	Axillary heads	60.0 c ± 3.00	21.0 cd ± 1.05
Alt	Axillary clusters	75.0 b ± 5.25	9.0 fgh ± 0.63
Eug	Cyathium	9.0 i ± 0.27	8.0 fgh ± 0.24
Bod	Umbels in terminal corymbose panicles	30.0 e ± 1.8	13.0 efg ± 0.78
Ach	Terminal spikes	21.0 g ± 1.26	36.0 b ± 2.16
Bln	Heads in erect terminal cymes	25.33 f ± 1.01	13.0 efg ± 0.52
Aca	Androgynous spikes	23.0 fg ± 1.61	178.3 a ± 12.48
Tum	Dense terminal and leaf opposed cymes	90.0 a ± 2.7	4.0 h ± 0.12
Cab	Terminal raceme	8.66 i ± 0.43	14.0 ef ± 0.7
Cfl	Axillary subsessile pairs of flowers	9.33 i ± 0.27	14.33 ef ± 0.42
Bdn	Corymbose panicled heads	15.66 h ± 0.93	16.66 de ± 0.99
Raw	Umbelliform cyme	9.66 i ± 0.67	7.33 gh ± 0.51
Opl	Panicle	2.0 j ± 0.08	24.0 c ± 0.96
p=		<0.001	<0.001

Table 3. (a) Reproductive features of invasive and native weeds

The results on reproductive capacity of invasive and native weeds revealed that the highest reproductive capacity was recorded in *Cassia obtusifolia,* which was followed by *Synedrella.* While *Achyranthes* and *Cassia uniflora* were on the third rank. The least reproductive capacity was reported in *Boerhaavia* (Table 3b).

Weed species	No. of fruits / inflo.	No. of fruits / plant	No. of seeds / fruit	No. of seeds / plant	Reproductive Capacity
Cul	9	2736 f ± 109.44	8	21888 d ± 875.52	18604 c ± 744.16
Snl	21	26460 a ± 1323	1	26460 b ± 1323	23814 b ± 1190.7
Alt	9	6075 d ± 425.25	1	6075 gh ± 425.25	3037.5 fg ± 212.62
Eug	8	576 h ± 17.28	3	1728 k ± 51.84	691.2 h ± 20.73
Bod	13	5070 e ± 304.2	1	5070 hi ± 304.2	2028 g ± 121.68
Ach	32	24192 b ± 1451.52	1	24192 c ± 1451.52	19353.6 c ± 1161.21
Bln	13	4280.77 e ± 171.23	1	4280.77 ij ± 171.23	2996.539 fg ± 119.86
Aca	1	4100.9 e ± 287.063	3	12302.7 e ± 861.18	6679.89 e ± 467.59
Tum	12	4320 e ± 129.6	2	8640 f ± 259.2	3247.72 f ± 97.43
Cab	14	1697.36 g ± 84.86	4	6789.44 g ± 339.472	3394.72 f ± 169.73
Cfl	14	1915.91 fg ± 57.47	32	62266.9 a ± 1868.007	28020.11 a ± 840.60
Bdn	37	9653.14 c ± 579.18	1	9653.14 f ± 579.18	8205.05 d ± 492.30
Raw	7	495.65 h ± 34.6955	2	991.309 k ± 69.39	7183.4 e ± 502.83
Opl	24	1152 gh ± 46.08	1	1152 k ± 46.08	6220.8 e ± 248.83
p=		<0.001		<0.001	<0.001

Table 3. (b) Reproductive features of invasive and native weeds

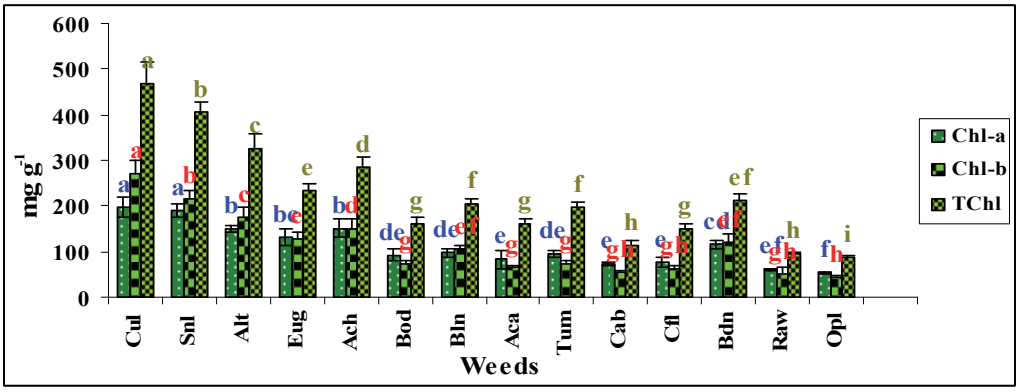


Fig. 1. Photosynthetic pigments in the invasive and native weeds

The role of reproductive capacity of weeds from arid zones was carried out by Sen (1981). Many researchers like Khanh et al. (2009) had studied the reproductive biology of Russian thistle, barnyardgrass, *Solidago canadensis* and *Bidens pilosa* respectively. Some have investigated the allelopathic potential of reproductive organs (flowers and fruits) of *Lantana camara* and explained their role in successful invasion, encroachment and dominance of it.

The role of reproductive capacity of weeds from arid zones was carried out by Sen (1981) and Kanchan and Jayachandra (1977) for *Parthenium hysterophorus*. The seed output and other propagules was studied by Stevens (1957) for different weed species. Many researchers like Young (1991), Norris (1992), Huang Hua et al. (2007) and Khanh et al. (2009) had studied the reproductive biology of Russian thistle, barnyardgrass, *Solidago canadensis* and *Bidens pilosa* respectively. Hu and Wang (2001) studied the reproduction of two weedy vines. Qiaoying Zhang et al. (2009) had investigated the allelopathic potential of reproductive organs (flowers and fruits) of *Lantana camara* and explained their role in successful invasion, encroachment and dominance of it.

The reproductive characters are the probable indicators of plants' invasiveness and aggressiveness in the ecosystem. Not only this, but they are also the factors deciding their density and abundance. Dekker (2005) had also opined similarly indicating the role of reproductive features in plant invasion. Bhan et al. (1976) also emphasized on the role of reproductive behavior of *Phalaris minor* in its dominance and invasion. In the present study also, the comparison made on the basis of morphological and reproductive characters between invasive and native weeds revealed that the selected invasive weeds were superior in all the above-mentioned features, reflecting on their dominance over natives in the campus of Pune University. Friedman and Waller (1983) also stated that the seeds of plants act as allelopathic agents, releasing the different types of allelochemicals in their surrounding environment and which help to establish their dominance.

4.6 Light harvesting components in invasive and native weeds

The results shown in Figure 1 revealed that amongst invasive weeds *Cassia uniflora* and *Synedrella* had higher chlorophyll a, b and total chlorophyll contents, followed by *Alternanthera.* Amongst invasive weeds *Bidens* and *Blainvillea* are at par for chlorophyll contents. Amongst the natives *Triumfetta* was following *Achyranthes* for photosynthetic pigments. Remaining invasive and native weeds were at intermediate state for the contents of photosynthetic pigments. Whilst, *Oplismenus* and *Rauwolfia* had shown the lowest chlorophyll contents. The invasive weeds like *Cassia uniflora, Synedrella* and *Alternanthera* had shown maximum amount of chlorophyll a, chlorophyll b and total chlorophylls.

The results recorded on photosynthetic pigments in different invasive and native weeds were corroborating with that of photosynthetic rate and other gas exchange parameters (Table 4 and Figure 1). Ghayal et al. (2009) have quantified the photosynthetic pigments in different invasive, native, aquatic as well as terrestrial weeds. Pawar (2004), Jadhav (2006), Castillo et al. (2007), Vaidya (2009) have quantified the photosynthetic pigments in different invasive, native, aquatic as well as terrestrial weeds. Wang et al. (2004) also recorded significant contents of chlorophylls and better photosynthetic rate in *Eupatorium.* Bhalerao (2003) had made similar observations regarding photosynthetic pigments in fern species like *Tectaria* and *Pteridium* from Mahabaleshwar area.

Photosynthetic pigments are the master molecules in carbon assimilation process, which govern photosynthetic efficiency. Sampietro et al. (2007) explained that the growth, development, dominance and allelopathic potential of any plant species mostly depend on its physiological, biochemical and enzymological characteristics. The growth, development, dominance and allelopathic potential of any plant species mostly depend on its physiological, biochemical and enzymological characteristics. The allelochemicals in it are also important along with above aspects. The amount of photosynthetic pigments usually correlates with photosynthetic rate, which is directly or indirectly reflected into

accumulation of reserved food materials like starch and dry matter accumulation rate and dry biomass. Considering this the dominance of *Cassia, Synedrella* and other native weeds can be correlated with higher amount of chlorophyll contents, enhanced photosynthetic rate and increased accumulation of reserved food materials.

4.7 Carbon assimilation rate of invasive and native weeds

The selected invasive weeds like *Cassia, Synedrella* and *Alternanthera* had shown the highest photosynthetic rate, stomatal conductance and transpiration rate as compared to native weeds such as *Achyranthes* and *Boerhaavia* (Table 4). Amongst the natives *Achyranthes* was superior in all above mentioned parameters over other weeds like *Triumfetta, Boerhaavia, Cassia absus* and *Rauwolfia*.

The third leaf area was maximum in *Cassia obtusifolia*, which was followed by *Rauwolfia* and *Triumfetta*. There was no significant difference in water use efficiency (WUE) amongst the investigated invasive and native weeds. On the basis of dry matter accumulation rate (DMAR) *Cassia uniflora* was topmost, which was followed by *Synedrella* and *Alternanthera.* Similarly the results for dry weight per plant (Table 4) indicated highest values for *Cassia obtusifolia* followed by *Cassia uniflora* and *Boerhaavia.*

According to McDowell (2002) the success of invasion and dominance of invasive weeds in any ecosystem over co-occurring species can be ascribed to their superiority in physiological attributes like photosynthetic rate, stomatal conductance, third leaf area and DMAR, but all such parameters mostly remained unexplained and unexplored. Understanding of such factors may give valuable insight to resolve the problem of invasion. Photosynthesis is the key catabolic process in the life cycle of any plant, which synthesizes various types of photo-assimilates and reflects into overall productivity and metabolic status of that plant. The secondary metabolites acting as allelochemicals are mainly derived from carbohydrates synthesis during photosynthesis.

Research workers like Durand and Goldstein (2001) and Ewe and Sternberg (2003) have also recorded the significant difference in photosynthetic rate, stomatal conductance, transpiration rate and total leaf area for the various invasive weeds and claimed that these weeds were highly dominant over the associated natives because of their superiority in above parameters.

According to Pattison et al. (1998) successful invasive species should have elite morphological and physiological traits, which increase their photon capturing ability and light utilization efficiency. But unfortunately this type of invagination has remained obscure due to paucity of experimental work, except few reports on light capturing mechanism, photon-saturated photosynthetic rate, specific leaf area (SLA) investigated in some invasive weeds by Ewe and Sternberg (2003). According to Durand and Goldstein (2001) invasiveness of alien species is dependent on photosynthetic efficiency. They further claimed that invasive species have a higher ability to capture solar radiations at the minimum cost of energy (ATP), diverting more resources for their growth, development, reproduction and yield. Coupled with the above distinctive features of photosynthesis, the invasive weeds have very high abiotic stress tolerance capacity, which enables them to survive and reproduce successfully, under extremely harsh environmental conditions like drought.

The water use efficiency (WUE) is always positively co-related with the rate of photosynthesis, however in present studies it has not shown any significant differences,

amongst the invasive and native weed species investigated. Funk and Vitousek (2007) have reported the positive co-relation with leaf traits and WUE in some weeds. Blicker et al. (2003) had also reported higher water use efficiency in the invasive weed *Centaurea maculosa* over three native grasses.

Weed Sps.	Photosynthetic rate µmol cm^{-2} sec^{-1}	Stomatal conductance mmol cm^{-2} sec^{-1}	III leaf area cm^2	Transp. rate mmol cm^{-2} sec^{-1}	WUE µmolm mol^{-1}	DMAR / m^2/ day	Dry weight / plant g
Cul	29.55 a ± 4.84	0.353a ± 0.064	20.41 d ± 0.82	1.181 a ± 0.21	25.02 ± 1.00	19.16 a ± 0.77	12.40 b ± 0.50
Snl	29.122 ab ± 6.15	0.351ab ± 0.038	14.90e ± 0.75	1.152 ab ± 0.242	25.28 ± 1.26	5.90b ± 0.30	4.10g ± 0.21
Alt	27.3ab ± 0.43	0.34a ± 0.072	12.80ef ± 0.90	1.053 abc ± 0.13	25.93 ± 1.81	4.18c ± 0.29	8.30f ± 0.58
Eug	27.137 ab ± 3.06	0.318a ± 0.027	18.41d ± 0.55	1.048 bcd ± 0.033	25.89 ± 0.78	2.66d ± 0.08	4.60g ± 0.14
Ach	27.237 ab ± 2.01	0.324a ± 0.027	12.20fg ± 0.73	1.052 abc ± 0.095	25.88 ± 1.55	2.58d ± 0.15	10.20d ± 0.61
Bod	22.1 bc ± 3.79	0.256bc ± 0.041	11.80fg ± 0.71	0.874 cde ± 0.16	25.29 ± 1.52	2.62d ± 0.16	10.70cd ± 0.64
Bln	23.308 bc ± 8.59	0.272bc ± 0.10	13.00ef ± 0.52	0.968 cd ± 0.28	24.08 ± 0.96	0.46gh ± 0.02	1.43jk ± 0.06
Aca	21.833 bc ± 0.45	0.253bcd ± 0.006	10.50g ± 0.73	0.845 cde ± 0.020	25.84 ± 1.81	0.85fg ± 0.06	1.75ij ± 0.12
Tum	22.483bc ± 5.01	0.266bc ± 0.058	33.66c ± 1.01	0.889 cde ± 0.19	25.29 ± 0.76	1.46e ± 0.04	11.35c ± 0.34
Cab	19.5c ± 0.7	0.178de ± 0.014	18.83d ± 0.94	0.731 def ± 0.038	26.68 ± 1.33	0.26h ± 0.01	3.02h ± 0.15
Cfl	21.533bc ± 0.51	0.21cde ± 0.018	45.00a ± 1.35	0.832 cde ± 0.032	25.88 ± 0.78	0.58gh ± 0.02	17.43a ± 0.52
Bdn	26.542ab ± 3.88	0.309a ± 0.044	33.10c ± 1.99	1.016 bcd ± 0.15	26.12 ± 1.57	1.09ef ± 0.07	2.35hi ± 0.14
Raw	17.867c ± 1.53	0.143e ± 0.033	42.66b ± 2.99	0.702 ef ± 0.0603	25.45 ± 1.78	0.32h ± 0.02	9.50e ± 0.66
Opl	15.283d ± 1.38	0.125e ± 0.015	3.50h ± 0.14	0.592 f ± 0.047	25.82 ± 1.03	0.16h ± 0.01	0.98k ± 0.04
	<0.001	<0.001	<0.001	<0.001	0.81	<0.001	<0.001

DMAR – Dry Matter Accumulation Rate

Data are the pooled means of three estimates each over two years **±** standard deviation. 'p-value' denotes the significance of difference between the means by one way ANOVA statistics. a The values followed by different letters differ significantly by Duncan's multiple range test at p=0.05.

*** Cul**: *Cassia uniflora* Mill.non Spreng ; **Snl**: *Synedrella nodiflora*(L) Gaertn; **Alt**: *Alternanthera tenella* Colla; **Eug**: *Euphorbia geniculata* Orteg.; **Ach**: *Achyranthes aspera* L.; **Bod**: *Boerhaavia erecta* L.; **Bln**: *Blainvillea acmella* L.; **Aca**: *Acalypha ciliata* Forsk.; **Tum:** *Triumfetta rhomboidea* Jacq.; **Cab**: *Cassia absus* L.; **Cfl:** *Cassia obtusifolia* L.; **Bdn**: *Bidens biternata* Lour.; **Raw:** *Rauwolfia tetraphylla* L.; **Opl:** *Oplismenus compositus* P.Beauv.

Table 4. Photosynthetic parameters of invasive and native weeds

Photosynthesis is the unique process that governs the plant productivity and biomass production. Hence the increased photosynthetic rate in invasive weeds might be able to increase biomass as the rate of dry matter accumulation rate resulting into higher biomass production. All these factors might be responsible for the higher dominance of selected invasive weeds in the study area. The invasive weeds studied in the present investigation like *Cassia uniflora, Synedrella* and *Alternanthera* had shown higher rate of photosynthesis, stomatal conductance, transpiration and dry matter accumulation rate over other invasive and native weeds, which might be the basic reason for the success of invasion and dominance in the campus of University of Pune (M.S.)

4.8 Biochemical nature invasive and native weeds

The results recorded on organic constituents like total sugars and starch indicated that *Cassia uniflora* had highest contents, which was followed by *Synedrella, Alternanthera* and *Bidens.* The remaining invasive and native weed species were on par for the contents of total sugars and starch, while *Cassia absus, Rauwolfia* and *Oplismenus* were at par but lower than the above mentioned weeds for starch and total sugars (Figure 2).

The invasive weeds like *Cassia uniflora* had shown highest contents of proteins and free amino acids, which was followed by *Synedrella* and *Alternanthera.* While *Bidens, Blainvillea* and *Euphorbia* were at par. The native weed *Achyranthes* had maximum contents of proteins and free amino acids, followed by *Triumfetta* and *Boerhaavia.* While remaining weed species had comparatively very less contents of proteins and free amino acids (Figure 3).

The dominant invasive and native weeds were having comparatively higher contents of reducing and total sugars, starch, proteins etc. They further explained that the superiority in organic constituents was contributing for the luxuriant growth and allelopathic potential. The chemicals released from damaged roots, root exudates and leaf leachates such as amino acids and carbohydrates may not directly act as allelopathic agents, but they can modify the activities of allelochemicals. The maximum contents of organic constituents like total sugars and starch have also indicated the better photosynthetic efficiency of these weeds over co-existing ones. The contents of primary metabolites like sugars, carbohydrates, amino acids, proteins etc. in plants also have allelopathic potential. The higher contents of all above organic constituents in *Cassia* and *Synedrella* might be responsible for their allelopathic potential.

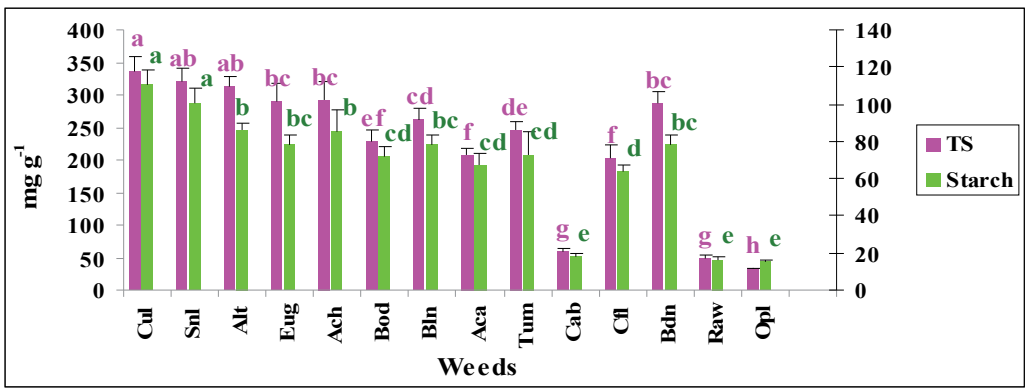
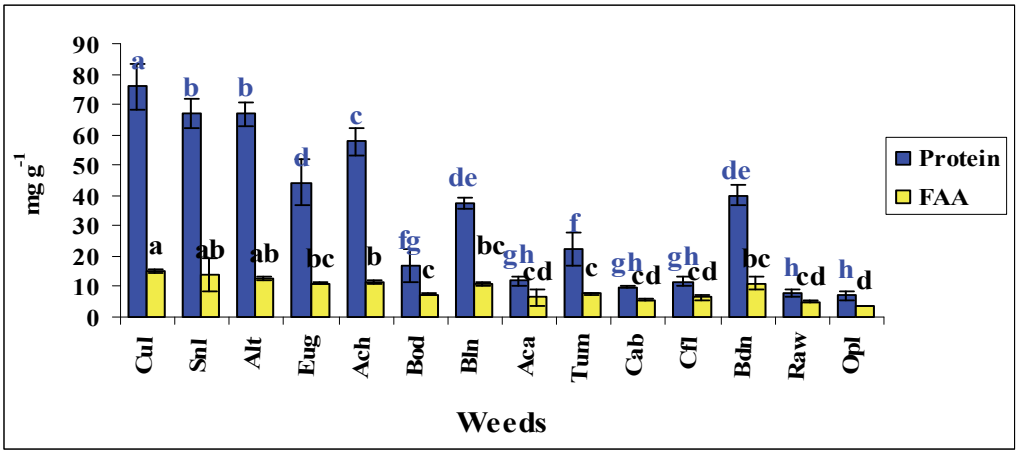


Fig. 2. Total sugars and starch contents in the invasive and native weeds



#Data columns are the pooled means of three replicates over two years with standard deviation as error bars. a Different letters at the data points denote significant difference by Duncan's multiple range test at $p<0.05$.

Fig. 3. Protein and free amino acid contentsin the invasive and native weeds

According to Blum (1996, 1997) and Inderjit and Nilsen (2003), allelopathic action can be explained by investigating the organic compounds in plants, because they may have additive effect or joint action on various biosynthetic pathways of primary and secondary metabolites. The contents of various organic constituents existing in plants may indicate their allelopathic potential, luxuriant growth and aggressive nature. Similar explanations may be applicable for the luxuriant growth and aggressive nature, faster encroachment and dominance of *Cassia* and *Synedrella* at all the four selected sites in Pune University campus, as both the weeds were highly superior in photosynthetic pigments, different photosynthetic parameters and organic constituents like sugar, starch, proteins and free amino acids.

4.9 ROS scavenging mechanism of invasive and native weeds

As it is well documented that invasive and native weeds have stress tolerant abilities and sustenance to withstand harsh ecological conditions due to the presence of osmolytes, antioxidants and ROS scavenging enzymes, an attempt was made for analysis of these parameters.

The results recorded in Table 5 on various antioxidants/ osmolytes/ compatible solutes like proline, glycine betaine and phenolics revealed that *Cassia uniflora* was having highest contents. It followed by *Synedrella* and *Alternanthera.* However the results on glycine betaine were not significant and for phenolics all the three weed species were at par.

The analysis of MDA content (lipid peroxidation) revealed that *Cassia uniflora* had lowest value, which was followed by *Synedrella* and *Alternanthera.* While *Achyranthes* was lowest amongst the native weeds.

The results on MSI showed that the index was highest in *Cassia uniflora* which was followed by *Synedrella* and *Alternanthera.*

The results reported in Table 5 on the relative water content of invasive and native weeds showed that here also *Cassia uniflora* was topmost, succeeded by *Synedrella* and *Alternanthera.*

Weed species	Proline m moles/g DW	Glycine Betaine mg/g	Phenols mg/g	Lipid Peroxidation T bars	MSI	RWC %
Cul	38.28 a ± 4.68	0.42 a ± 0.01	8.43 a ± 2.75	0.03 d ± 1E-04	75.64 a ± 5.67	64.8 a ± 5.83
Snl	34.48 b ± 2.63	0.37 a ± 0.01	8.22 a ± 0.61	0.03 cd ± 0.002	70.11ab ± 9.93	49.5 b ± 12.63
Alt	25.57 c ± 1.02	0.33 a ± 0.21	8.19 a ± 0.53	0.04 d ± 1E-04	65.63 bc ± 2.45	41.57 c ± 5.56
Eug	11.82 de ± 0.35	0.30 a ± 0.18	6.59 abc ± 1.36	0.04 cd ± 0.061	60.76 cd ± 4.55	27.9 de ± 3.90
Ach	12.44 d ± 0.74	0.334 a ± 0.023	7.36 ab ± 0.47	0.05 bcd ± 0.002	61.13 cd ± 3.97	36.1 cd ± 5.05
Bod	6.54 gh ± 0.32	0.27 a ± 0.01	4.59 bcd ± 1.38	0.05 bcd ± 0.03	48.44 f ± 2.99	17.6 fg ± 1.40
Bln	9.264ef ± 2.87	0.303 a ± 0.009	6.17 abc ± 2.85	0.08 bcd ± 0.08	57.36 de ± 3.15	23.4 efg ± 3.51
Aca	6.27 h ± 0.43	0.26 a ± 0.19	4.06 d ± 0.38	0.101 bcd ± 0.003	47.53 f ± 4.51	15.34 gh ± 2.14
Tum	8.37 efg ± 0.58	0.28 a ± 0.01	4.75 cd ± 0.26	0.1 bcd± 0.005	50.45 ef ± 3.095	19.65 efg ± 1.37
Cab	12.41 d ± 0.21	0.24 ab ± 0.017	5.82 bcd ± 1.75	0.103 bcd ± 0.004	45.53 f ± 1.15	15.69 gh ± 0.62
Cfl	20.84 c ± 0.29	0.26 a ± 0.18	6.66 abc ± 0.27	0.13 bc ± 0.008	47.38 f ± 3.554	18.83 ef ± 2.61
Bdn	9.486 def ± 0.379	0.305 a ± 0.015	6.34 abc ± 2.74	0.13 bc ± 0.15	57.94 de ± 4.34	25.9 ef ± 3.88
Raw	15.24 cd ± 0.31	0.059 b ± 0.002	5.69 bcd ± 0.202	0.17 b ± 0.007	38.66 g ± 2.51	15.52 gh ± 0.071
Opl	7.54 fgh ± 0.22	0.054 b ± 0.003	4.41 cd ± 0.61	0.36 a ± 0.02	34.57 g ± 2.593	12.01 h ± 0.261
	<0.001	0.024	<0.001	<0.001	<0.001	<0.001

Data are the pooled means of three estimates each over two years ±standard deviation. 'p-value' denotes the significance of difference between the means by one way ANOVA statistics. [a] The values followed by different letters differ significantly by Duncan's multiple range test at p=0.05.
*** Cul**: *Cassia uniflora* Mill.non Spreng ; **Snl**: *Synedrella nodiflora*(L) Gaertn; **Alt**: *Alternanthera tenella* Colla; **Eug**: *Euphorbia geniculata* Orteg.; **Ach**: *Achyranthes aspera* L.; **Bod**: *Boerhaavia erecta* L.; **Bln**: *Blainvillea acmella* L.; **Aca**: *Acalypha ciliata* Forsk.; **Tum:** *Triumfetta rhomboidea* Jacq.; **Cab**: *Cassia absus* L.; **Cfl:** *Cassia obtusifolia* L.; **Bdn**: *Bidens biternata* Lour.; **Raw:** *Rauwolfia tetraphylla* L.; **Opl:** *Oplismenus compositus* P.Beauv.

Table 5. Osmolytes and antioxidants in invasive and native weeds

The alien species like *Cassia uniflora, Synedrella* and *Alternanthera* have higher contents of different types of antioxidants as compared to the co- occurring invasive and native weeds at the selected sites of Pune University campus (Table 5). Along with this lower values of lipid peroxidation and higher MSI and RWC might be offering them additional mechanisms for abiotic stress tolerance. As a result of this the selected invasive weeds might have succeeded to invade and encroach over the native plants of Pune University campus even during the harsh environmental conditions.

The enhancement in various antioxidants was reported by many allelopathy workers like Tambussi et al. (2000), Horling et al. (2003), Guha et al. (2004), Yang and Lu (2005) in different types of invasive plants growing in terrestrial and boreal forest communities of North America. The role(s) of different antioxidants and osmolytes existing in the invasive and native weeds of forest and cropland ecosystems are very much important. They have explained that the antioxidants were helpful for these weeds to become dominant over co-occuring plant species.

The free radicals are constantly generated under stress conditions which are quenched by an efficient antioxidant network in the plant body. The complex network of such adaptive mechanisms at physiological and molecular levels cause changes in the synthesis and accumulation of various osmolytes, antioxidants and antioxidant enzymes, which provide stress tolerance to the plants (Bagul et al. 2005, Bhattacharya et al. 2009).

Proline is a major organic osmolyte accumulating in a variety of plant species in response to biotic and abiotic stresses, though its actual role in plant osmo-tolerance remains controversial. It is also thought to help in stabilization of sub-cellular structures (e.g. membranes and proteins), and to scavenge free radicals under stress conditions. Proline is known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses (Kavi Kishore et al. 2005).

The rapid breakdown of proline upon relief of stress may provide sufficient reducing agents that support mitochondrial oxidative phosphorylation and generation of ATP for recovery from stress and repairing of stress induced damages (Zhu 2002). In response to drought or salinity stress in plants proline also helps for cytoplasmic osmotic adjustment. Accumulation of proline under stress in many plant species has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress - tolerant than in stress - sensitive plants (Ashraf and Harris 2004, Ashraf and Foolad 2007). Comparatively higher amount of proline accumulation in *Cassia* and *Synedrella* might be functioning as mentioned above providing stress tolerance to these weeds, as a result of which both the weeds were able to survive throughout the year and producing large no. of seeds even under unfavourable stress conditions. These outnumbering seeds of both the invasive weeds when germinate during favourable season, naturally establish the monothickets or pure stands which caused substitution of many natives resulting into loss of phytodiversity of Pune University campus.

Malondialdehyde (MDA) is a product of lipid peroxidation by a thiobarbituric acid reaction. During drought conditions high activities of antioxidant enzymes are associated with lower concentration of MDA, being linked to drought tolerance (Gao et al. 2008). Like proline lowest values of MDA in *Cassia* and *Synedrella* can be linked with drought tolerance and better survival in extremely adverse environmental conditions.

One of the most common responses in plants to abiotic stresses is overproduction of different types of compatible organic solutes (Serraj and Sinclair 2002), which protect the plants from stress injuries by cellular osmotic adjustment, detoxification of ROS, protection of membrane integrity and stabilization of enzymes/ proteins. The antioxidants also protect cellular components from dehydration injury. These solutes include proline, sucrose, polyols, trehalose and quaternary ammonium compounds (QACs) such as glycine-betaine, alanine-betaine, proline-betaine, choline *O*-sulfate, hydroxyproline-betaine and pipecolate-betaine (Rhodes and Hanson 1993).

Amongst the many quaternary ammonium compounds known in plants, glycine betaine occurs most abundantly in response to dehydration stress (Venkatesan and Chellappan 1998, Mansour 2000). GB is abundant mainly in chloroplast where it plays a vital role in adjustment and protection of thylakoid membrane, thereby maintaining photosynthetic efficiency. The results of present investigation on antioxidants indicated more accumulation of GB in *Cassia* and *Synedrella* as compared to the native weeds at selected sites of Pune University campus. Along with high proline and low MDA, GB might also be contributing for stress tolerance and thereby maintaining the photosynthetic efficiency of both the dominant invasive weeds as suggested by Genard et al. (1991).

RWC has special significance in physiological activities of plants. The results of the present investigation indicated that *Cassia* and *Synedrella* had significantly higher RWC as compared to other invasive and native weeds. It may be the additional physiological adaptation for drought tolerance along with above mentioned antioxidants.

Likewise MSI decides the extent of membrane perturbations in structure and dysfunctioning in the cellular activities during the stress conditions. The membrane stability index (MSI) is very important parameter that gives idea about the stress tolerance ability of invasive and native weeds. The MSI of weeds under investigations agree with this. Increase in allelochemicals in these weeds might also be helping the weeds to get more stress tolerance. Membranes are barriers isolating aqueous compartments of the cells and the membrane proteins participate in signal reception and in transport of specific solutes giving them stability and thereby afford stress tolerance to the plants (Ramadevi et al. 1997). The higher values of MSI in both the invasive weeds recorded in the present investigation may be having similar role as mentioned above, because of which these weeds are tolerating extreme environmental conditions, survive comfortably and invade successfully in the new habitats. On the contrary the native weeds are not able to tolerate the stress conditions and hence make the place for highly tolerant invasive weeds. This results in to loss of native phytodiversity in that particular ecosystem.

4.10 Antioxidant enzymes in invasive and native weeds

The activities of antioxidant enzymes like PPO (Polyphenol oxidase), POX (Peroxidase) and SOD (Superoxide dismutase) (Figure 4, 5), were stimulated in *Cassia uniflora* followed by *Synedrella* and *Alternanthera.* The remaining invasive and native weeds followed the above mentioned weed species. The difference in stimulation of these enzymes might be due to the difference in stress tolerance ability of these weeds. More accumulation of antioxidants and stimulated activities of antioxidant enzymes might be becoming helpful for stress tolerance to these weeds. The results of the present investigations are in agreement with the findings of Bhalerao (2003), Jadhav (2006), Vaidya (2009) and Ghayal et al. (2009). They have also reported comparatively higher stimulation of antioxidant enzymes like PPO, POX and SOD in some invasive as well as native weeds of forest, aquatic and terrestrial ecosystems. They further concluded that more accumulation of antioxidants and stimulated activities of antioxidant enzymes were helpful for stress tolerance to these weeds.

Antioxidative enzymes, such as superoxide dismutase (SOD), peroxidases (POD) and polyphenol oxidase (PPO), are the most important components in the ROS scavenging system. SOD dismutates O_2^- to H_2O_2, POD and PPO subsequently scavenge the H_2O_2. The activities of antioxidant enzymes are usually stimulated on exposure to oxidative stress, for protecting the plants, because these enzymes scavenge the reactive oxygen species (Tanaka 1994). The invasive and native weeds studied in the present investigation had shown very

high stimulation in the activities of above mentioned enzymes in response to stress conditions, which might be responsible for survival of these weeds in extreme ecological conditions existing in the campus of Pune University. Ping Lu et al. (2007) supported the above view.

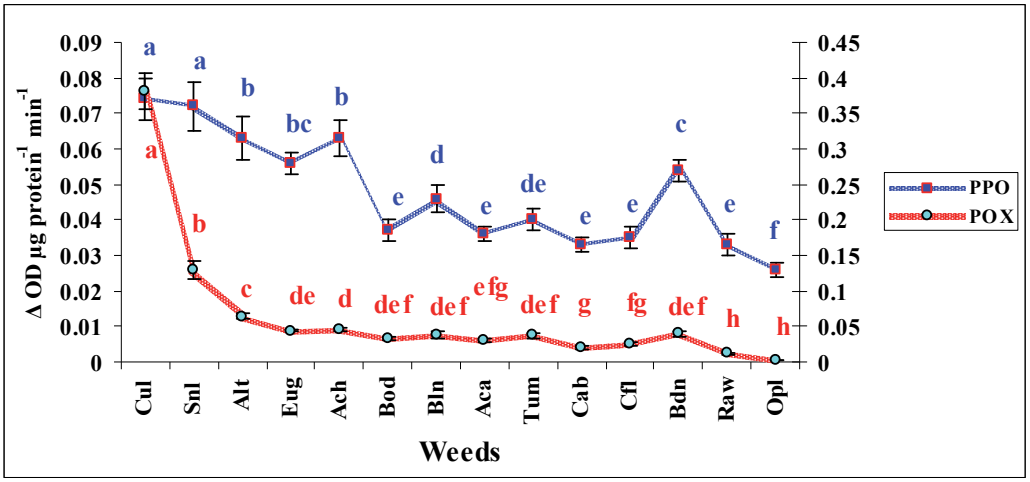
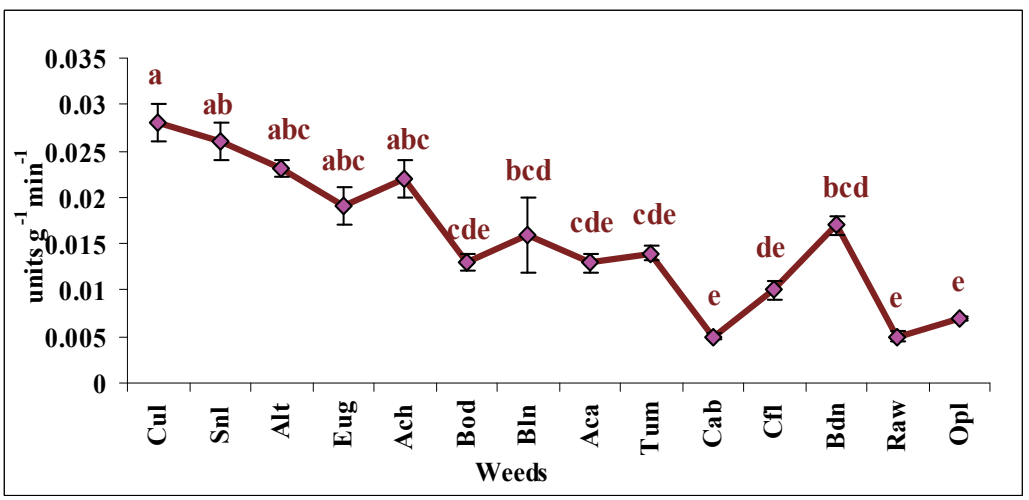


Fig. 4. Activity of polyphenol oxidase and peroxidase in the invasive and native weeds



#Data points are the pooled means of three replicates over two years with standard deviation as error bars. a Different letters at the data points denote significant difference by Duncan's multiple range test at $p<0.05$.

Fig. 5. Activity of superoxide dismutase in the invasive and native weeds

5. Allelobiogenesis of invasive weeds

To understand the allelopathic nature of any plant, extraction, identification and characterization of allelochemicals in its roots, stems and leaves has predominant role. In fact, all the interpretations in allelopathy are mostly based on such investigations. However, collection, isolation and complete identification, characterization and quantification of allelochemicals is difficult and a challenge to the allelopathy scientists. The allelochemicals like terpenes, steroids, flavonoids, alkaloids etc. have major impact on physiology of recipient plants, right from gene to organism level e.g. the monoterpenes which are the main constituents of the essential oils from many higher plants, interfere with basic biological processes like DNA replication, respiration, enzyme activities, seed germination and plant growth. These monoterpenes have allelopathic action. Triterpenes from many different weeds like *Cassia*, *Lantana, Mikania* are known for their allelopathic responses and great ecological significance with respect to invasion Ghayal et al. (2007a).

The allelochemicals like terpenoids, steroids, phenols and bitter essential oils present in roots, stems and leaves of *Cassia* and *Synedrella* might be released in to their environment, through various processes in the form of extracts, leachates, root exudates and even residues of all above plant parts which in due course of time become allelopathic to associated invasive and native weeds as a result of which they were suppressed slowly and substituted by *Cassia* and *Synedrella*. This phenomenon was observed at all the four sites of Pune University campus.

5.1 GC-MS study

The phytosociological dominance of *Cassia uniflora* and *Synedrella nodiflora* at the four selected sites in Pune University campus recorded previously and the inhibitory weed-weed interaction between these invasive weeds and co-occuring native weeds can be attributed to the different types of allelochemicals existing in them which are detected with GC-MS. The allelopathic potential exhibited by both the weeds might be due to different types of allelochemicals existing in them.

The distribution, quantity and type of allelochemicals depends on various factors such as age of the plant, growing season, vegetative or reproductive phase, environmental conditions and habitat. The allelopathic influence of extracts, leachates or residues of such plants is due to the different types of allelochemicals such as salts, esters, fatty acids, alkaloids, glycosides, terpenoids, flavonoids and steroids present in them. Their solubility in different solvents and mechanism of actions of such allelochemicals mostly depend on their chemical nature. These allelochemicals might be exuded, excreted or released from the plants. The chemical nature of such allelopathic compounds governs the process of invasion, dominance, distribution and encroachment over co-occurring species in any ecosystem.

Many researchers have isolated more than ten thousand low molecular weight secondary metabolites from higher plants and fungi. These compounds or their analogs are new sources of allelochemicals. Drager (2002), Mashhadi and Rodosevich (2003), Bhalerao (2003), Haig (2004), Elzaawely et al. (2005), and Alonso-Amelot (2006) had detected different types of allelochemicals from various weeds and fern species with GC-MS technique and studied their allelopathic activity. Ru Bai et al. (2009) also reported many allelopathic compounds by GC-MS in root exudates of *Malus prunifolia*. Qiaoying Zhang et al. (2009) detected allelopathic potential of flowers and fruits of *Lantana camara* which was ascribed to the allelochemicals by GC-MS. Seal et al. (2009) had also identified different allelochemicals

in weed species like Shepherd's purse (*Capsella bursa-pastoris*) by GC-MS and studied its phytotoxicity.

5.2 IR, NMR, and MASS spectra studies

The dominance, negative weed-weed interaction, encroachment over native weeds as well as successful invasion of *Cassia uniflora* and *Synedrella* recorded at all the four selected sites in the campus of Pune University can be attributed to the existence of different types of allelochemicals in the leaves of both the weeds such as 2(4H)- Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl (Dihydroactinidiolide), 2-pentadecanone, Isobutyl phthalate, 4,4,8-trimethyltricyclo [6.3.1.0(1,5)]dodecane-2,9-diol, Hexadecanoic acid, Phytol, Dioctyl phthalate, Neophytidiene, Caryophylene oxide and Di-isooctyl phthalate. The presence of above allelochemicals in both the invasive weeds was well documented by Ghayal et al. (2007). The presence of allelochemicals such as Dodecane-4- yl butyrate in *Cassia* leaves and 3-(5-(1-(3-methylpentyloxy) propyl)-tetrahydro-2-oxofuran-3-yl)-dihydrofuran-2(3*H*)-one in *Synedrella* leaves were detected for the first time in the present investigation.

Many allelopathy researchers such as An et al. (2000), Orr et al. (2005) and Santos et al. (2007) separated, identified and quantified the allelochemicals from different weeds and tested their phytotoxicity. Yang et al. (2006) also have separated and identified allelochemicals by NMR in *Ageratina adenophora* and studied their allelopathic activity on rice seedlings. Isolation and bioactivity of withaferin A from *Withania somnifera* roots was done by Kannan and Kulandaivelu (2007). Leicach et al. (2007, 2009) have used different methods of chromatography and spectroscopy for alkaloid separation and identification from different plants and elaborately discussed the importance of extraction, separation and identification of allelochemicals from different plants having allelopathic activity. Ma et al. (2009) and Li et al. (2009) have attempted the isolation and identification of allelochemicals by NMR and Mass in invasive plants *Ipomoea cairica* and *Polygonatum odoratum* respectively.

The results of the present investigation are in conformity with the above findings.These allelochemicals usually had greater adverse impact on the physiological as well as biochemical processes, enzymological activities, nutrient uptake and assimilation, reproductive abilities, growth and development of recipient plant species. The changes induced by allelochemicals at molecular level are also expressed in phenotypes. The antimicrobial activity in leaf leachates and extracts of *Cassia* and *Synedrella* might be due to the presence of allelochemicals such as terpenoids, steroids, flavonoids, pungent and bitter essential oils and various types of phenols present in them.

6. Conclusions

The present investigations attempted on phytosociology, physiology, biochemistry and enzymology of selected weeds, phytochemicals and allelochemicals existing in them, their allelopathic potential tested through seed germination bioassays, seedling growth and physiological, biochemical and enzymological changes including treated seedlings of test-crops due to leachates of selected invasive weeds, clearly revealed that the basis for all such events was allelopathic nature of *Cassia* and *Synedrella.*

The weed – crop interactions at molecular, cellular and whole plant level were also attempted with special emphasis, as these weeds in future are likely to invade agroecosystems and croplands. At present slowly they are spreading from wastelands to agricultural lands and competing with the crops of interest and cause significant yield loss.

The studies on impact of leachates, extracts and even residues of *Cassia* and *Synedrella* revealed that higher concentrations had severe and very dreadful influence on physiology, biochemistry and enzymology of test crops and such negative changes were also manifested on growth and yield attributes of crops interacting with weeds.
These investigations have also thrown a light on successful invasion of both the weeds in the campus of Pune University, their dominance, aggressiveness and encroachement over native and even other invasive weeds. The ecological and morphological superiority enabled them to do so very efficiently and effectively. The exclusive dominant nature of selected invasive weeds and their allelopathic potential resulted into the loss of native phytodiversity, which is the major threat of such invasion to any ecosystem. Such investigations may become the basis for exploring environmental and ecological degradations in nature.

7. Future research

The need for research and development in allelopathy for the improvement of agriculture, forestry and different types of ecosystems, community structures and functioning is of extreme urgency, because the understanding of allelopathy has major role(s) in the interactions between invasive/ exotic and native weeds, weeds-crops, crops-crops etc. These studies are of utmost importance in agriculture, forestry and environmental degradation. Many of these weeds cause damages to agroecosystems and disturb natural phytodiversity. Their dominance, luxuriant growth, persistence throughout the year, tolerance to biotic and abiotic stress conditions and allelopathic potential might be the probable factors of successful invasion in new habitats.
The use of naturally produced huge weed biomass for weedicidal, cytotoxic, larvicidal, insecticidal and antimicrobial activity is gaining ground in sustainable agriculture. With this view many research workers have reported the antimicrobial activity in different plant parts and extracts, leachates or residues of large number of plant species available in plenty.
The richness of bioactive compounds, secondary metabolites and variety of allelochemicals present in these weeds and other co-dominant weeds can give enticement to screen their cytotoxic, genotoxic, larvicidal, antimicrobial activities etc. The results of such experiments could be positive, if the analyses of their bioactive compounds, antioxidants and antioxidant enzymes is given due importance. The studies on genomics and proteomics of different weeds, having biotic and abiotic stress tolerance can be exploited with the aid of biotechnological tools, to have such type of agronomic traits in various crops. Only the coupling of all the aspects of studies can give an applied touch to the entire field of allelopathy.

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3

Alteration of Abiotic Stress Responsive Genes in *Polygonum minus* Roots by Jasmonic Acid Elicitation

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1. Introduction

Plants are continuously exposed to both biotic and abiotic stress in their natural environment. Unlike animals, plants are immobilized organisms which tend to be vulnerable to various environmental stresses. In order to survive, plants have evolved a wide range of defense mechanism to cope with these stresses. Both biotic and abiotic stresses might share some common signaling pathway in triggering the defense system in plants. Recent researches have revealed that phytohormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are intermediate molecules which play key roles in the crosstalk between biotic and abiotic signaling network (Fujita et al. 2006). In this chapter, we highlight the effects of exogenous applied jasmonic acid in triggering the synthesis of some molecules and activating their respective biosynthetic genes in plants as a response towards abiotic stresses.

Abiotic stress is defined as non-living external factors, usually environment conditions, which could reduce plant growth and cause huge devastation on agricultural productivity. Some of these major adverse environmental factors are drought, salinity, heavy metals, extreme temperatures, nutrient poor soils and other source of natural disasters. To our knowledge, these abiotic stresses have account for major crops lost worldwide where more than 50% of their average yields were decreased yearly (Rodriguez et al. 2005). However, not all effects are detrimental. Plants are able to exhibit various molecular mechanisms as a defense system and these responses could be generally divided into three main groups. Firstly, signalling of stress-activated molecules leading to changes of osmotic and ionic homeostasis as well as detoxification mechanism. Secondly, up-regulation of different gene expression leading to synthesis of specific proteins (e.g. heat-shock proteins and LEA proteins) and some protective molecules (e.g. sugars, polyalcohols and amino acids). Thirdly, generation of reactive oxygen species (ROS) and activation of antioxidant systems by synthesizing secondary metabolites such as flavonoids and phenolic compounds (Boscaiu et al. 2008). Among these changes, synthesis of secondary metabolites is at the highest interest because it has a wide range of functions, ranging from plant defense against abiotic stresses to human benefits.

Plants have the ability to produce vast variety of secondary metabolites naturally. Secondary metabolites have been defined as compounds that did not play a vital role in

plant growth and development but are important in the interaction between plant and its environment (Namdeo 2007). These molecules function primarily in plants adaptation towards their environment such as biotic and abiotic stress and also serve as a major source for pharmaceutical products (Ramachandra Rao & Ravishankar 2002). Secondary metabolites are usually release by plants as a type of defense system against insects feeding; herbivory effects and pathogens attack such as virus, bacteria and fungi. They also protect plants against abiotic stress such as draught, salinity, UV light, heavy metals, extreme temperatures, nutrient poor soils and other environmental factors. Several other functions of secondary metabolites include attracting pollinators for plants reproduction and serving as signaling molecules and hormones in plant cells secondary metabolism (Korkina 2007). Up to date, thousands of different secondary metabolites structures have been identified in plants. The bioactive compounds extracted from various plant parts were usually used in the pharmaceutical, agrochemical, cosmetic, perfumery, food flavouring and pesticide industries (Balandrin & Klocke 1988). For instance, morphine and codeine extracted from the latex of opium poppy are the commercial anesthesia available in market today whereas ginsenosides isolated from ginseng roots have been proven to be the stimulant for health and longetivity (Sticher 1998). Apart from that, alcohols, aldehydes, ester, free fatty acids, ketones and phenolic compounds purified from plants are also being used in the foods and beverages industries. The food flavouring that are succesfully marketed are apple (Drawert et al. 1984), cocoa (Townsley 1972), caryophylene (Longo & Sanroman 2006), flavanol (Nakao et al. 1999) and vanillin (Dornenburg & Knorr 1996). Many of these phytochemicals, especially the volatile compounds are secreted by plants as an indirect defense mechanism against herbivory and some other abiotic stress (Yuan et al. 2008).

The exploitation of novel secondary metabolites and their functions have gained the interest of many scientists worldwide and extensive studies have been done since the past 50 years. More than 80% of 30,000 known natural products were originated from plants (Fowler & Scragg 1988; Phillipson 1990). Although the advancement of computational biology has shedded light to medical field as new drugs could be designed base on predicted chemical structure, plants-derived compounds still serve as the model for drugs synthesis due to the complexity of their chemical structures (Pezzuto 1995). In fact, the world market for plant-derived drugs will be expected to achieve more than $26 billion in year 2011 (Saklani & Kutty 2008). However, there are some major drawbacks associated with phytochemicals production. Naturally these phytochemicals present at a much lower concentration compare to primary metabolites. The production of secondary metabolites is approximately 1% of the plant dry weight. Depending on the type of environmental stresses surrounding the plants, the type and level of secondary metabolites produce in plants changing from time to time, and from one place to another (Dixon 2001; Oksman-Caldenteyl & Inze 2004). Besides, the widespread of deforestation and instability of geopolitics make it difficult to extract pure secondary metabolites in whole plants (Shilpa et al. 2010). Fortunately, the advancement of biotechnology has made it possible to alter the production of secondary metabolites by means of plant cell cultures technology. The major advantages of plant cell cultures are that it could produce a continuous and more reliable source of plant pharmaceuticals (Vijaya et al. 2010). Though many efforts have been done since four decades ago, little success was achieved. Only the production of Shikonin from *Lithospermum erythrorhizon* cell culture and Taxol or Paclitaxel from *Taxus* cell cultures meeting the satisfactory yields for commercialization (Sekar & Kandavel 2010). Some other successful cases are such as rosmarinic acid production from *Anchusa officinallis*, indole alkaloids and catharantine

production from *Catharanthus roseus* and anthraquinone synthesize from *Morinda citrifolia* (Vanisree et al. 2004).

Up to now, many approaches have been done to increase the yield of secondary metabolites from plants. Strategies that have been accepted for used are manipulation of culturing media such as glucose, nitrate, phosphate and plant growth regulators concentration; screening and selection of cell lines which have the ability to produce better yield; optimization of culturing conditions such as temperature, light, pH and aeration; and addition of precursor or elicitor (Ramachandra Rao & Ravishankar 2002; Vanisree & Tsay 2004). Since the main roles of plant secondary metabolites are to increase plant adaptation to abiotic stresses and enhance plant defense system against pathogen attack, it is better to investigate some strategies to alter the production of metabolites based on this principle (Sekar & Kandavel 2010). In fact, elicitation by using molecules that involve in plant defense mechanism is the most efficient strategy to increase the productivity of secondary metabolites in plants (Roberts & Shuler 1997). Many biotic and abiotic elicitors have been employed to increase the production of desired compounds in plants (Barz et al. 1988), namely methyl jasmonate (MeJA), jasmonic acid (JA), salicylic acid (SA), fungus polysaccharide, yeast extract, heavy metal etc. Extensive researches have been done to study the roles of JA as the key signaling molecules in signal transduction system regulating the alteration of plant defense genes against environmental stresses. For instance, the expression of *pin* genes was activated by JA or its derivative, MeJA, in mechanically wounded tomato and potato (Farmer & Ryan 1992). The expression of *vsp* genes was also activated by MeJA as a defense mechanism towards wounded cells of soy bean (Creelman et al. 1992). Besides, exposure of *Hypericum perforatum* L. suspension culture to JA had activated the genes that expressed phenylalanine ammonia lyase (PAL) and chalcone isomerase (CHI) enzymes. Activation of these genes had increased the production of phenylpropanoids such as phenolic, flavanol and flavonol a 6-fold in cells treated with JA (Gadzovska et al. 2007). JA also altered the synthesis of ajmalicine and catharantine in *Catharanthus roseus* (Vazquez-Flota & De Luca 1998), rosmarinic acid and shikonin in *Lithospermum erythrorhizon* (Yazaki et al. 1997), scopoletin dan scopolin in *Nicotiana tabacum* (Sharon et al. 1998) and taxol dan paclitaxel in *Taxus* spp. (Palazon et al. 2003). Thus, jasmonate has been shown to be key molecules in the elicitation process leading to de novo transcription and translation that resulted in the enhancement of secondary metabolites biosynthesis in *in vitro* plants (Gundlach et al. 1992).

It is clear that harsh environmental conditions would activate the expression of certain abiotic stress related genes which involve in the biosynthesis pathway of secondary metabolites in plants. Therefore it becomes a crucial need to identify the stress responsive genes and study their signal transduction pathways not only for a better understanding of plants response and adaptation towards abiotic stress, but also for further development of strategies for commercial production of valuable compounds by manipulating certain metabolic pathways based on gene expression (Shilpa et al. 2010). A significant amount of studies have been done on application of elicitation to plant cultures, either to enhance secondary metabolites production or to discover novel compounds. Though many of these showed positive and encouraging results, the stress responsive genes which involved in the reactions of defense-related pathway and secondary metabolites biosynthesis pathway remain largely unexploited. Many molecular approaches such as mRNA differential display, representation difference analysis, RNA fingerprinting, cDNA microarray and suppression subtractive hybridization (SSH) technique have been applied to identify and characterize the

genes which were expressed differentially during stress condition. Of all, SSH is a more effective and efficient method compared to others especially when non-model organism is of concern because genome sequences information is not required (Huang et al. 2007). By combining normalization and suppression PCR in a single step, SSH technique could reduce the excessive target cDNA sequences; at the same time enriched the low amount differential expressed transcripts up to 1000 – 5000 times in the sample population (Diatchenko et al. 1996). Hence, it is rational to identify the JA responsive genes by SSH technique because these genes may be involved in the synthesis pathways of valuable metabolites and/or abiotic stress tolerance. In order to support this hypothesis, we had demonstrated the effects of jasmonic acid (JA) as the elicitor in altering secondary metabolites synthesis in a type of local herb called *Polygonum minus* and the expression of JA-responsive genes have been identified by subtractive cDNA library construction.

2. Elicitation

2.1 The concept of elicitation

In general, plants respond towards abiotic stress stimuli by regulating signaling cascade followed by modulating gene expression machinery which could lead to the synthesis of stress responsive protein or valuable bioactive compounds. When stress signal received by the receptor on plant membrane, the small endogenous signaling molecules such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) will regulate defense system, both synergistically and antagonistically. It has been proven scientifically that exogenous application of these signaling molecules on plant cultures could alter the expression of genes involved in biosynthesis of different classes of secondary metabolites. This approach is known as elicitation, a process that involves the application of chemicals or addition of physical stress to plant cultures or whole plants as a way to produce secondary metabolites which are normally absent in the plants (Bourgard et al. 2001; Roberts & Shuler 1997). Elicitors are defined as chemicals from various sources which could trigger the physiological and morphological changes or phytoalexin accumulation in plants as a defense mechanism against stresses (Sekar & Kandavel 2010). They could mimic the mode of action of natural stress stimuli and thus create a stress environment for plants growth and development. They are able to trigger the normal metabolism in plant cells to synthesize enzymes that catalyze the defense-related pathways which ultimately leading to secondary metabolites production.There are a few ways to classify type of elicitors. It could be categorized according to its nature, which is known as biotic and abiotic elicitors or based on its origin, which is known as exogenous and endogenous elicitors. Abiotic elicitors are substances that derived from non-living thing such as inorganic salts, heavy metal ion, UV radiation, high pH level and so on whereas biotic elicitors are derived from living organims, for instance, pectin and selulose from plant cell walls, and chitin and glucan from microorganism. On the other hand, as defined by the prefix “exo-” and “endo-”, exogenous elicitors are substances derived from outer cellular compartment such as polisaccharide, polyamine and fatty acids whereas endogenous elicitors are molecules present in the inner cellular compartment, such as glucuronide or hepta-β-glucoside (Namdeo 2007). Table 1 summarizes the classification of elicitors.

A handful of experiments have been done extensively to study the effects of abiotic elicitors in enhancing the production of secondary metabolites in whole plants or plant cell cultures. However, the mechanisms that actually take place in the elicited cells remain unclear. Till

now, a few hypotheses have been proposed as the biochemical responses of plants towards the challenges of elicitors. For examples, ion Ca2+ influx changes in the cytoplasm (Gelli et al. 1997) and significant changes in protein phosphorylation and kinase proteins activation (Romeis 2001). Besides, some scientists also hypothesized that deactivation of H^+-ATPase will result in acidic cytoplasm (Armero & Tena 2001). Generation of reactive oxygen species (ROS) such as superoxide anion and H_2O_2 may have direct antimicrobial effect against pathogen attack, in the case of biotic elicitors. These ROS could trigger the formation of bioactive fatty acids derivatives in plants (Apostol et al. 1989). Furthermore, ROS could act as secondary signals in the activation of plant defence genes expression (Low & Merida 1996). Thus, it could be concluded that the mechanism of elicitors is almost the same according to the origin, specificity, concentration, physio-chemical environment, stages in plant's life cycle and nutrient assimilation of plant (Namdeo 2007).

Nature-based elicitors		**Origin-based elicitors**	
Biotic elicitors	**Abiotic elicitors**	**Exogenous elicitors**	**Endogenous elicitors**
Enzyme, cell wall pieces, pectin, chitosan, glucan.	UV light, denatured protein, heavy metal, chemical.	Glucomannose, glucan, chitosan, poly-$_L$-lysin, polyamine, glycoprotein, polygalacturonase, selulase.	Dodeca-β-1,4-$_D$-galacturonide, hepta-β-glucoside, alginate oligomer

Table 1. Classification of elicitors according to their nature or origin

2.2 JA biosynthesis and its roles in plant secondary metabolism

Jasmonic acid (JA) or its methyl ester, Methyl jasmonate (MeJA) are endogenous signalling molecules derived from lipid and distributed widely in plants. They function primarily in plant response towards biotic and abiotic stress and a variety of plant growth and development processes, including flowering, fruit ripening, and root growth. It has also been recognised as promoter for senescence, growth inhibitor and elicitor for secondary metabolism in many plant species. It is synthesized from oxylipin by lipoxygenase pathway in plant cells. The signalling of oxilipin molecules which include JA, MeJA, JA and amino acid conjugate and other JA derivatives, are regulated by the fatty acid residues that form plant membranes (Wasternack 2007). Oxilipins are originated from either α-linolenic acid (α-LeA, 18:3) or linoleic acid (18:2) released by chloroplast membrane. JA biosynthesis is initiated with the formation of (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT) and (9S)-hydroperoxyoctadecatrienoic acid (9-HPOT) from α-LeA. Product formed from linolenic acid is (13S)-hydroperoxyoctadecadienoic acid (13-HPOD) whereas linoleic acid will produce (9S)-hydroperoxyoctadecadienoic acid (9-HPOD). This reaction is catalysed by lipoxygenase (LOX) enzyme (Feussner & Wasternack 2002). 13-HPOT will then be converted by allene oxide synthase (AOS) to 12-13-epoxy-octadecatrienoic acid (12, 13-EOT) which is very instable (Song et al. 1993). 12, 13-EOT will be converted to *cis*(+)-12-oxophytodienoic acid (OPDA) by allene oxide cyclase (AOC), and consequently to 3-oxo-2(2′-pentenyl)-cyclopentane-1-heptanoic acid (OPC) by 12-oxophytodienoic reductase acid (OPR). For the final step in JA biosynthesis, OPC will undergo three β-oxidation cycles in peroxysomes (Miersch & Wasternack 2000). Every gene that coded for JA biosynthesis enzyme could be

alter by JA itself (Wasternack 2006). Till now, many promoters have been tested and it was found that the activities of promoters have been increase upon exposure to JA (Kubigsteltig et al. 1999). This observation suggested that the biosynthesis of JA is regulated by positive feedback reaction.

JA is recognised as the signalling molecule in elicitation process that leads to *de novo* transcription and translation and eventually activates the secondary metabolites in plant cell cultures (Gundlach et al. 1992). It has also been known as molecule involves in the signal transduction pathway that leads to the activation of plant defense against pathogen, insect and herbivore attack (Menke et al. 1999). There are also some studies which shown that JA activities are not restricted to one type of secondary metabolite only but it is able to alter the production pathways of various classes of secondary metabolites such as phenylpropanoids, alkaloids and terpenoids (Zhao et al. 2005; Pauwels et al. 2009). Many studies were done using JA as the elicitor to induce the production of important metabolites. For example, the *Hypericum perforatum* (St. John's wort) cell cultures showed significant increase in the production of hypericin after treated with JA (Walker et al. 2002). This result was supported by another group of scientists where they have successfully increase 6-8 times the production of phenylpropanoids like phenolic compounds, flavanol, flavonol; and naphtodianthrones like hypericin in *H. perofatum* cell culture after elicited by JA (Gadzovska et al. 2007). Besides, the production of anthraquinone in *Morinda elliptica* cell cultures was also enhanced by JA treatment (Chong et al. 2005), so as to antioxidant like carotenoids, vitamin C and vitamin E (Chong et al. 2005). Furthermore, MeJA was showed to increase the accumulation of silymarin products in *Silybum marianum* (L.) Gaertn cell cultures (Sanchez-Sampedro et al. 2005) and shikonin in *Lithospermum* cell cultures (Yazaki et al. 1997).

In a recent study, we are interested in finding the genes involved in the biosynthesis of aromatic compounds or other valuable metabolites found in *P. minus* roots using the elicitation strategy. Since most of the secondary metabolites induced by elicitor present *de novo* in plant cells (Pare & Tumlinson 1997) and involved certain enzymes activities induction (Bouwmeester et al. 1999; Degenhardt & Gershenzon 2000), the production of volatile compounds triggered by JA in kesum roots will reflect the enzyme activities which catalysed the biosynthesis of those compounds. The enzyme activity is proportional with the mRNA transcripts related to the compounds production. By combining the analysis of metabolomics and differentially expressed genes dataset, we hope to have a better understanding in the correlation between plant defence system against abiotic stress and secondary metabolites production.

2.3 Case study: Elicitation of *Polygonum minus* roots with JA

We have recently conducted an experiment to evaluate the effect of JA elicitation on the production of volatile compounds in *Polygonum minus* roots by gas chromatography coupled with mass spectrometry, as well as using SSH technique to identify the transcripts responsive to JA stress. *Polygonum minus* Huds., or commonly known as kesum, is a type of local herbs rich in essential oils. It has been recognized by the Malaysian government in the Herbal Product Blueprint as an essential oil-producing crop (Wan Hassan 2007). Previous studies reported that the essential oil from kesum leaves was found to contain about 76.59% aliphatic aldehydes that consisted of two dominant compounds, decanal and dodecanal, and contained 0.18% b-caryophyllene, a sesquiterpene (Karim 1987). Other studies have successfully identified valuable compounds from *Polygonum* roots that possessed medicinal

properties. For example, researchers have found phytoestrogens from *P. cuspidatum* and *P. hydropiper* roots (Matsuda et al. 2001), phytohormones, and anthraquinones from *P. multiflorum* roots (Yu et al. 2006) and indigo from *P. tinctorium* roots (Chae et al. 2000). We decided to target roots as the organ for elicitation process because of their ability to import and export molecules between root cells and the rhizosphere (Gleba et al. 1999). In addition, roots also possess some advantages against other plant parts. As they are physically unprotected in the soil environment, they are surrounded by many types of microorganisms. Hence, they may produce a vast amount of metabolites that possess antimicrobial or aromatic properties to ensure plant survival (Poulev et al. 2003). The application of elicitors on plant shoots has serious limitations because the hydrophobic surfaces and impermeable characteristics of leaves result in low uptake of chemical elicitors. On the contrary, elicitors can be easily added into growth media and absorbed by roots and can easily harvest and screen for bioactive compounds. Roots also contain low levels of pigments and other compounds found in leaves, such as tannins, which may interfere in chemical screening and extraction (Gleba et al. 1999; Poulev et al.2003).

In our experiment, *P. minus* roots harvested from *in vitro* plantlets were used for elicitation process and subtractive cDNA library construction. *P. minus* were micropropagated by internode culture and subculture in MS solid medium at every 2 months interval. The cultures were incubated at 26 $\pm$ 2 ^{o}C with 16 hours photoperiod and 20 µM/m2/s light intensity. Prior to adding JA solution, *P. minus* were transferred to MS liquid medium supplemented with different concentrations of JA (50µM, 100 µM, 150 µM and 200 µM). The non-treated plantlets were used as control. *P. minus* roots were harvested from JA-treated plantlets at day-1, day-3 and day-6 for GC-MS analysis. Each experiment was performed in triplicate where each treatment contained 10 plantlets. The experimental designed was done based on factorial 5 x 3 (JA concentration x treatment period). The volatile compounds and other secondary metabolites induced by JA stress were extracted from 2g of roots by Solid Phase Micro Extraction (SPME). The extracted compounds were then purified and separated by gas chromatography in Shimadzu AQ5050P with HP-5MS non-polar column (30m x 0.25mm x 0.25µM) and detected by quadrupole mass spectrometry. The parameters for GC-MS analysis were as follow: injection temperature 220^{o}C; detector temperature 280^{o}C; column temperature 50^{o}C – 3 min, 20^{o}C/min - 100^{o}C, hold 3 min, 30^{o}C/min - 250^{o}C, hold 3 min; flow rate 1.3ml/min; injection volume 1µl; injection method – split ration and mass spectrometry was operated in scan mode. Finally the compounds detected by MS were compared against the GC-MS Nist. 147 library according to similarity index (SI) and retention time (RT). Only compounds with SI unit more than 80 and present consistently in two or more replicate were considered for further analysis. The results from qualitative analysis showed β-caryophyllene present abundantly and consistently in the sample. Hence, it was selected as a marker compounds which is known as single point external standard for quantitative analysis. The data obtained were analysed with SAS (Statistical Analysis Systems) statistic program version 9.0 at significant level $p < 0.05$. General Linear Modelling (GLM) and Duncan analysis were performed according to experimental design and the appropriate data obtained.

2.3.1 Phytochemical analysis of *P. minus* roots

The chemical compounds from some species in the Polygonum family have been studied decades ago, for example *P. minus* (Karim 1987) and *P. odoratum* (Dung et al. 1995; Hunter et al. 1997). From our experiment, 30 compounds were successfully detected by GC-MS from

the *P. minus* roots extract based on the comparison of similarity index (SI) and retention time (RT) with the database in NIST 147 library. The chromatogram profile for non-treated plant was shown in Figure 1.

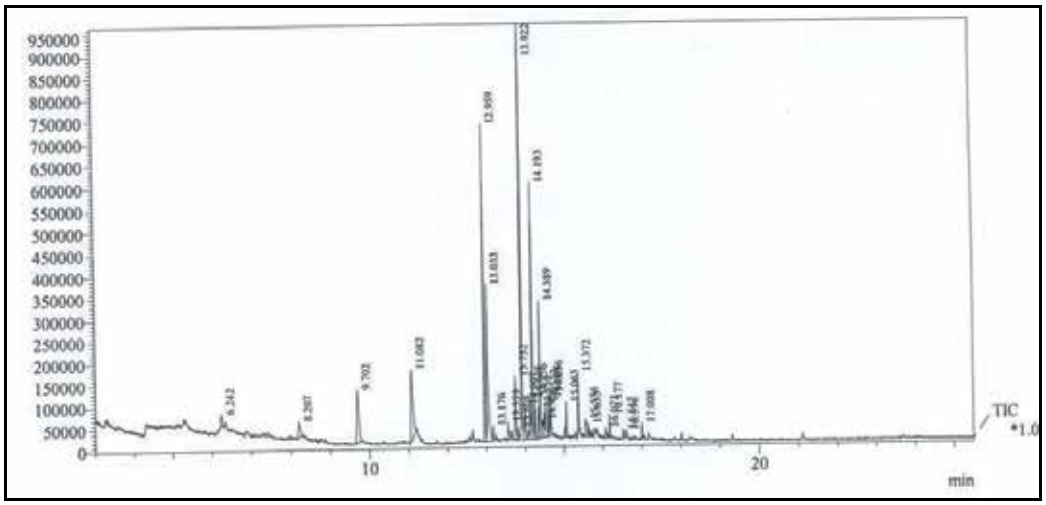

Fig. 1. Chromatogram profile for volatile compounds extracted from non-treated kesum roots extract using HP-5MS non-polar column (30m length x 0.25mm diameter x 0.25µM thickness). Injection temperature: 220ºC; Detection temperature: 280 ºC; Column temperature: 50 ºC, 3 min; 20ºC/min - 100ºC, 3 min; 30ºC/min - 250ºC, 3 min. Flow rate: 1.3ml/min. Injection volume: 1µl. Injection method: split ratio. Type of detector: quadrupole. Mass spectrometry: scan mode. TIC: Total ion chromatogram.

Of 30 compounds detected, only 16 compounds were found to have SI value more than 80. These compounds were shown in Table 2 according to their own RT. The dominant compounds found in kesum roots extract are β-caryophyllene (17.57%), acetic acid (11.07%), α-caryophyllene (9.50%), octadecanal (3.08%), β-farnesene (2.84%), phenol (2.73%) and trans-α-bergamotene (2.13%). The volatile extracts consisted of 32.85% sesquiterpenes (β-caryophyllene, trans-α-bergamotene, β-farnesene, α-caryophyllene and α-panasinsene) and 5.58% alkanes (nonane, heptanes, octane and undecane). Only one aliphatic aldehyde was detected in kesum root extracts, which is known as octadecanal (3.07%).

2.3.2 The effect of JA elicitation on the production of volatile compounds in *P. minus* roots

We were looking into two factors that will affect JA elicitation on kesum, which is the JA concentration and treatment period. Four concentrations of JA (50µM, 100µM, 150µM and 200µM) and three treatment period (1, 3 and 6 days) were tested to ensure that the concentration of JA is not too high or the treatment period is not too long to cause plant death. This is because JA was proven to be the negative regulator for plant growth and development by creating a stress environmental condition to plants (Gadzovska et al. 2007). The optimum JA concentration and treatment period need to be carefully determined to ensure that the volatile compounds could be altered to the maximum level of production compared to control sample. Only the combination of JA concentration and treatment

period that could alter and increase the production of volatile compounds significantly, which is more than 2-fold compared to control will be chosen for subsequent subtractive screening. This step was to ensure that the differentially expressed genes are the genes coded for enzymes involved in volatile compounds induced by JA. We divided the compounds induced by JA into three categories. First group represents the compounds that increase in kesum roots treated with JA (nonane, heptane, β-caryophyllene, trans-α-bergamotene, β-farnesene, α-caryophyllene and pentanoic acid). Second group represents compounds that decrease or not detected in kesum roots after JA treatment (p-benzoquinone, phenol, α-panasinsen, octane, undecane and 1,2-benzenedicarboxylic acid) whereas the third group shows compounds that have slight increment after JA elicitation (octadecanal). The chromatogram profile for JA-induced compounds was shown in Figure 2.

Retention Time	Compounds	Total Peak Area (%)
8.027	Nonane	1.65
13.599	Heptane	1.11
13.752	Octadecanal	3.08
13.922	β-caryophyllene	17.57
14.007	Trans-α-bergamotene	2.13
14.128	β-farnesene	2.84
14.193	α-caryophyllene	9.50
14.256	p-benzoquinone	1.85
14.532	Phenol	2.73
14.656	α-panasinsen	1.82
15.063	Pentanoic acid	1.47
15.556	Octane	1.42
15.633	Heptane	0.44
16.072	Undecane	0.52
16.517	1,2-benzenedicarboxylic acid	0.52
16.596	Nonane	0.44

Table 2. Volatile compounds detected in kesum root extracts.

Among the elevated compounds, β-caryophyllene was found to be the most dominant sesquiterpene compound in kesum roots and it presents consistently in every experiment replicate. It has also been found in many other plant species such as *Elsholtzia argyi* flower (Peng & Yang 2004), *Salvia officinalis* flower (Dewick 2001), carrot seed oil (Ozcan & Chalchat 2007) and *Artemisia absinthium* essential oil (Judpentiene & Mockute 2004). This compound is the major ingredient for plant aroma (Peng & Yang 2004) and it is always used in the perfumery and aromatherapy industry (Dewick 2001). Therefore it was chosen as a marker compounds for further quantitative analysis where JA concentration and treatment period could be determined. For this purpose, pure β-caryophyllene was purchased from Sigma Ltd. as an external standard and diluted to 100ppm to obtain the standard peak area. The number of β-caryophyllene analytes from the JA-treated root samples was calculated by comparing their respective peak area with the standard peak area. All data collected were performed with ANOVA analysis. The increment of β-caryophyllene analytes was considered significant at the significant level of $p \leq 0.05$. Figure 3 below shows the effect of

JA elicitation on the production of β-caryophyllene analytes in both JA-treated and non-treated kesum roots.

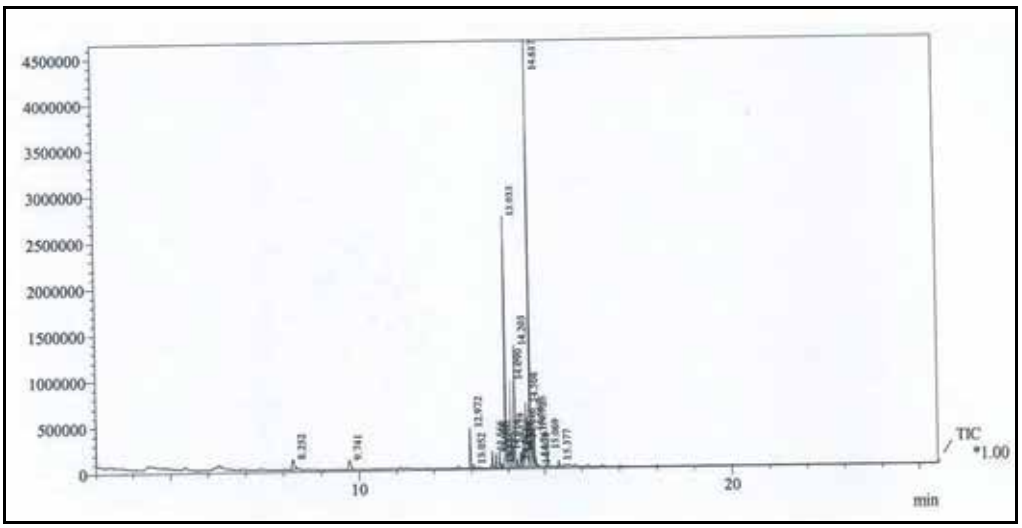

Fig. 2. Chromatogram profile for volatile compounds extracted from 150µM JA-treated kesum roots extract using HP-5MS non-polar column (30m length x 0.25mm diameter x 0.25µM thickness). Injection temperature: 220°C; Detection temperature: 280 °C; Column temperature: 50 °C, 3 min; 20°C/min - 100°C, 3 min; 30°C/min - 250°C, 3 min. Flow rate: 1.3ml/min. Injection volume: 1µl. Injection method: split ratio. Type of detector: quadrupole. Mass spectrometry: scan mode. TIC: Total ion chromatogram.

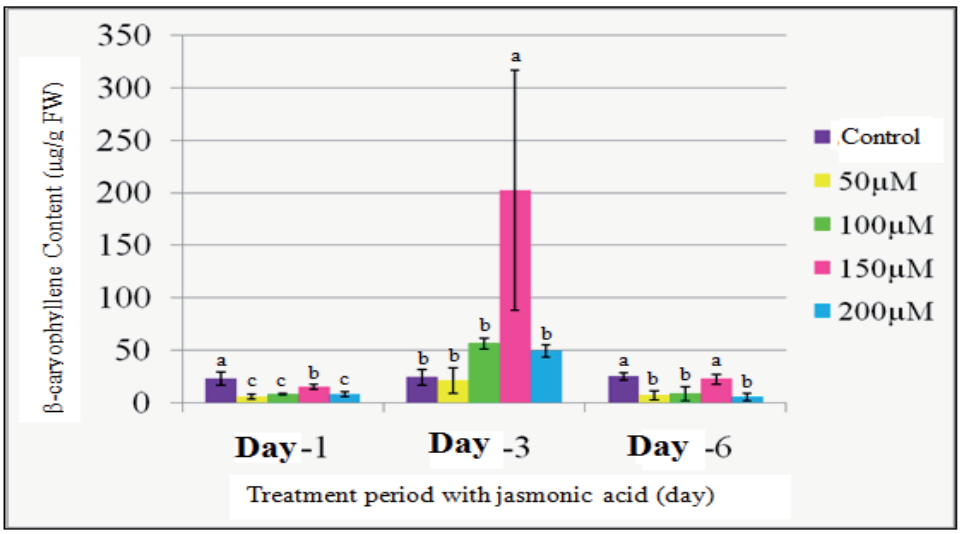

Fig. 3. The effect of JA treatment on β-caryophyllene in kesum roots. The values with different alphabet (a-c) are different significantly ($p \leq 0.05$). Data represent mean for triplicate with standard deviation.

Day-1 observation indicate that the production of β-caryophyllene was occur at a much lower level and inconsistent because the standard deviation between replicates differ greatly (coefficient of variance = 27.86). The overall production of this sesquiterpene compound at all JA concentration was lower than control. The ANOVA analysis showed that no significant increase ($p > 0.5$) was observed in the production of β-caryophyllene in kesum roots treated with all four JA concentrations compared to control. This might be because of the kesum plantlets might need time to adapt to the stressful environment. The development of kesum root cells might be retarded after treated with JA which would suppress the primary metabolism and subsequently activated the secondary metabolism in root cells (Chen & Chen 2000; Wang et al. 2001). The level of production in day-3 was much higher and could be found in all three replicates for each JA concentration tested. The production of β-caryophyllene increased after treated with JA from 50μM to 150μM but decrease again at 200μM. Significant increase ($p \leq 0.05$) was seen when *P. minus* was treated with 150μM JA, which is 203.45 + 114.79μg/g compared to 25.59 + 8.96μg/g in control roots (coefficience of variance = 73.28). The production of this sesquiterpene compound increase about 1.2-fold, 2.1-fold and 8 fold in the roots treated with 50μM, 100μM and 150μM JA respectively compared to control sample when kesum plantlets established defense mechanism against abiotic stress created by JA elicitation. However, exposure of kesum to higher concentration of JA inhibited the production of β-caryophyllene where a decrease of 1.4-fold of this metabolite was observed in 200μM JA treatment. Necrosis was observed when the leaves and roots of kesum turned brownish as JA is an inhibitor to roots growth (Wasternack 2007). In day-6, β-caryophyllene present consistently in two or more replicates in every treatment. However, ANOVA analysis showed that the level of production decrease significantly ($p > 0.05$) compared to day-3 in all four treatments (coefficience of variance = 34.21). This situation might be caused by over exposure of kesum plantlets to JA stress. Our observation is similar to a study done by Sanchez-Sampedro et al. (2005) where they found that the production of silymarin in *Silybum marianum* increased to the maximum level after 3 days of MeJA treatment but decrease significantly after 7 days. Therefore, we concluded that kesum roots treated with 150μM JA for 3 days could produce β-caryophyllene at maximum level and we assume that the biosynthesis of other metabolites such as alkanes, aldehydes, alcohols and acids could also be enhance. Hence the RNA extracted from this treatment was subtracted against the non-treated kesum roots RNA to identify and clone the transcripts regulated by JA stress.

3. Differentially expressed genes induced by JA

3.1 Identification of JA-responsive genes

Subtractive screening is an efficient approach to clone the genes which are being expressed in one population but not being expressed or slightly expressed in another population. In this study, cDNA derived from kesum roots treated with 150μM JA for 3 days was served as tester whereas the cDNA derived from non-treated kesum roots was served as driver for subtracted cDNA library construction. Only forward subtraction was done as we were interested in identifying genes up-regulated by JA. A total of 1,344 white colonies were randomly picked from the subtracted cDNA library and screened by PCR using M13 forward and reverse universal primers to confirm the presence of cDNA inserts. PCR amplification revealed that 960 colonies were single stranded clones with the insert sizes ranging from 250bp to 1,200bp. These clones were subsequently hybridized against

unsubtracted tester cDNA and unsubtracted driver cDNA by Reverse Northern hybridization to reduce false positives. Our results showed that of these 960 clones, 195 clones hybridized strongly, 213 clones hybridized moderately, while 552 clones hybridized weakly with the unsubtracted tester cDNA whereas almost all of the clones showed weak or no signal when compared with the unsubtracted driver probe (Figure 4).

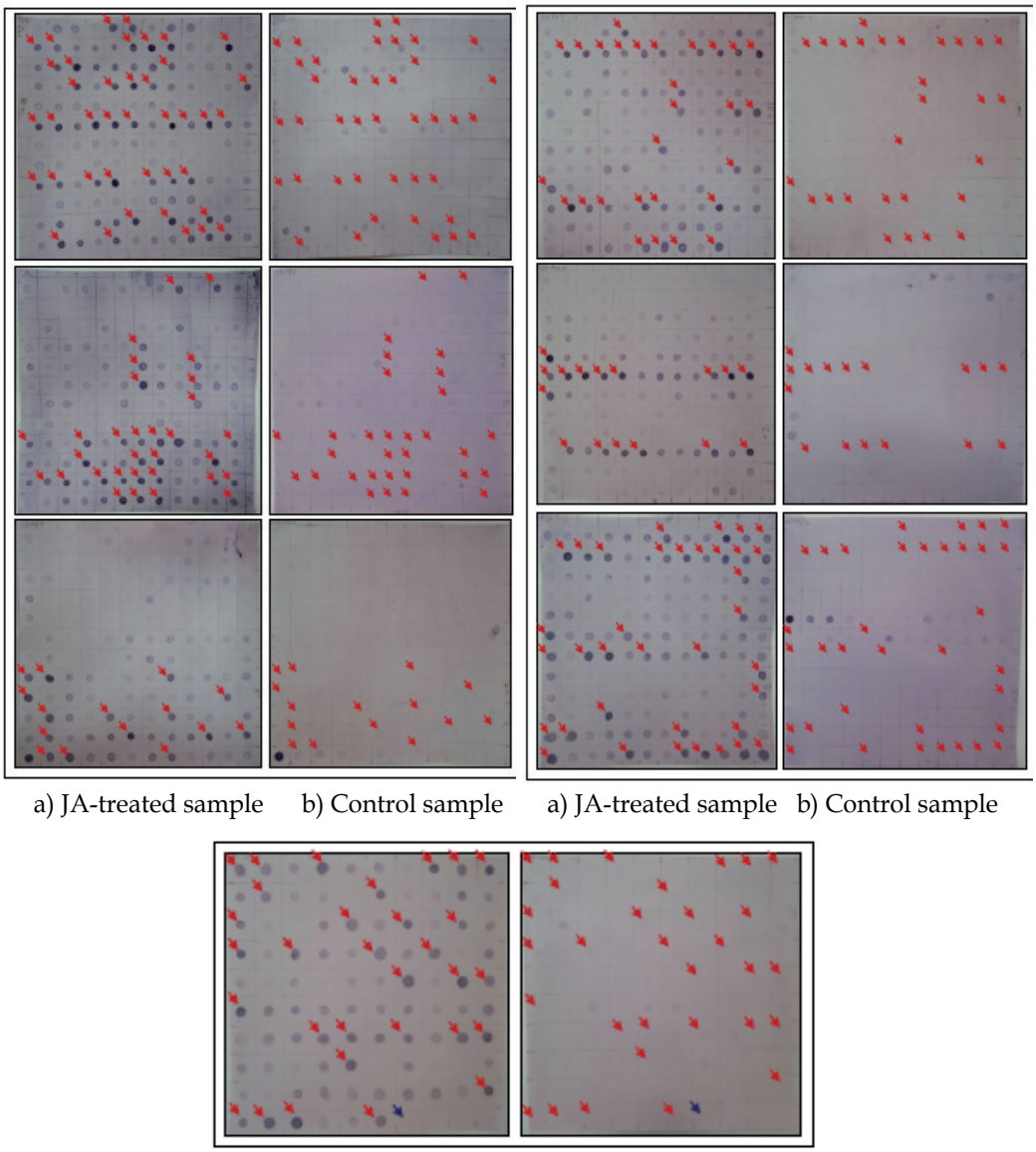

Fig. 4. Reverse Northern analysis showing differential screening for putative cDNA clones altered by JA. (a) PCR products hybridized with DIG-labelled tester cDNA. (b) PCR products hybridized with DIG-labelled driver cDNA. Clones showing significant differential expression were pointed with red arrows. Negative control was pointed with blue arrow.

190 strongly hybridized clones were picked from Reverse Northern results and sent for sequencing. Of these, 174 clones were readable sequence in which 130 clones were unigenes that showed significant similarity to cDNA sequences in the NCBI database (E-value $\leq 10^{-5}$), 18 clones did not show any similarity to any known sequences and 26 clones had no significant results (Table 3). All of the 130 unigenes were deposited into NCBI and could be found in dbEST with accession numbers starting from GR505448 to GR505519. These clones were then classified into 11 categories according to their putative molecular functions (Figure 5). The largest set of genes was assigned to the stress-related genes (25%). This was followed by the following groups: other metabolism (20%), unknown genes (9%), transcription factors (8%), amino acid metabolism (6%), signal transduction and kinase (5%), carbohydrate metabolism (2%), transporter (2%), energy (2%), and regulation of gene expression (2%). Clones that showed similarity to the cDNA sequences from other plant species, but did not have any specific function, were assigned to the 'other' category (19%). The functional categories showed that these cDNAs might be involved in different biological processes. Clones that showed no similarity to any sequences in the GenBank were classified as hypothetical proteins and their putative structure and functions were predicted using bioinformatics software, which will be discussed later in this chapter. These clones may represent unique genes that were transcribed in response to the JA treatment that are involved in the metabolic pathway elicited by JA. We focused further analysis on clones that represent genes involved in biosynthesis of aromatic compounds in kesum, either under natural conditions or JA stress.

The largest group (25%) was assigned to stress-related genes. Exogenous JA application has been known as a stress treatment to plants and served as a stimulus to activate the expression of genes involved in the synthesis of plant secondary metabolites. As expected, a large number of clones encoded for stress-related genes were identified upon JA elicitation in this study. The complexity of kesum roots response towards JA elicitation suggested that many genes involved in plant defence mechanism against stress. A few clones were found to have homology with abiotic stress related genes as a defence response of kesum root cells towards JA treatment, including glutathione S-transferase from *Glycine max* (GR505459) and putative glutathione S-transferase T1 from *Lycopersicon esculentum* (GR505461), heat shock protein (GR505458), anionic peroxidise H from *Zea mays* (GR505463) and peroxidise 1 from *Scutellaria baicalensis* (GR505464), ELI3-1 (GR505453) and auxin induced protein (GR505454). Generally plant response towards pathogen or herbivore attack would activate a series of mechanism, including synthesizing anthocyanin and oligolignol, pathogenesis proteins (PR), generation of reactive oxygen species and formation of plant cell walls (Pauwels et al. 2009). The increase of gluthathione S-transferase (GST) was associated with hormone homeostasis or anthocyanin isolation in vacuole because GST played a role as auxin, cytokinin and anthocyanin transporter. When kesum roots were exposed to JA, excessive anthocyanin will be synthesized. The equilibrium of hormone in kesum root cells could be achieved by transporting the excessive anthocyanin into vacuole to be removed. This step was catalyzed by glutathione S-transferase enzyme (Moons 2003). On the other hand, peroxidase transcripts could be linked to generation of reactive oxygen species such as H_2O_2 and other metabolites like phenylpropanoids (Thimmaraju et al. 2006). Therefore we predicted that the peroxidase induced by JA was responsible for volatile compounds production such as sesquiterpenes in kesum roots. ELI3-1 gene is a type of elicitor activated gene and it responds to a wide range of elicitors (Ellard-Ivey & Douglas 1996). In this study, ELI3-1 was induced by JA, as well as heat shock protein which functions as a defence

Gene Bank Accession	Size (bp)	Similarity	Organism	Number of clones	E-Value
Transcription factor					
GR505449	265	F-box containing protein	*Populus tremula*	1	$3e^{-12}$
GR505460	595	Kelch repeat-containing F-box family protein	*Arabidopsis thaliana*	1	$1e^{-22}$
GR505470	423	GAMYB-binding protein (gbp5)	*Hordeum vulgare*	1	$2e^{-25}$
GR505481	298	ERF-like transcription factor	*Coffea canephora*	1	$2e^{-09}$
GR505492	583	BURP domain containing protein	*Solanum tuberosum*	2	$9e^{-05}$
GR505503	583	BURP domain containing protein	*Phaseolus vulgaris*	4	$8e^{-06}$
Signal transduction & kinase					
GR505514	285	Protein kinase	*Malus domestica*	2	$1e^{-55}$
GR505518	563	Multicopy supressor IRA1 (MSI1)	*Arabidopsis thaliana*	1	$3e^{-48}$
GR505519	712	Calmodulin-binding protein	*Arabidopsis thaliana*	2	$7e^{-116}$
Stress-related					
GR505451	666	MeJA-elicited root cell suspension culture	*Medicago trunculata*	1	$2e^{-18}$
GR505452	539	MeJA-elicited hairy roots culture	*Panax ginseng*	4	$3e^{-22}$
GR505453	503	ELI3-1 (ELICITOR ACTIVATED GENE 3)	*Arabidopsis thaliana*	1	$2e^{-29}$
GR505454	589	Auxin-induced protein	*Nicotina tabacum*	7	$6e^{-13}$
GR505455	269	EST from mild drought-stressed leaves	*Populus tremula*	1	$5e^{-16}$
GR505456	267	cDNA clone from senescing leaves	*Populus tremula*	1	$3e^{-05}$
GR505457	406	Cell cultures in osmotic stress	*Bouteloua gracilis*	3	0.0
GR505458	669	Gene for heat-shock protein	*Glycine max*	2	$3e^{-17}$
GR505459	656	Glutathione S-transferase (GST14)	*Glycine max*	7	$2e^{-21}$
GR505461	570	Putative glutathione S-transferase T1	*Lycopersicon esculentum*	3	$3e^{-10}$
GR505462	716	cDNA clones from water stress seedlings	*Zea mays*	1	$2e^{-07}$
GR505463	747	Anionic peroxidase H	*Zea mays*	1	$1e^{-09}$
GR505464	546	Peroxidase 1	*Scutellaria baicalensis*	1	$3e^{-48}$
Carbohydrate metabolism					
GR505465	684	Alcohol dehydrogenase	*Prunus armeniaca*	1	$3e^{-68}$
GR505448	603	Alcohol dehydrogenase 1 (adh1)	*Zea mays*	1	$1e^{-75}$
GR505466	436	β-fructofuranosidase	*Arabidopsis thaliana*	1	$2e^{-14}$

Acid amino metabolism					
GR505467	457	S-adenosyl-L-methionine synthetase	*Beta vulgaris*	3	$1e^{-135}$
GR505468	446	S-adenosyl-L-methionine synthetase	*Actinidia chinensis*	3	$2e^{-29}$
GR505469	443	S-adenosyl-L-methionine synthetase	*Elaeagnus umbrellata*	1	$6e^{-18}$
GR505471	741	S-adenosyl-L-homocystein hydrolase	*Mesembrayanthemum crystallinum*	1	0.0
Other metabolism					
GR505473	313	Glyoxal oxidase-related mRNA	*Arabidopsis thaliana*	2	$5e^{-06}$
GR505474	328	Dihydrolipoamide dehydrogenase 1	*Arabidopsis thaliana*	1	$3e^{-20}$
GR505475	460	Nodulin-35 (N-35) gene encoding a subunit of uricase II	*Glycine max*	1	$3e^{-09}$
GR505476	742	Nodulin family protein (NLP	*Gossypium hirsutum*	1	$4e^{-49}$
GR505477	520	Glycosyltransferase family protein 47	*Arabidopsis thaliana*	1	$2e^{-05}$
GR505478	524	Cytochrome oxidase subunit 1 (COI) gene	*Persicaria maculosa*	4	0.0
GR505479	391	Cytochrome oxidase subunit 1 (COI)	*Plumbago* sp.	3	$6e^{-163}$
GR505480	557	Cytochrome c oxidase	*Gossypium barbadense*	3	0.0
GR505482	401	NADH dehydrogenase	*Beta vulgaris*	7	0.0
GR505483	344	Urate oxidase	*Vitis vinifera*	1	$1e^{-20}$
GR505484	748	Glucan-endo-1,3-beta-glucosidase	*Nicotina tabacum*	1	$5e^{-29}$
GR505472	952	Lipoxigenase (lox gene)	*Capsicum annuum*	1	$7e^{-47}$
Energy					
GR505485	430	Type-AAA ATPase family protein	*Arabidopsis thaliana*	1	$1e^{-41}$
GR505486	393	Glyseraldehyde-3-phosphate dehydrogenase	*Zea mays*	2	$3e^{-11}$
Transporter					
GR505487	399	Root-specific metal transporter	*Lycopersicon esculentum*	1	$5e^{-12}$
GR505488	291	Auxin efflux carrier protein	*Zea mays*	2	$1e^{-11}$
Regulation of gene expression					
GR505489	377	Ribosomal protein S12	*Fagopyrum esculentum*	1	$8e^{-162}$
GR505490	675	18S rRNA gene	*Polygonum* sp. Soltis	2	0.0

Table 3. Putative JA-induced cDNA sequences in *P. minus* roots.

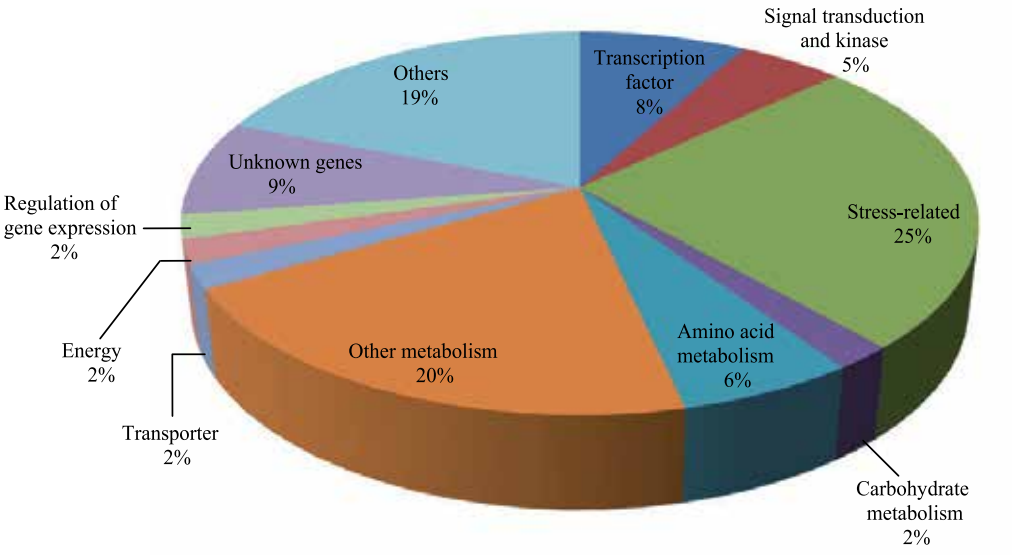

Fig. 5. Classification of clones based on their putative molecular functions.

response towards environmental stress. Besides, JA is known to be a phytohormone that regulates many plant physiological processes and it could interact with other hormone such as salicylic acid, absisic acid, auxin and giberellin in controlling plant growth and development (Creelman & Mulpuri 2002; Pauwels et al. 2009). Apart from that, some clones also showed homology to cDNA sequences in hairy roots of *Medicago trunculata* (GR505451) and *Panax ginseng* (GR505452) treated with MeJA. Other clones that showed homology to cDNA sequences of plants grown under drought stress (GR505455), osmotic stress (GR505457), water stress (GR505462) and leaves senescence (GR505456) were also identified. This result implies that many different stress factors will lead to the same gene expression (Sandermann et al. 1998). This result also suggests that JA, or its derivative MeJA, are signal molecules that regulate kesum defense responses in response to stressful environments, resulting in the activation of defense-related genes in plants.

The next group of transcripts which showed putative functions in plant growth and development were categorised under other metabolism (20%). Any changes in the primary metabolism will lead to plant defence response to stress (Ingram 7 Bartels 1996). Transcripts that were induced by JA in kesum roots include glyoxal oxidase (GR505473), dihydrolipoamide dehydrogenase (GR505474), Nodulin-35 gene (GR505475), nodulin family protein (NLP) (GR505476), cytochrome oxidase subunit I from *Persicaria maculosa* (GR505478) and *Plumbago sp.* (GR505479), cytochrome c oxidase from *Gossypium barbadense* (GR505480), urate oxidase (GR505483), glucan-endo-1,3-beta-glucoxidase (GR505484), glycosyltransferase family protein 47 (GR505477) and NADH dehydrogenase (GR505482). It was predicted that the Nodulin-35 and nodulin family protein were being expressed to ensure the normal development of root nodules as JA might inhibit roots elongation (Gadzovska et al. 2007). Glucan-endo-1,3-beta-glucosidase and glycosyltransferase family protein 47 were also being expressed to form kesum root cell walls. Besides, it was predicted that the generation of reactive oxygen species or secondary metabolites had affected the respiration process in kesum root cells and thus leading to the increase of cytochrome

oxidase transcripts. JA elicitation was also believed to induce the expression of urate oxidase and activates the production of H_2O_2 that resulted in hypersensitive cell death. The functions of glyoxal oxidase and dihydrolipoamide dehydrogenase in kesum roots under JA stress were yet to be discovered. Another unigene that encoded for lipoxygenase, the first enzyme in the oxylipin pathway for JA biosynthesis (Devitt et al. 2006) was also found in this study. It has been identified as the enzyme that involved in the production pathways of volatile compounds as the indirect plant defensive response to herbivory (Kessler and Baldwin 2001). Thus, it is believed that the lipoxygenase expression was associated with other volatile compounds detected by GC-MS, such as the alkanes, aldehydes and alcohols. This result suggests that there is a crosstalk between abiotic stress triggered by JA and other herbivory biotic stress.

Another group of cDNA sequences were associated with transcription factor (8%). For example, F-box containing TIR1 protein (GR505449), Kelch-repeat containing F-box family protein (GR505460), GAMYB-binding protein (gbp5) (GR505470), ERF-like transcription factor (GR505481) and BURP domain containing protein from *Solanum tuberosum* (GR505492) and *Phaseolus vulgaris* (GR505503). The F-box containing TIR1 protein (Parry & Estelle 2006) and Kelch-repeat containing F-box protein have been proven to be activated by JA in plant cells. Naturally F-box containing TIR 1 protein is a receptor to auxin. In plants, auxin is activated by auxin responsive factor (ARF) but inhibited by Aux/IAA protein. It was predicted that the expression of F-box containing TIR1 transcripts could activate the degradation of Aux/IAA protein so that auxin could be synthesized to equilibrate the hormones content in kesum roots. The Kelch-repeat containing F-box family protein was thought to be interacted with other proteins which involved in protein degradation process through ubiquitin-dependent pathway. Protein degradation is an important process in regulating cell cycle, transcription and signal transduction as a defence mechanism in kesum (Sun et al. 2007). The GAMYB-binding protein, BURP domain and ERF-like transcription factor induced by JA in this study were believed to be elements that regulate JA signalling in kesum roots.

Genes that were categorised into amino acid metabolism (6%) include cDNA clones coded for enzymes involved in phenylpropoanoids biosynthesis pathway, namely S-adenosyl-L-methionine sinthase from *Beta vulgaris* (GR505467), *Actinidia chinensis* (GR505468) and *Elaeagnus umbrellata* (GR505469), and S-adenosyl-L-homocystein hydrolase (GR505471) (Dewick 2001). The results of this study suggested that S-adenosyl methionine synthase and S-adenosyl homocystein hydrolase induced by JA could activate the production of aromatic compounds in kesum roots using aromatic amino acids as precursor. Carbohydrate metabolism (2%) or carbon-containing compounds covered alcohol dehydrogenase gene from *Prunus armeniaca* (GR505568), alcohol dehydrogenase 1 from *Zea mays* (GR505568) and β-fructofuranosidase (GR505466). The up-regulation of transcription of alcohol dehydrogenase may be associated with the biosynthesis of phenylpropenes, another important group of volatiles (Devitt et al. 2006). This result suggests that the genes involved in the biosynthesis of secondary metabolites can be induced by JA elicitation in kesum roots.

Genes categorized under signal transduction and kinase (5%) include kinase protein (GR505514), Multicopy suppressor IRA1 (MSI1) (GR505518) and calmodulin-binding protein (CaMBP) (GR505519). Kinase protein could modify other proteins or enzymes through phosphorylation of serine, threonine or tyrosine residues (Zheng et al. 2004). The kinase proteins found in this study might function in phosphotylation protein as a response

towards JA elicitation. Another plant response against stress is the increased of free calcium in cytosol as Ca^{2+} ion plays an important role in signal transduction that activated plant defence genes (Reddy et al. 2008). Ca2+ ion could activate calmodulin protein (CaM). The interaction between Ca2+/CaM and target molecules could lead to plant response towards environmental stress by activation of calmodulin-binding protein (CaMBPs) (Zielinski 1998). Besides, MSI1 has also been proven to function in signal transduction mechanism (Zheng et al. 2004). Therefore, the increase expression of these transcripts could possibly be linked to JA-induced signal transduction pathway.

AAA-type ATPase family protein mRNA (GR505485) and glyceraldehydes-3-phosphate dehydrogenase (GR505486) were categorized under energy group (2%). The increment of these transcripts was mainly due to energy consumption in regulation of plant metabolisms under JA stress. Root-specific metal transporter (GR505487) and auxin efflux carrier protein (GR505488) were classified into transporter group (2%). The discovery of root-specific metal transporter in JA-treated kesum roots may suggest that *P. minus* could be a suitable plant for phytoremediation of metal contamination in soil and further investigation need to be done by focusing to this aspect. The expression of auxin efflux carrier protein could be linked to auxin induced protein and F-box protein and it was predicted that the interaction of these three components could stabilize JA content in kesum roots by auxin synthesis. Next, S12 ribosomal protein (GR505489) and rRNA 18S gene (GR505490) were classified into regulation of gene expression group (2%). A few cDNA clones which represent ribosomal proteins such as 60S, 40S and 30S were known to be gene sequences respond towards stress. These ribosomal proteins play important roles in *de novo* protein synthesis (Machida et al. 2008).

Clones which have no significant similarity with any sequences in the databases were categorized as unknown genes (9%) and into others group (19%) that include cDNA clones that have no similarity with any nucleotide, mRNA or EST sequences in NCBI database (GR505498, GR505499, GR505500, GR505501, GR505502, GR505504, GR505505, GR505506, GR505507, GR505508, GR505509, GR505510, GR505511, GR505512, GR505513, GR505515, GR505516, GR505517), These sequences could be considered as novel genes induced by JA in kesum roots and further characterization must be done to identify their function in plant stress response, JA signalling and secondary metabolites production. The relationship between these clones and JA elicitation is yet to be identified and bioinformatics analysis has been carried out to investigate possible classification of these unknown sequences.

3.2 Discovery of unknown and novel cDNA sequences discovered during JA elicitation

A substantial fraction of the genes in the EST dataset encode for unknown proteins (also termed as hypothetical proteins) and about half the proteins in most genomes are candidates for hypothetical proteins (Minion et al. 2004). Hypothetical proteins are proteins that are predicted from nucleic acid sequences but have no corresponding experimental protein (Lubec et al. 2005). They are characterized by low identity to known annotated proteins. Thus, their functions remain unknown, and they pose a challenge to functional genomics and biology in general (Galperin 2001). Hypothetical proteins are utmost importance to complete genomic and proteomic information. Detailed knowledge on hypothetical proteins offers presentation of new structures and functions, contributing to the rising of new domains and motifs, revelation on a series of additional pathways hence completing fragmentary knowledge on the mosaic of proteins intrinsically.

The comparison of DNA or protein sequences from various organisms using computational methods is a powerful tool in protein study. By finding similarities between sequences, functional inference of newly sequenced genes can be achieved, new members of gene families can be predicted and evolutionary relationship can be explored. Computational analysis can quickly analyze and assign hypothetical proteins and able to generally predict their tentative biochemical functions (Lubec et al. 2005, Galperin 2001, Hoskeri 2010). Fundamentally, the prediction of functional inference is achievable by standard homology-based gene annotation complemented by genomic-context approaches (von Mering et al. 2003, Mellor et al. 2002, Marcotte et al. 1999) and, in some cases, requires structural intervention (Kolker et al. 2004). The combination of these approaches is intuitive and usually applies to various circumstances. Even though predictions can sometimes reliably infer the function of hypothetical proteins (Aravind and Koonin 1999), predictions do not provide necessary information regarding the exact biochemical function of a protein. Thus, predictions must still be validated through wet-lab experiments. However, computational analysis provides a faster and cheaper alternative to wet-lab experiments. Here, we cover the computational predictions of a set of hypothetical proteins obtained from the substracted cDNA library of a *P. minus* root that was treated with jasmonic acid (Gor et al. 2010).

3.2.1 Computational analysis of unknown genes

The unknown protein dataset discovered from a subtracted cDNA library of *P. minus* roots elicited with jasmonic acid were first translated to protein sequences for detailed bioinformatic analysis. The sequences were then examined for the existence of signal peptides using a signal peptide prediction tool. Knowledge of the existence of a signal peptide in a protein sequence is essential to defining and characterizing the protein. If there is a detectable signal peptide in a sequence, the signal peptide region must be cut off before the sequence can be used for further bioinformatics analysis. The sequences were compared to the databases of non-redundant proteins to detect any homologous sequences. The sequences with no significant outputs from the similarity search were further analyzed using preliminary structure-prediction analysis to identify a possible fold category. This information provided useful insights into the functional inference of these sequences. The analysis was performed using an in-house analysis portal called the Hypothetical Protein Analysis System (HPAS), which provided a systematic functional annotation procedure. The HPAS consisted of various tools for signal peptide prediction (SignalP 3.0) (Bendtsen et al. 2004), analysis of physicochemical properties (ProtParam (Gasteiger et al. 2003) and ProtScale (Yu et al. 2010)), topology analysis (Psortb (Bagos et al. 2008), SOSUI (Hirokawa et al. 1998), HMMTOP (Tusnady and Simon 2001), SignalP (Bendtsen et al. 2004), LipoP (Rahman et al. 2008)) and similarity search and annotation (NPSA@BLASTP (Altschul et al. 1990), NPSA@PSI-BLAST (Altschul et al. 1997), MPSrch (Agarwal et al. 1998), SSEARCH(Mazumder et al. 2008) and InterProScan (Zdobnov et al. 2001)). The HPAS covered all of the possible aspects of a protein sequence and, through a series of analytical tools, used all of the protein's characteristics to determine the protein's predicted functions.

3.2.2 Protein characterization by physicochemical properties

ProtParam was used to compute the physicochemical properties of these hypothetical proteins. Here, a few selected physicochemical properties were highlighted; molecular

weight, pI, instability index, aliphatic index and GRAVY (grand average of hydropathy). A GRAVY index greater than zero indicates a hydrophobic protein (Kyte and Doolittle 1982). Notably, only one sequence in this dataset (GR505502) had a GRAVY value (0.528) greater than zero. The other proteins were predicted to be hydrophilic. The aliphatic value refers to the relative volume occupied by aliphatic side chains (Ala, Val, Ile and Leu) and is considered to be a positive factor for increased thermal stability of globular proteins (Ikai 1980). Both GR505495 and GR505502 had the highest aliphatic indices (115.98 and 111.2, respectively). The stability index provides an estimate of the stability of a protein *in vitro*. An instability index higher than 40 indicates an unstable protein. Our results showed that five sequences were predicted to be unstable (GR505491, GR505497, GR505498, GR505499 and GR505502). Different protein localizations usually imply different biological functions. The prediction of subcellular localization is relevant to inferring possible functions, annotating genomes, designing proteomics experiments and characterizing pharmacological targets (Lubec et al. 2005). The prediction of the protein type from its primary sequence or the determination of whether an uncharacterized protein is a membrane protein is important in both bioinformatics and proteomics. For this purpose, a few programs were used (Psortb (Bagos et al. 2008), SOSUI (Hirokawa et al. 1998), HMMTOP (Tusnady and Simon 2001), SignalP (Bendtsen et al. 2004), LipoP (Rahman et al. 2008)) to predict the subcellular localizations of the hypothetical proteins. Two sequences were predicted to be membrane proteins (GR505495 and GR505450, with two and one transmembrane helices, respectively). A polypeptide can be a membrane protein if it contains at least one transmembrane helix. HMMTOP predicted transmembrane regions for both sequences, at residues 106-130 and 137-159 for GR505495 and residues 39-62 for GR505450. Table 4 shows the physicochemical analysis of the *P. minus* Huds hypothetical proteins achieved using various tools from HPAS and public databases. The consensus results were significant and were selected for further analysis (namely, the molecular responses of *P. minus* Huds roots to jasmonic acid induction).

3.2.3 Similarity search

Four programs are consecutively used for a similarity search analysis. Table 5 provides all results from the analysis. In the first round, BLAST was used to find sequences that were similar to the hypothetical proteins. If BLAST did not find any significant hits for the hypothetical sequences, then Psi-BLAST was used. MPSrch and SSearch were then used for the sequences that had no significant matches from the previous program. BLAST was able to reveal similarities to BURP-domain-containing protein 3 for GR505494, GR505505, GR505506, GR505507, GR505508, GR505509, GR505510, GR505512, GR505515 and GR505517. The sequence motif of the BURP-domain-containing protein family has been described previously (Hattori et al. 1998), and many plant species (but not other organisms) that contain this domain have been identified. The BURP-domain-containing protein consists of several modules, such as an N-terminal hydrophobic transit peptide, a short conserved segment, an optional segment consisting of repeating units that are unique to each protein and the BURP domain at the C-terminus. The BURP-domain-containing protein consists of four typical members, BNM2, USP, RD22 and PG1β. Thus far, this domain has been found only in plants, suggesting that its function may be plant-specific. The BURP-domain-containing protein family has been found in various plant species, but their specific functions are still being explored. Based on their existence in various plants at various stages and in various locations, many BURP family members are involved in maintaining normal plant metabolism and development. For example, in the oilseed rape (*Brassica napes* L.),

BNM2 is induced at the beginning of microspore embryogenesis, whereas the corresponding protein remains confined to the seed, where it is localized in the protein storage vacuoles (Zheng et al. 1992, Boutilier et al. 1994. Teerawanichpan et al. 2009, Treacy et 1997). USP from *Vicia faba* L., known as an abundant non-storage seed protein, is expressed during the early stage of zygotic embryogenesis (Bassuner et al. 1998) and at the very beginning of in vitro embryogenesis (21). However, PG1β is a non-catalytic β-subunit of the polygalacturonase isozyme from the ripening tomato and plays an important role in regulating pectin metabolism (Zheng et al. 1992, Watson et al. 1994). In contrast, RD22 is a drought-induced protein in Arabidopsis thaliana and is often used as a reference for drought-stress treatment in different plants (Yamaguchi-Shinozaki et al. 1993). To date, this is the first report of the prediction of the BURP-domain-containing protein from the hypothetical proteins of P. minus Huds.

Psi-BLAST was then used to identify remote homologs, especially for the sequences with no significant hits from the BLAST search output. A detailed sequence analysis of these sequences allowed us to provide a tentative characterization of GR505450. A distant homolog was identified as an elongation factor 1-gamma from *Danio rerio* (zebrafish) with an e-value of 2e-30. Unlike the BLAST search, Psi-BLAST identified the homolog for the hypothetical proteins GR505494, GR505505, GR505506, GR505507, GR505508, GR505509, GR505512, GR505515 and GR505517 as BURP-domain-containing protein 17 (B9G9L9). The homolog of GR505510 was BURP-domain-containing protein 5 (Q0JEP3), and the homolog of GR505511 was BURP-domain-containing protein 3 (Q942D4). Notably, for GR505511, the BLAST search identified a similarity to dehydration-responsive protein RD22 (Q08298). In this case, the BLAST output was favorable because the percentage of sequence identity between GR505511 and Q08298 is 39%. In general, Psi-BLAST was able to successfully identify distant homologs from the same protein family group and, hence, corroborate the output of the BLAST search.

Furthermore, there were a few more sequences (GR505491, GR505493, GR505495, GR505496, GR505497, GR505498, GR505499, GR505500, GR505502, GR50513 and GR505516) that had no significant hits from either the BLAST or Psi-BLAST runs. These sequences were then analyzed with two different similarity software packages (MPSrch and SSearch). The significance of the MPSrch results depends on the score value (i.e., a higher score implies more significant results). However, for the SSearch results, both the e-value and the SW score play important roles in choosing a significant hit. Both of these programs identified a putative uncharacterized protein from *Oryza sativa subsp. indica* (A2XQP1_ORYSI) and *Sorghum bicolor* (C5XJ38) for GR505494 and from *Oryza sativa subsp. indica* (A2XQP1_ORYSI) and *Glycine max* (C6TCE4) for GR505510. For GR505491, MPSrch identified a putative uncharacterized protein (D0ND35_PHYIN) from *Phytophthora infestans T30-4* as its homolog, while SSearch found no matches. GR505493 had no significant matches from either the BLAST or Psi-BLAST runs. Notably, MPSrch and SSearch detected N-acetylglucosaminyltransferase (Q70MW8) from *Bradyrhizobium sp. ISLU256* as similar to GR505493. N-acetylglucosaminyltransferase (EC.2.4.1.-) is a resident Golgi-enzyme that is essential for the processing of high mannose to hybrid and complex glycans (Strasser et al. 2005). For GR505500, the first two programs failed to detect any similarity matches, but MPSearch identified a match with a predicted protein (B7G4S6_PHATR) from *Phaeodactylum tricornutum CCAP 1055/1*, and SSearch identified galactoside-O-acetyltransferase (Q465V0) from *Methanosarcina barkeri*. This enzyme is usually found in bacteria, and it is interesting to experimentally investigate the existence of this protein in a plant. There were no significant

Peptide ID	ProtParam							
	Amino acid count	Molecular weight	pI value	Negative residues	Positive residues	Instability index	Aliphatic index	GRAVY
GR505450	121	14082.1	4.73	21	14	33.85 (Stable)	67.6	-0.269
GR505491	85	9656.2	6.39	7	6	69.9 (Unstable)	56.24	-0.5
GR505493	63	6679.5	9.52	4	7	23.04 (Stable)	76.19	-0.148
GR505494	201	22009.5	6.06	23	19	35.5 (Stable)	69.25	-0.277
GR505495	164	18007.6	6.98	13	13	23.11 (Stable)	115.98	0.346
GR505496	91	10096.5	5.45	13	12	36.17 (Stable)	83.41	-0.176
GR505497	151	16836.1	9.46	9	15	41.19 (Unstable)	66.56	-0.541
GR505498	43	4594	6.91	4	4	43.57 (Unstable)	43.02	-0.723
GR505499	87	9811.1	9	10	13	43.43 (Unstable)	50.46	-1.064
GR505500	164	18010.1	5.18	18	12	25.48 (Stable)	70.18	-0.367
GR505502	50	5511.6	4.46	6	4	46.33 (Unstable)	111.2	0.528
GR505505	174	19184.4	7.11	20	20	26.11 (Stable)	71.61	-0.29
GR505506	167	18241	6.89	16	16	31.5 (Stable)	73.47	-0.12
GR505507	166	18520.6	6.04	19	16	34.5 (Stable)	75.66	-0.245
GR505508	167	18383.3	5.83	20	16	32.49 (Stable)	74.01	-0.205
GR505509	159	17609.2	6.18	19	17	35.51 (Stable)	67.99	-0.348
GR505510	157	16928.4	6.83	16	16	27.45 (Stable)	69.55	-0.202
GR505511	135	14905.9	6.03	18	15	28.6 (Stable)	61.26	-0.485
GR505512	159	17609.2	6.18	19	17	35.51 (Stable)	67.99	-0.348
GR505513	126	13267.9	8.96	8	11	23.75 (Stable)	78.17	-0.06
GR505515	167	18256	7.58	16	17	33.9 (Stable)	71.14	-0.166
GR505516	127	13339	8.96	8	11	24.97 (Stable)	78.35	-0.045
GR505517	171	18937.5	7.9	18	19	26.83 (Stable)	81.4	-0.16

Table 4. Physicochemical properties of hypothetical proteins from the root of *P. minus*.

Peptide ID	BLAST	Psi-BLAST	MPSrch	SSearch
	Description	Description	Description	Description
GR505450	No significant match	Elongation factor 1-gamma; Organism = *Danio rerio* (Zebrafish)	Putative uncharacterized protein; Organism = *Glycine max* (Soybean)	Probable glutathione S-transferase; Organism = *Nicotiana tabacum* (Common tobacco)
GR505491	No significant match	No sequence selected in the PSI-BLAST model	Putative uncharacterized protein; Organism = *Phytophthora infestans T30-4*	No significant match
GR505493	No significant match	No sequence found by PSI-BLAST	N-acetylglucosaminyltransferase; Organism = *Bradyrhizobium sp. ISLU256*	N-acetylglucosaminyl transferase; Organism = *Bradyrhizobium sp. ISLU256*
GR505494	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein Sb03g033760; Organism = *Sorghum bicolor* (Sorghum)
GR505495	No significant match	No sequence selected in the PSI-BLAST model	Predicted protein; Organism = *Nematostella vectensis* (Starlet sea anemone)	Predicted protein; Organism = *Nematostella vectensis* (Starlet sea anemone)
GR505496	No significant match	No sequence selected in the PSI-BLAST model	Putative uncharacterized protein; Organism = *marine gamma proteobacterium HTCC2148*	Putative uncharacterized protein; Organism = *marine gamma proteobacterium HTCC2148*
GR505497	No significant match	No sequence found by PSI-BLAST	Putative uncharacterized protein; Score = 93; Identity = 11.4; Identifier = A8URE8_9AQUI;	Putative uncharacterized protein; E-value = 0.45; SW Score = 127; Bit Score = 38.3; Identifier =

			Organism = *Hydrogenivirga sp. 128-5-R1-1*	A5Z3S2; Organism = *Eubacterium ventriosum ATCC 27560*
GR505498	No significant match	No sequence found by PSI-BLAST	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein; Organism = *Ruminococcus gnavus ATCC 29149*
GR505499	No significant match	No sequence selected in the PSI-BLAST model	Putative uncharacterized protein; Organism = *Sphingomonas wittichii* (strain RW1 / DSM 6014 / JCM 10273)	Putative uncharacterized protein; Organism = *Sphingomonas wittichii* (strain RW1 / DSM 6014 / JCM 10273)
GR505500	No significant match	No sequence selected in the PSI-BLAST model	Predicted protein; Organism = *Phaeodactylum tricornutum CCAP 1055/1*	Galactoside-O-acetyltransferase; Organism = *Methanosarcina barkeri* (strain Fusaro / DSM 804)
GR505502	No significant match	No sequence found by PSI-BLAST	Predicted protein; Organism = *Paracoccidioides brasiliensis (strain Pb03)*	No significant match
GR505505	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein Sb03g033760; Organism = *Sorghum bicolor* (Sorghum)
GR505506	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing; protein 17 Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	RD22-like protein; Organism = *Polygonum sibiricum*
GR505507	BURP-domain-containing protein 3;	BURP-domain-containing protein 17;	Putative uncharacterized protein;	RD22-like protein; Organism = *Polygonum*

	Organism = *Oryza sativa subsp. japonica*	Organism = *Oryza sativa subsp. japonica*	Organism = *Oryza sativa subsp. indica* (Rice)	*sibiricum*
GR505508	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein Sb03g033760; Organism = *Sorghum bicolor* (Sorghum)
GR505509	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	RD22-like protein; Organism = *Polygonum sibiricum*
GR505510	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 5; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein; Organism = *Glycine max* (Soybean)
GR505511	Dehydration-responsive protein RD22; Organism = *Arabidopsis thaliana*	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	BURP-domain-containing protein; Organism = *Brassica napus* (Rape)
GR505512	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	RD22-like protein; Organism = *Polygonum sibiricum*
GR505513	No significant match	No sequence selected in the PSI-BLAST model	Putative coat protein; Organism = *Elderberry latent virus*	Putative coat protein; Organism = *Elderberry latent virus*
GR505515	BURP-domain-containing protein	BURP-domain-containing protein 17;	Putative uncharacterized protein;	RD22-like protein; Organism = *Polygonum*

	Organism = *Oryza sativa subsp. japonica*	Organism = *Oryza sativa subsp. japonica*	Organism = *Oryza sativa subsp. indica* (Rice)	*sibiricum*
GR505516	No significant match	No sequence selected in the PSI-BLAST model	Putative coat protein; Organism = *Elderberry latent virus*	Putative coat protein; Organism = *Elderberry latent virus*
GR505517	BURP-domain-containing protein 3; Organism = *Oryza sativa subsp. japonica*	BURP-domain-containing protein 17; Organism = *Oryza sativa subsp. japonica*	Putative uncharacterized protein; Organism = *Oryza sativa subsp. indica* (Rice)	Putative uncharacterized protein Sb03g033760; Organism = *Sorghum bicolor* (Sorghum)

Table 5. Results of the similarity search using four similarity search programs (BLAST, Psi-BLAST, MPSrch and SSearch)

matches for GR505502, except for one match from MPSrch, but the score was low. Thus, thissequence is a good candidate for a structure-prediction approach for making a functional inference. Neither GR505513 nor GR505516 had matches from the BLAST or Psi-BLAST runs. Using MPSrch and SSearch, both sequences were predicted to be similar to a putative coat protein (Q911J7) from the elderberry latent virus. Even though the scores from both programs were reasonably low, at least the output can provide some insight into the functions of these hypothetical proteins and a basis for experimentation. A number of hypothetical proteins obtained from the roots of *P. minus* Huds were computationally identified as similar to at least one fully characterized domain. However, the functional interpretation of these proteins is limited. Notably, despite our best efforts, we were unable to provide functional annotations for GR505498 or GR505502. In addition, functional changes over evolutionary time (Devos and Valencia 2000, Todd et al. 2001) and database errors (Brenner 1999) confound the reliable computational predictions of the precise functions of these newly discovered genes. Further experimental evidence is required to successfully deduce their molecular roles.

3.3 Effect of JA elicitation on gene expression

RT-PCR analysis was performed to compare the transcripts expression between control root sample and JA-treated root samples. To verify whether the gene expression corresponding to the cDNA sequences generated by SSH were differentially expressed in *P. minus* under JA stress, four clones involved in the biosynthesis of aromatic compounds, three clones related to abiotic stress, and one clone representing a transcription factor were examined. Those clones were selected based on the nearest E-value to zero and molecular functions identified by BLAST. The clones that showed similarity to genes associated with aromatic compound biosynthesis were GR505472 (lipoxygenase, LOX), GR505465 (alcohol dehydrogenase, ADH), GR505467 (S-adenosyl-L-methionine synthetase, SAMS) and GR505471 (S-adenosyl-L-homocysteine hydrolase, SHH). The clones that were similar to abiotic stress response genes were GR505453 (ELI3-1), GR505459 (glutathione S-transferase, GST) and GR505464

(peroxidase, POD) and the clone involved in protein degradation pathway was GR505460, which had a cDNA sequence similar to the kelch-repeat containing F-box family protein (F-box). Ubiquitin 11, an endogenous gene expressed constitutively in plant was selected as internal control for normalization of gene expression. In general, the expression patterns were consistent with the results of the Reverse Northern analysis. The expression patterns can be divided into three types: (1) strong upregulation in JA-treated roots and slight up-regulation in normal roots, i.e., GR505453 and GR505459; (2) strong up-regulation in JA-treated roots but very little or no expression in normal roots, i.e., GR505465, GR505467, GR505471, GR505464, and GR505460; and (3) slight upregulation in JA-treated roots compared to non-treated roots, i.e., GR505472. The expression level of Ubiquitin 11 did not differ between samples (Fig. 6).

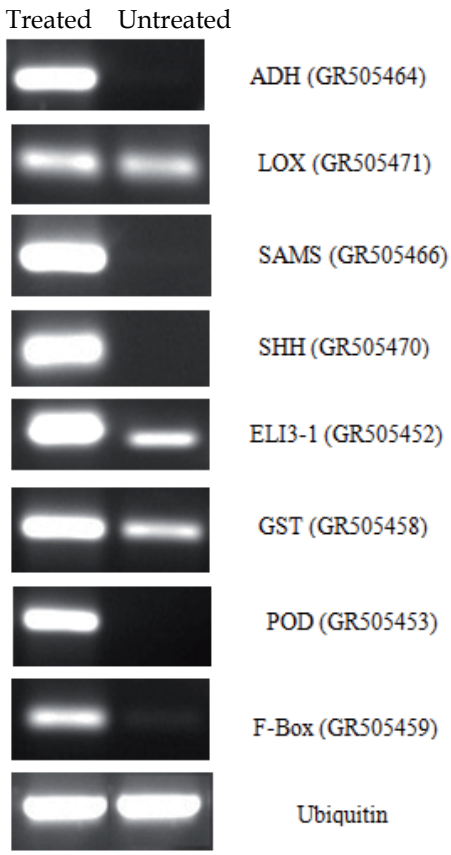

Fig. 6. Semi-quantitative RT-PCR analysis of expression patterns of genes responsive to jasmonic acid metabolism of JA-treated P. minus roots subtracted library. Expression pattern for each selected clone was examined in both JA-treated and non-treated roots' samples. Ubiquitin 11 was used as a control to demonstrate equal cDNA used as templates. Clones involved in aromatic compounds biosynthesis are GR505465 (ADH alcohol dehydrogenase); GR505472 (LOX lipoxygenase); GR505467 (SAMS S-adenosyl-L-methionine synthetase) and GR505471 (SHH S-adenosyl-L-homocysteine hydrolase). Clones related to abiotic stress are GR505453 (ELI3-1 ELI3-1 gene), GR505459 (GST glutathione S-transferase) and GR505464 (POD peroxidase). GR505460 is a clone similar to F-box protein (kelch repeat containing F-box family protein)

3.4 Correlation study between phytochemistry profiling and transcriptomic dataset

3.4.1 Genes involved in aromatic compounds production

A total of 11 cDNA sequences found in this study showed significant similarity with enzymes associated with biosynthesis of volatile compounds from plants. These sequences are found to be involved in acyl lipid catabolism pathway (2 ADH clones and 1 LOX clone) and shikimate pathway (7 SAMS clones and 1 SHH clone). GR505471 clone showed 81% identity similar to lipoxygenase gene isolated from *Capsicum annuum*. Lipoxygenase (LOX, EC 1.13.11.12) catalyzes deoxygenation of linoleic and linolenic acid to hydroperoxide in oxylipin pathway (Devitt 2006). Oxylipin is a common name for oxidized compounds derived from fatty acid though enzymatic reaction. Examples of oxidized compounds are hydroperoxide fatty acid, hydroxyl fatty acid, epoxy fatty acid, keto fatty acid, volatile aldehyde and cyclic compounds. This oxylipin pathway will affect plant aroma and taste (Yilmaz 2000). Lipoxygenase has been isolated from cucumber infected by spider mite and the expression of this gene was linked with the production of volatile compound named (Z)-3-hexynyl acetate (Mercke et al. 2004). It has also been isolated from papaya (Devitt et al. 2006) and apple (Dixon & Hewett 2000). Thus, we the expression of lipoxygenase was believed to be associated with the production of aldehyde in kesum, such as octadecanal which contribute to the aromatic flavour of kesum. Beside, GR505464 clone showed 72% identity similar to alcohol dehydrogenase from *Prunus armeniaca*. Alcohol dehydrogenase (ADH, EC 1.1.1.1) identified in this study might be involved in phenylpropanoids formation, another group of volatile aromatic compounds (Devitt et al. 2006). All the alkanes identified by GC-MS in kesum root extract could be oxidized into alcohols, aldehydes and acids homolog to the alkanes by using NAD and NADH as cofactor (Figure 7) (Dixon & Hewett 2000). ADH has also been identified in papaya (Devitt et al. 2006), apple (Dixon & Hewett 2000), corn (Walker et al. 1987) and grapevine (Torregrosa et al. 2008). It was believed that the expression of both LOX and ADH were involved in the oxidation of alkanes into alcohols, aldehydes and acids in kesum roots.

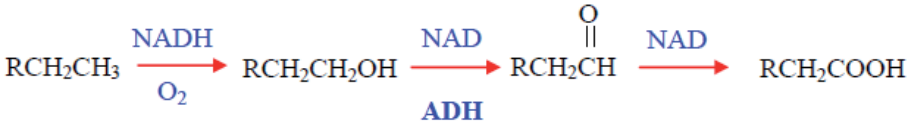

Fig. 7. Oxidation pathway of alkanes.

The identification of lipoxygenase and alcohol dehydrogenase in our study have further confirmed the results obtained by Karim (1987), who reported that approximately 76% of the essential oil in kesum leaves were comprised of aliphatic aldehydes, such as decanal and dodecanal (Karim 1987). Our results also strengthened the GC–MS data previously reported for *P. odoratum* leaves (Du˜ng et al. 1995; Hunter et al. 1997). Naturally these two genes may occur in kesum roots but are not routinely expressed. However, our study showed that their expression in roots could be up-regulated by exposure to JA. According to a model proposed by Yilmaz (2001) in a tomato-ripening study, lipoxygenase will catalyze the deoxygenation of linoleic and linolenic acid into hydroperoxides and subsequently to aldehydes and alcohols. Alcohol dehydrogenase will then catalyze the oxidation of aldehydes to the respective alcohols or vice versa (Figure 8) (Yilmaz 2001). In this study, JA elicitation may have caused the release of free fatty acids from the root cell membranes and

these fatty acids may have served as substrates for the production of volatile aromatic compounds in kesum.

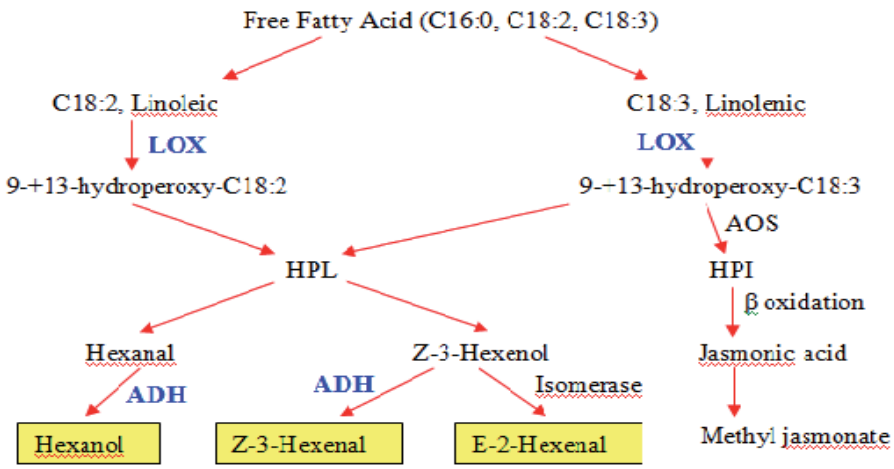

Fig. 8. Alcohol and aldehyde production by oxylipin pathway in tomato. (Source: Baldwin et al. 2000).

Apart from that, seven clones showed similarity to S-adenosyl-L-methionine synthetase (87% identity similar to *Beta vulgaris*) and one clone was similar to S-adenosyl-L-homocysteine hydrolase (87% identity similar to *Mesembrayanthemum crystallinum*). These clones were involved in the synthesis of aromatic shikimic acid through the shikimate pathway. Both of these clones demonstrated the type 2 expression pattern, where their expression in kesum roots was only up-regulated upon JA elicitation and no expression was observed under control conditions. Shikimic acid is the precursor for phenylpropanoid biosynthesis, another important group of secondary metabolites (Dewick 2001). Both S-adenosyl-L-methionine synthetase (SAMS, EC 2.5.1.6) and S-adenosyl-L-homocysteine hydrolase (SHH, EC 3.3.1.1) are enzymes that play a role in the synthesis of S-adenosyl-L-methionine (SAM), the main donor of the methyl group for many specific methyl transferase reactions, such as the transmethylation of alkaloids (Kutchan 1995). In plants, SAM is also associated with the production of phenylpropanoids (Kawalleck et al. 1992). Again, our data corroborate previous reports that identified flavonoids in leaves of the Polygonum family (including P. minus), which are synthesized by the phenylpropanoid metabolic pathway (Urones et al. 1990), P. stagninum (Datta et al. 2002) and P. hydropiper (Peng et al. 2003). The data suggest that the genes that are normally expressed in leaves could be triggered by JA in roots. On the other hand, SAM is also the intermediate molecule in ethylene and polyamine biosynthesis (Ravanel et al. 1998). Nevertheless, the expression of these two genes was also shown to be up-regulated in the petunia flower and they were linked to the production of benzenoid, a compound that contributes to the flower's scent (Schuurink et al. 2006). Hence, it was believed that the expression of the SAMS and SHH genes found in this study was related to the production of phenylpropanoids, alkaloids, ethylene or polyamine after JA elicitation. The activated-methyl cycle was predicted to be closely related to JA signaling and must be further investigated.

3.4.2 Genes related to abiotic stress

Clones encoding the genes related to abiotic stress,such as glutathione S-transferase (GST), ELI3-1 and peroxidase (POD) were evaluated to investigate the correlation between JA stress and other stress factors. Both GST and ELI3-1 demonstrated a type 1 expression pattern. Ten clones were similar to GST in this subtracted library. GST (EC 2.5.1.18) is a cytosolic enzyme found in all eukaryotes. This gene is always detected in stressed plants, such as heavy metal and salt-treated rice seedlings (Moons 2003), water-stressed maize seedlings (Zheng et al. 2004), drought-stressed horse gram (Chandra Obul Reddy et al. 2008) and fungus-elicited rice seedlings (Xiong et al. 2001). Therefore, and not surprisingly, the expression of GST was observed in normal kesum roots because the in vitro culture itself was a stress condition for kesum plantlets. It was strongly up-regulated in JAtreated roots as a defense mechanism, suggesting an overlap in the plant responses of plants to various stress factors. Also, GST catalyzes the conjugation between synthetic electrophilic compounds and the glutathione tripeptide (c-glutamyl-cysteinyl-glycine, GSH). The polar S-glutathionylated product will be actively transported into the vacuole by an ATP-binding cassette. Thus, GST is part of the detoxification mechanism in plants. In fact, it is the main ingredient for a variety of commercial herbicides (Andrews et al. 2005). Therefore, it is crucial to investigate kesum as a potential plant for phytoremediation purposes. ELI3-1 is another gene related to the abiotic stress caused by JA elicitation that will lead to phytoalexin and pathogenesis-related protein accumulation, phenolic compound production, and cell wall reconstruction. Seventeen of the ELI genes were identified in parsley cells cultured and treated with the Phytophthora megasperma fungus (Trezzini et al. 1993). In addition, MeJA elicitation has successfully activated ELI3, TyrDC, HRGP, and BMT genes in parsley (Ellard-Ivey and Douglas 1996). This observation proved that the expression of ELI3-1 could also be induced by JA elicitation. Peroxidase (POD, EC 1.11.1.7) plays an important role in the oxidation process, such as in peroxidative ooxidative oxidation and catalytic hydrosilylation (Umaya and Kobayashi 2003; Veitch 2004). It is an enzyme that catalyzes the oxidation of phenylpropanoids (Thimmaraju et al. 2006). It also functions in the plant-defense system, and it could be triggered by an elicitor (Go´mez-Va´squez et al. 2004; Perera and Jones 2004). The clones that were similar to POD in this study showed a type 2 expression pattern in the RT-PCR analysis. Its expression has been shown to induce the production of terpenoids in cucumbers infected by spider mites via oxidative degradation (Mercke et al. 2004). Our observation corroborates the results reported in kesum (Karim 1987) and *P. odoratum* (Du˜ng et al. 1995; Hunter et al. 1997), where various sesquiterpene hydrocarbons and their oxygenated compounds were identified. Therefore, it was predicted that stress stimuli, such as JA, could regulate the induction of important classes of plant secondary metabolites in kesum. In addition, its oxidative reaction showed that POD can be used as a component in the reagent for clinical diagnosis and various laboratory experiments (Thimmaraju et al. 2006) and thus increases the value of kesum.

3.4.3 Transcription factor activated by JA

Interestingly, the kelch-repeat containing F-box family protein and the TIR1 protein that is contained in the F-box found in this subtractive cDNA library are induced by JA (Craig and Tyers 1999; Parry and Estelle 2006). The kelchrepeat containing F-box family protein is involved in the protein–protein interaction in the ubiquitin protein degradation process via the ubiquitin-mediated pathway. The protein degradation process is important to regulate

the cell cycle, transcription, and signal transduction, as a mechanism for the root cells to adapt to JA elicitation stress (Sun et al. 2007). The functions of these proteins have been demonstrated in Arabidopsis and they may serve as transcription factors in genes expressed in response to JA treatment. While these proteins all play the same role in regulating JA, they also regulate species-specific secondary metabolite pathways (Pauwels et al. 2009). These proteins must be characterized and examined for the mechanism that drives secondary metabolites production in response to JA.

4. Conclusion

Our results showed that there is a close relationship between abiotic stress and the expression of genes involved in the biosynthesis of secondary metabolites. The subtractive cDNA library data set presented here provides the first collection of a set of JA responsive genes that may be involved in the secondary metabolite production in *P. minus* roots and also those participating in plant-defense mechanisms. These genes include dehydrogenase, lipoxygenase, S-adenosyl-L-methionine synthetase, S-adenosyl-L-homocysteine hydrolase, glutathione S-transferase, peroxidase, ELI3-1, and a transcription factor, F-box family protein. Identification of genes associated with flavour volatiles and the production of other aromatic compounds will provide a better understanding of the secondary metabolite biosynthetic pathways and their regulation in *P. minus*. Furthermore, the observed stress-related genes induced by JA elicitation indicate that plants respond to abiotic stresses in parallel with the biosynthesis of certain secondary metabolites. Characterization of these genes will be studied in details using *E. coli* expression system. Besides, their functions could be explored with GC or HPLC to confirm the synthesis of the corresponding compounds whereas their ability to cope with abiotic stress could be performed by culturing *P. minus* plantlets in various stress conditions. In this study, we concluded that the JA-responsive genes might be the genes associated with volatile compounds production as a defence response against abiotic stress.

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4

Transcriptomics of Sugarcane Osmoprotectants Under Drought

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1. Introduction

Sugarcane (*Saccharum* spp.) is an alogamous plant from the Poaceae family and the Andropogoneae tribe (Daniels & Roach, 1987). This crop covers more than 23 million hectares worldwide, representing about 0.5 % of the total global area used for agriculture, with a production of 1.6 billion metric tons of crushable stems (FAOSTAT, 2009). Brazil is the world's largest producer, contributing with two-thirds of total sugar production - about 31 million tons per year - of which 19.5 million tons are exported (UNICA, 2009). Sugarcane, its derivatives and by products have received great attention, due to their multiple uses, with emphasis on the ethanol production, representing an important renewable biofuel source. It has been estimated that sugarcane ethanol fuel may replace up to 10.0 % of the world's refined petroleum products consumption in the next 15 to 20 years (Goldemberg, 2007). Despite its importance and similarity to other important agronomic crops, the sugarcane production has been adversely influenced by many environmental factors such as harsh climate and soil conditions.

Abiotic stresses are among the main causes of losses in the productivity of the major crops worldwide (Bray *et al*., 2000), a scenario where drought figures as the most significant stress, causing negative impacts on crop adaptation and productivity. Besides, this condition can exacerbate the effect of other stresses (biotic or abiotic) to which the plants may be submitted. Although breeding activities have provided significant progress for the understanding of the physiological and molecular responses of plants to water deficit, there is still a large gap between yields in optimal and stressful conditions (Cattivelli *et al*., 2008). Essays regarding plant responses to drought stress have been published applying technologies of functional genomics (Wang *et al.,* 2011). These evaluations provided important insights into molecular and biochemical mechanisms in the study of drought tolerance in various crops and model species. Plants are able to ''perceive'' the external stimuli by multiple sensors, recognizing adverse situations and invoking signal transduction cascades and consequently secondary messengers, activating stress responsive genes (Grennan, 2006), resulting in both molecular and physiological responses. Among the mechanisms developed by plants to face the adverse conditions generated under drought, the accumulation of osmoprotectants compounds are often recognized as a mitigation mechanism of the negative consequences of water deficit (Choluj *et al*., 2008).

Osmoprotectants are small solutes used by cells of numerous water-stressed organisms and tissues to maintain cell volume (Yancey, 2001), and may play other roles regarding tolerance, as proteins stabilizing and antioxidant action (Rathinasabapathi, 2000). They include sugars, mainly fructose and sucrose, sugar alcohols (like myo-inositol), complex sugars (like trehalose and fructans) and charged metabolites (like glycinebetaine, proline and ectoine) (Yancey, 2005).

Previous information regarding sugarcane osmoprotectants under stress was reported. For example Suriyan & Chalermpol (2009) analyzed diverse parameters in sugarcane submitted to iso-osmotic salt and water-deficit stresses. Among the physiological alterations, an increase in the proline content in stressed-leaves was positively correlated to the reported stresses, indicating a key role of proline in osmoregulation and antioxidant defense mechanisms. Also Rasheed *et al*. (2010) investigated the possible roles of proline and glycine betaine (GB) in mitigating the effect of chilling stress in the sprouting nodal buds of sugarcane. To accomplish this evaluation, they performed a pre-treatment with proline bud chips and GB, obtaining a substantial reduction in the H_2O_2 production and an increase in the synthesis efficiency of soluble sugars, protecting developing tissues from the effects of chilling stress. Recent molecular research works, regarding drought and salinity performance in sugarcane, were carried out using techniques based on molecular hybridization such as SSH [Subtractive Hybridization Suppressive] (Patade *et al*., 2010) and micro/macroarrays (Rodrigues *et al*., 2009). In a general view, the main limitations of these methods regard their low sensibility and specificity (Shimkets, 2004). Moreover, in relation to micro/macroarrays, in spite of their high performance and broad use, the inability to analyze and discover new genes have been reported (Wang *et al*., 2009). Techniques based on sequencing [i.e. SAGE (Velculescu *et al*., 1995) and derivatives] take advantage of the available frequencies of fragments (tags) representing transcripts expressed in the sample, by the assumption that a short and defined tag contains the information needed to identify the corresponding cDNA (Velculescu *et al*., 1995). Thus, besides constituting an open architecture analysis (i.e., allowing the discovery of new genes), the abundance of tags found for a given gene provides an estimative of their transcription in the sample. In this context, the SuperSAGE technique (Matsumura *et al*., 2003) stands out for its efficiency in generating transcription profiles, especially with the actual association to the high performance sequencing platforms [Pyrosequencer (454 Roche®), Solexa (Illumina®) and SOLiD (Applied Biosystems®)].

The SuperSAGE method is characterized by the generation of 26 bp tags by using the type III restriction enzyme *Eco*P15I (Matsumura *et al*., 2003). This technique presents itself as one of the most modern tools of functional genomics and provides some advantages such as improvements in tag-to-gene annotation, simultaneous analysis of two interacting eukaryotic organisms, full-length cDNAs amplification using tags as primers, potential use of tags via RNA interference (RNAi) in gene function studies, identification of antisense and rare transcripts, and identification of transcripts with alternative splicing (Matsumura *et al*., 2006). It also provides a global and quantitative transcriptomic analysis based on the study of the entire transcriptome produced in a given time under a given stimulus. This technique has been successfully applied in plant species such as rice (Matsumura *et al*., 2003), banana (Coemans *et al*., 2005), chili pepper (Hamada *et al*., 2008), chickpea (Molina *et al*., 2008; 2011), tobacco (Gilardoni *et al*., 2010) and tropical crops (cowpea, soybean, sugarcane; Kido *et al*., 2010). Some of them using the association of SuperSAGE with a high-throughput sequencing platform (HT-SuperSAGE; Matsumura *et al*., 2010). In the present work we

profit from the high resolution power of SuperSAGE® coupled to the Illumina® sequencing in a SuperTag Digital Gene Expression (STDGE) profile (GenXPro GmbH, Frankfurt, Germany) trying to characterize the transcriptome of drought-stressed sugarcane roots after 24 hours of submission to this stress, aiming to elect a best group of tags to be validated by RTqPCR. For this purpose, a high-throughput transcriptome project as a joint Brazilian initiative from UFPE (Federal University of Pernambuco) and CTC (Sugarcane Technology Center) was carried out. The project generated a large amount of gene candidates from different important categories considering the response against this kind of stress. In the present chapter an overview regarding the identification, categorization and differential expression of osmoprotectants will be evaluated in sugarcane, compared with the up to date knowledge concerning this crop and related species. Considering its role in world's economy and biotechnological potential, the identification and expression profile of responsive osmoprotectant coding genes in sugarcane may be helpful to unravel the basic mechanisms of stress tolerance, bringing valuable evidences for sugarcane improvement.

2. Drought: Understanding the problem

Biological stress may be defined as an adverse environmental condition that inhibits the normal operation of a biological system such as plants (Jones & Jones, 1989). The life in the terrestrial condition represents a challenge to plants, and often have to occupy environments that are not the most appropriated for their development, being also subjected to frequent environmental changes in their native conditions. A large number of abiotic factors associated with plant-water relations – such as drought, salinity, chilling, frost and flooding – negatively affect the overall growth of terrestrial plants, leading to stunted form, metabolic changes, reduced yields, germination problems and even plant death under extreme conditions (Smith and Bhavel, 2007). Among the mentioned factors, drought stands out, bringing the most serious threat in view of the existing freshwater shortage in many regions of the world, bringing serious limitations to the agriculture (Jury & Vaux, 2005).

Notwithstanding the availability of more than 150 definitions for drought in the literature (Boken, 2005), it is often defined in terms of available humidity as compared with a normal value, with the severity correlating in function to the time and magnitude of the exposition to a deficient humidity (Smith & Pethley, 2009). Among the diverse types reported, some can be highlighted as meteorological, hydrological, socioeconomic and agricultural drought (Boken, 2005; Smith & Pethley, 2009). According to Boken (2005), the meteorological drought occurs when seasonal or annual precipitation falls below its long-term average; the hydrological drought when the meteorological drought is prolonged and causes local shortage of surface and groundwater; the socioeconomic drought is a manifestation of continued drought of severe intensity that shatters the economy and sociopolitical situation in a region, while the agricultural drought sets, due to soil moisture stress, a significant decline in crop yields (production per unit area).

The agricultural drought is the most important nowadays and will be here focused. Agriculture is by far the largest consumer of water, representing for 80.0 % of the freshwater consumption worldwide (Jury & Vaux, 2005). Economic losses associated to water availability reached about one billion dollars, in 2009, only in the United States (Anderson *et al.*, 2009). Actually, one-third of the world population lives in areas with water shortages. This is specially serious considering other adverse factors such as the high levels of

atmospheric CO_2, climate change scenarios and predictions of future global warming, all of them increasing drought incidence, frequency and severity. Significant further problems are predicted for food production due to the limited availability of suitable freshwater for agriculture.

According to Hazell & Wood (2008), the climate change will affect different localities in different ways, with potential benefits to some important food growing areas as the Canadian Prairies, but making agriculture more difficult in some other regions as many drought prone areas in Africa and Americas. Such predictions also reinforce that agricultural systems have considerable capacity to adapt to climate change, but this poses many challenges that are not yet fully understood, with urgent research efforts necessary to identify the best ways to adapt.

3. Sugarcane and drought: Current outlook and use of science in search of solutions

Sugarcane is one of the world's major food crops, providing about 75.0 % of the sugar harvested for human consumption [Food and Agriculture Organization (FAO) statistics]. The crop is closely associated with sustainability as it is presented as a renewable energy source including ethanol and electricity (Tew & Cobill, 2008). Despite this status, sugarcane suffers severe losses due to the availability of soil water. In Brazil, the largest global sugarcane producer (Henry, 2010), the state of São Paulo accounts for about 60.0 % of the country production, suffering losses of around 10.0 % due to periods of drought, as reported for harvests in 2010 (UNICA, 2011). Thus, it is necessary to generate new tolerant varieties to this stress. Currently, breeding programs for sugarcane are being developed in different countries by public and private institutions, and also by cooperative systems formed by producers. Most projects involve the performance of controlled crossings which are costly and bring long term results, reaching sometimes more than 15 years (Cesnik & Miocque, 2004).

An alternative way to promote the breeding is associated with the analysis and identification of specific genes involved in given metabolic processes. The traditional method for identifying a gene responsible for a particular trait includes the initial demonstration that the trait is inheritable, followed by isolation of a candidate gene that is postulated to be responsible for that trait. This "single gene" approach is fundamentally flawed for many traits since it is assumed that every trait is governed by a single gene. According to Casu *et al*. (2010) genomics and all of the related "omics" techniques, e.g. transcriptomics, break this formula and rely on the in-depth assembly of large amounts of data followed by data-mining to determine connections between a particular trait and any number of associated genes.

Transcriptomics data are available for sugarcane, and have been generated mainly by EST (Expressed Sequence Tag) sequencing, or using methodologies based on probe hybridization arrays, using known genes from other crops. Among the available collections of ESTs for sugarcane, the sequences generated by the SUCEST project should be highlighted. This project regards a large consortium of Brazilian researchers who sequenced approximately 238,000 redundant ESTs from 26 diverse cDNA libraries (Vettore *et al*., 2001), representing the largest effort to generate information using new technologies for this species. This endeavor brought a broader panel when compared to previous available ESTs produced by other consortia in countries like Australia (Casu *et al*., 2003, 2004; Bower *et al*., 2005) and the US (Ma *et al*., 2004).

Additionally, microarray platforms have been also used to evaluate sugarcane expression profile. The first ones were used to investigate gene expression differences between immature and maturing stems of sugarcane (Casu *et al*., 2003; 2004). Using glass microarrays the authors assayed up to 4,715 non-redundant random ESTs derived from immature and maturing stems, and also from roots. The most recent report using this method made use of a custom cDNA microarray (3,598) to profile the effect of elevated CO_2 on sugarcane leaves (De Souza *et al*., 2008). Regarding open architecture transcriptomics technologies – which analyze the entire population of transcripts produced in a given time under a given stimulus – a single literature report is available for sugarcane, in an approach developed by Calsa & Figueira (2007). The authors used standard 14 bp SAGE to characterize the sugarcane mature leaf transcriptome, generating 9,482 valid tags, with 5,227 unique sequences, from which 3,659 (70.0 %) matched at least one sugarcane assembled sequence with putative function.

Despite the relative data abundance for sugarcane transcriptome covering different conditions, there is still a restricted number of publications regarding the analysis of molecular behavior under drought, whilst most available evidences come from technologies based on hybridization or cDNA (EST) sequencing. Rocha *et al*. (2007) in a cDNA microarrays approach to profile expression of 1,545 genes involved in signaling processes in plants submitted to drought, phosphate starvation, herbivory and N_2-fixing endophytic bacteria, identified 485 differentially expressed candidates after exposition of the plants to water shortage. In another approach Gupta *et al*. (2010) using cDNA libraries associated to the RTqPCR validation of drought related genes, revealed differences greater than 2-fold regarding the expression of given genes during dehydration stress. The most recent report was carried out by Iskandar *et al*. (2011) that investigated whether the degree of expression of eight stress-related genes - *P5CS, OAT, AS, PST5, TF1, LEA, POX* and *dehydrin* – was correlated with the sucrose content in the sugarcane culm, and whether such genes were also responsive to water deficit stress. Almost all selected genes were upregulated, with exception of *POX* that was downregulated after 15 days of water deficit stress. However, subsequent analysis revealed a different transcriptional profile to that, showing a correlation with the sucrose accumulation. For example, genes with homology to late embryogenesis abundant-related proteins and dehydrin were strongly induced under water deficit, but did not correlate with sucrose content. The expression of genes encoding proline biosynthesis was associated with both sucrose accumulation and water deficit, but amino acid analysis indicated that proline was negatively correlated with sucrose concentration.

Since drought is a complex feature discovery of genes associated to tolerance processes is urgent, being one of the most important and difficult challenges. A larger number of studies is made necessary, aiming to understand this issue in sugarcane, enriching the knowledge on the metabolic pathways involved in acclimation process to water deficit.

4. Drought and osmoprotectants in plants

Water deficit, caused by "lack of water" or by other environmental stresses like extreme temperatures or salinity (Bartels & Souer, 2004), has been great problems for agriculture, affecting virtually every aspect of plant physiology and metabolism. As a consequence of these stresses, a range of adaptive responses including morphological (Jaleel *et al*., 2009), physiological (Harb *et al*., 2010) and biochemical changes (Ahmadi *et al*., 2010) enabled plants to tolerate and survive at such adverse conditions. Similar cellular and molecular adaptive responses include a significant accumulation of compatible solutes.

Compatible solutes regard a variety of low-molecular-weight organic compounds, electrically neutral molecules, soluble in water and nontoxic at high cellular concentrations (Yancey, 2001). Such osmolytes include a variety of simple sugars (e.g. fructose and glucose), sugar alcohols (glycerol and methylated inositols) and complex sugars (trehalose, raffinose and fructans), while other include quaternary amino acid derivatives (proline, glycine betaine, b-alanine betaine, proline betaine), tertiary amines (ectoine; 1,4,5,6-tetrahydro-2-methyl-4-carboxy-lpyrimidine) and sulfonium compounds (choline o-sulfate, dimethyl sulfonium propironate) (Rhodes & Hanson, 1993; Vinocur & Altman, 2005). Compatible solute accumulation in response to osmotic stress is a ubiquitous process in organisms as diverse as bacteria, plants and animals (Bohnert & Jensen, 1996). These osmoprotectants compounds are typically confined mainly to the cytosol, chloroplasts, and other cytoplasmic compartments (Rontein *et al*., 2002), protecting plants in different ways, including: stress defense by osmotic adjustment (helping the cells to maintain their hydrated state and turgor maintenance), stabilization of proteins and enzymes, induction of stress proteins and acceleration of reactive oxygen species scavenging systems (Bohnert & Jensen 1996; Ashraf & Foolad, 2007). In plants that naturally accumulate osmoprotectants, the level of these compounds are highest under stress extension (Rhodes & Hanson, 1993). So, changes in plant drought-induced gene expressions have been revealed, and many genes have been isolated from numerous species, playing important roles in both initial stress response and in establishing plant stress tolerance (Shinozaki & Yamaguchi-Shinozaki, 2007). Proline, glycine betaine, sugars and sugar alcohols are examples of compatible solutes encoded by some of these stress-inducible genes that function in cellular osmotic adjustment, promoting drought tolerance, guaranteeing plasma membrane integrity, without disrupting the protein function (Bartels & Sunkar, 2005). So, the osmotic adjustment by accumulation of these compounds has been proposed as an important mechanism to overcome the negative consequences of water deficit in crop production (Rathinasabapathi, 2000; Choluj *et al*., 2008). Based on accumulation of these compounds to be associated to high levels of tolerance in plants, and considering that their beneficial effects are generally not species-specific (Rontein *et al*., 2002), considerable progress has been achieved in investigations using transgenic plants overexpressing selected osmoprotectants conferring abiotic stress tolerance (Ashraf & Foolad, 2007; Chen & Murata, 2008). Some of them are reviewed below.

4.1 Glycine betaine

Glycine betaine (GB) is a quaternary ammonium compound (QAC) synthesized by a great variety of organisms, including plants, animals and microorganisms (Rhodes & Hanson, 1993). In most organisms GB is synthesized either by the oxidation (or dehydrogenation) of choline or by the N-methylation of glycine. However, the pathway from choline to glycine betaine has been the main GB-accumulation pathway in plant species (Weretilnyk *et al*., 1989). In this pathway choline is converted to betaine aldehyde by choline monooxygenase (CMO) (Rathinasabapathi *et al*., 1997), which is then converted to GB by betaine aldehyde dehydrogenase (BADH) (Vojtechova *et al*., 1997). Similarly to proline (and other osmoprotectants in plants) GB is one of the most extensively studied compatible solutes, being upregulated after drought (Ma *et al*., 2007), salinity (Kern & Dyer, 2004), low temperature (Zhang *et al*., 2010) and oxidative stresses (Liu *et al*., 2011). *In vitro* assays indicate that GB acts as an osmoprotector, stabilizing both the quaternary structure of proteins and the highly ordered membrane structure under adverse conditions (Gorham,

1995). Based on the correlation between GB accumulation and stress tolerance, progress in exogenous GB application (Jokinen *et al*., 1999), cloning and expression of GB encoding enzymes has been achieved (Sakamoto & Murata, 2002; Quan *et al*., 2004). Examples of these genes include *CMO* (Tabuchi *et al*., 2005), *BADH* (Wood *et al*., 1996); *CDH* and *BADH* (Landfald & Strøm, 1986), which were cloned from different organisms and introduced into transgenic plants. For example, in transgenic cotton (transformed with *bet*A gene) GB expression induced the protection of the cell membrane integrity from drought stress damage, being also active in osmotic adjustment (Lv *et al*., 2007). Huang *et al*. (2000) transformed three different species (*Arabidopsis thaliana, Brassica napus* and *Nicotiana tabacum*) with the *COX* gene from *Arthrobacter pascens*, an elucidative approach considering that these plants are non accumulators of this osmoprotector. The highest levels of betaine in independent transgenic plants were de 10- to 20-fold lower than the levels found in natural betaine producers. Further, it was observed that the supplementation of choline is necessary to allow the accumulation of physiologically relevant amounts of betaine. Despite of that, the authors reported the acquisition of a moderate stress tolerance (drought and salinity) in some but not all betaine-producing transgenic lines, based on the relative shoot growth; while the responses to salinity, drought, and freezing stresses were variable among the three transformed species. The results lead to the supposition that higher efficiencies would be achieved in species that naturally produce this osmoprotector (Huang *et al*., 2000). Similar results were achieved by Shirasawa *et al.* (2006) after transformation of rice plants (*Oryza sativa*) – also a non accumulator of GB - with the choline monooxygenase gene from spinach. Enhanced tolerance to salt stress and temperature stress in the seedling stage was observed, however the CMO-expressing rice plants were not effective for accumulation of GB and improvement of productivity. Considering sugarcane, Patade *et al*. (2008) observed the accumulation of free proline and glycine betaine in embryogenic sugarcane calli *(Saccharum officinarum* L.; cv. CoC-671) after NaCl stress. The gradual increase in glycine betaine was positively correlated with the concentrations of NaCl (up to 213.9 mM). Indeed, such osmoprotector was also higher as compared to proline content, in all stress conditions tested (NaCl treatments: 42.8 to 256.7 mM). However, in the higher NaCl concentration, proline was not observed.

4.2 Proline

Proline (Pro) is a proteinogenic amino acid essential for primary metabolism. It is considered one of the most important osmolytes, being accumulated in a large number of species in response to stress damage (for a review, see Hare & Cress, 1997). Under abiotic stress condition, proline accumulation is involved in the maintenance of turgor, promoting continued growth in case of low water potential in the soil (Mullet & Whitsitt, 1996). The accumulation of this important osmolyte, upon osmotic stress, is well documented in a large number of different organisms, including, protozoa (Poulin *et al*., 1987), eubacteria (Csonka, 1989) and marine invertebrates (Burton, 1991). In addition to its role in the osmotic adjustment mechanism, other important functions have been attributed to proline (Bartels & Sunkar, 2005) such as protection of plasma membrane integrity, enhancing of different enzymes activity (Sharma & Dubey, 2005; Mishra & Dubey, 2006), regulation of nitrogen and carbon reservoir (Kishor *et al*., 2005) and as scavenger of free radicals (Smirnoff & Cumbes, 1989).

In higher plants, proline biosynthesis may proceed from two different ways: either via glutamate, by successive reductions catalyzed by pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), respectively (Hu *et al*., 1992; Savouré *et al*.,

1995) or ornithine pathway, by ornithine d-aminotransferase (OAT) (Mestichelli *et al*., 1979). Here the first pathway will be discussed, since it is considered the main pathway during osmotic stress in plants (Bartels & Sunkar, 2005; Parida *et al*., 2008) especially considering the drought response. Under water deficit, proline is synthesized from the glutamate by two intermediates. In the first step, the glutamate is reduced to glutamic acid-5 semialdehyde (GSA) by P5CS. The GSA produced is converted into pyrroline-5-carboxylate (P5C) (Hu *et al*., 1992; Savouré *et al*., 1995) which is then reduced by P5CR to proline (Zhang *et al*., 1995).
Proline induction in response to abiotic stresses has been related for many angiosperms (Mohammadkhani & Heidari, 2008; Székely *et al*., 2008), revealing a positive relationship between proline accumulation and stress tolerance in this group. Kishor *et al*. (1995) reported the overexpression of a *Vigna aconitifolia P5CS1* gene in tobacco plants, leading to increased levels of proline (10- to 18-fold when compared to the control plants), enhancing root biomass, growth rhythm and tolerance under drought-stress. The importance of proline metabolism in the process of drought tolerance was evidenced by Ronde *et al*. (2000) in soybean plants (*Glycine max*). The authors reported the suppression of proline synthesis in transgenic soybean plants containing the *P5CS* gene in the antisense direction. Transformed plants presented increased sensitivity to water deficit, as compared with the wild type. In cotton under drought-stress, Parida *et al*. (2008) verified an induction of proline levels by the upregulation of P5CS and downregulation of proline dehydrogenase (PDH), indicating a possible involvement of proline production in the development of drought tolerance. Osmotic adjustment through proline accumulation was reported as a primary response of drought stressed sugarcane (*S. officinarum*) plantlets (Errabii *et al*., 2006).
On the other hand, reports suggested that the increase in proline concentration is related to protective symptoms under severe water stress rather than an osmoregulatory function. In transgenic wheat plants, the higher accumulation of proline (when compared to the wild type) conferred drought stress tolerance by increasing the antioxidant metabolism rather than increasing osmotic adjustment (Vendruscolo *et al*., 2007). In sugarcane transformed with the *V. aconitifolia P5CS* gene, it was observed that after nine days without irrigation proline content in transgenic plants was on the average 2.5-fold higher than in the controls. However, no osmotic adjustment was observed in plants overproducing proline during the water-deficit period, suggesting a role of proline as component of the antioxidative response system rather than as a promoter of osmotic adjustment (Molinari *et al*., 2007). Indeed, the hypothesis of the protective role played by proline under severe drought stress was also supported by Gomes *et al*. (2010), who evaluated the water stress effect on osmotic potential, proline accumulation and cell membrane stability in leaflets of the coconut palm (*Cocos nucifera* L.).

4.3 Myo-inositol

Inositol is a cyclohexanehexol, a cyclic carbohydrate with six hydroxyl groups, one on each carbon ring. Among the nine types of existing steroisomers, myo-inositol is the most abundant in the nature, being also important for the biosynthesis of a wide variety of compounds including inositol phosphates, glycosylphosphatidylinositols, phosphatidylinositides, inositol esters, and ethers in plants (Murthy, 2006). Besides the own myo-inositol, other related or derived molecules are also important osmoprotectors. Myo-inositol serves as a substrate for the formation of galactinol, the galactosyl-donor that plays a key role in the formation of raffinose family oligosaccharides (RFOs, raffinose, stachyose, verbascose) from sucrose. RFOs accumulate in plants under different stress conditions

(Kaplan *et al*., 2004; Peters *et al*., 2007). In the case of the halophyte *Mesembryanthemum crystallinum* (common ice plant) – that possesses a remarkable tolerance against drought, high salinity, and cold stress – inositol is methylated to D-ononitol and subsequently epimerized to D-pinitol. This plant accumulates a large amount of these inositol derivatives during the stress (Adams *et al*., 1992; Vernon *et al*., 1993).

Throughout the biological kingdom, myo-inositol is synthesized by a two-step pathway that is unofficially known as the "Loewus pathway". The first step is the conversion of D-glucose-6-P to D-myo-inositol (1)-Monophosphate, 1D-MI-1-P, which is catalyzed by an L-myo-inositol 1-phosphate synthase (MIPS) (Majumder *et al*., 1997), followed by its specific dephosphorylation to free myo-inositol by the Mg^{++} dependent L-Myo-inositol 1-phosphate phosphatase (IMP) (Parthasarathy *et al*., 1994). Due to the potential of myo-inositol, some transgenic plants expressing this substance have been generated, mainly using MIPS enzyme or inositol derived enzymes.

Majee *et al*. (2004) reported on the isolation of the *PINO1* gene (also known as *PcINO1*, encoding an l-myo-inositol 1-phosphate synthase) from the wild halophytic rice relative *Porteresia coarctata*. This gene was expressed in tobacco plants, conferring them the capacity of growth in 200–300 mM NaCl with retention of ~ 40–80 % of the photosynthetic competence with concomitant increased inositol production when compared with unstressed control. Additionally, *PINO1* transgenics showed *in vitro* salt-tolerance, confirming *in planta* functional expression of this gene.

Das-Chatterjee *et al*. (2006) carried out a functional introgression of *PcINO1* and *OsINO1* genes (this last regarding the corresponding homologue from the cultivated rice that encodes for a salt-sensitive MIPS protein) in distantly related organisms, as prokaryotes (*Escherichia coli*) to eukaryotes (yeast: *Schizosaccharomyces pombe*; plants: *Oryza sativa* and *Brassica juncea*) analyzing the tolerance of these transgenic lines under salinity stress. The results confirmed the role of the *PcINO1* gene, conferring salt tolerance to various levels of complexity, from prokaryotes to different eukaryotes, including higher plants, leading to an unabated production of inositol and survival under NaCl stress. Patra *et al*. (2010), in turn, held introgression and functional expression studies in tobacco plants using *PcINO1* (a) and *McIMTI* (b) [inositol methyl transferase, IMTI, from the common ice plant *M. crystallinum*] genes. After submission of the obtained transgenic lines to saline and oxidative stresses it was observed that all plants presented higher performances in terms of growth potential and photosynthetic activity and were less prone to oxidative and salt stresses when compared to the controls. Physiological experiments demonstrated the superiority of the *PcINO1–McIMT1* double transgenic plants to withstand the salt stress accompanied by the accumulation of both myo-inositol and methylated inositols in the system over the transgenic plants with either of the single gene(s).

4.4 Trehalose

Trehalose is a non-reducing α,α-1,1-linked glucose disaccharide that functions as an energy source and a storage form of more reactive glucose in lower organisms (Galinski, 1993). At least three different pathways for the biological synthesis of trehalose have been reported (Elbein *et al*., 2003). In plants, the synthesis of this sugar occurs normally by the formation of the trehalose-6-phosphate (T6P) from the UDP-glucose and glucose-6-phosphate, a reaction catalyzed by the trehalose 6-phosphate synthase (TPS). Afterwards the T6P is dephosphorylated by the trehalose-6-phosphate phosphatase (TPP) resulting in the formation of free trehalose (Wingler, 2002).

Although trehalose is widely distributed in the nature (including prokaryotes and eukaryotes) this sugar has been isolated from a few plant species, being identified in ripening fruits of species from the Apiacea family, in the leaves of *Selaginella lepidophylla* and its relatives (Goddijn & van Dun, 1999) as well as in *Arabidopsis thaliana* (Wingler *et al*., 2002). According to Elbein *et al*. (2003) in yeast and plants trehalose may serve as a signaling molecule to direct or control certain metabolic pathways or even to affect growth. In addition, it has been shown that trehalose can protect proteins and cellular membranes from denaturation caused by a variety of stress conditions, including desiccation. Kaushik & Bhat (2003) demonstrated that this sugar is an exceptional stabilizer of proteins, while Fait *et al*. (2006) considered its role in the maintenance of the conformation of both storage and housekeeping proteins during dehydration in seeds of *A. thaliana*.

Considering the evidences in favor of a positive role of this protein under abiotic stress, trehalose has been widely evaluated in expression assays. Garg *et al*. (2002) showed that trehalose overproduction has considerable potential for improving abiotic stress tolerance in rice transgenic plants, which accumulated increased amounts of it and showed high levels of tolerance to salt, drought, and low-temperature stresses, as compared with the non-transformed controls. Resurrection plants (*S. lepidophylla*) have the ability to withstand almost complete water loss in their vegetative tissues, being able to remain alive in the dried state for several years and regaining full functionality upon re-hydration (Scott, 2000). Such capacity is associated with an accumulation of trehalose in plant leaves (Iturriaga *et al*., 2000).

Almeida *et al*. (2005) transformed tobacco plants with the *AtTPS1* gene from *Arabidopsis*. The transgenic seeds were germinated on media with different concentrations of mannitol (0, 0.25, 0.5 and 0.75 M) and sodium chloride (0, 0.07, 0.14, 0.2, 0.27 and 0.34 M) to score their tolerance to osmotic stress. Additionally, the transgenic plants were submitted to drought, desiccation (measurement of water loss as a consequence leaf detaching) and temperature stresses (germination at 15 °C and 35 °C). The transformed plants revealed a reduced increase of drought tolerance and dehydration but exhibited a considerable tolerance to osmotic and temperature stresses, indicating that the heterologous expression of *TPS1* gene from *Arabidopsis* can be successfully used to increase abiotic stress in plants.

Zhang *et al*. (2005) transformed tobacco plants with the trehalose synthase gene from *Grifola frondosa*, submitting transformed plants to drought and salinity stresses (MS medium containing 1 % NaCl). Compared with non-transgenic plants, the transgenic ones were able to accumulate high levels of trehalose, which were increased up to 2.126–2.556 mg/g fresh weight, although levels were undetectable in non-transgenic plants. This trehalose accumulation resulted in increased tolerance to drought and salinity improving physiological performance, such as water content in excised leaves, malondialdehyde content, chlorophyll a and b contents, the activity of superoxide dismutase (SOD) and peroxidase (POD) in excised leaves.

Some evaluations of trehalose activity have been also carried out in sugarcane. Wang *et al*. (2005) transferred the trehalose synthase gene from *G. frondosa* to sugarcane, analyzing the tolerance of the transgenics to osmotic stress [PEG8000 17.4 % (w/v)]. While the non-transformed plants began turning yellow at the third day, with wilting and drying extending from old leaves to young leaves in seven days, all transgenic plants kept green and began turning yellow only at the seventh day, indicating their improvement regarding osmotic stress tolerance. Zhang *et al*. (2006) carried out a similar approach using the same gene in sugarcane, generating transgenic plants that accumulated high levels of trehalose

(up to 8.805–12.863 mg/g fresh weight), whereas trehalose was undetectable in non-transgenic plants. Trehalose accumulation in these plants resulted in increased drought tolerance, as shown by the drought physiological indexes, such as the rate of bound water/free water, plasma membrane permeability, malondialdehyde content, chlorophyll a and b contents, and activity of SOD and POD of the excised leaves.

5. SuperSAGE: Looking for osmoprotectants in sugarcane

Besides a review of the up to date evaluations, the present preview analyzes the sugarcane transcriptome under drought, using a combination of high-throughput transcriptome profiling by SuperSAGE with the Solexa® sequencing technology, allowing the *in silico* identification of potential tags related to osmoprotectants in response to this stress. In the scope of this report four libraries have been generated by the Bulked-Extremes SuperTag Digital Gene Expression (BE-STDGE) method (GenXPro GmbH, Frankufurt, Germany), using bulked root tissues from four drought-tolerant materials as compared with four bulked drought-sensible genotypes, aiming to generate a panel of differentially expressed stress-responsive genes. Both groups were submitted to the same experimental conditions at the glasshouses of CTC (a Brazilian Sugarcane Technology Center, in Piracicaba, state of São Paulo, Brazil), including 24 hours of water deficit stress as compared with non stressed controls. The SuperSAGE libraries produced 8,787,315 tags (26 bp) that, after exclusion of singlets, allowed the identification of 205,975 unitags. Most relevant BlastN matches ($42 \leq$ Score ≤ 52; intact CATG sequence and plus/plus alignments) comprised 567,420 tags, regarding 75,404 unitags with 164,860 different ESTs, most of them matching to sequences of the genus *Saccharum*. The coverage of the transcriptome by the tags, considering the number of tags per genotype in relation to the number of expected transcripts per cell (500,000; Kamalay & Goldberg, 1980), was 6.5 times for the tolerant and 5.8 for the sensible bulk, i.e., the number of expected transcripts in a single copy per cell should be represented by around six tags in each library. Coverage of this magnitude permits a comprehensive evaluation of a given transcriptome, including rare expressed transcripts.

5.1 Betaine aldehyde dehydrogenase (BADH)

Regarding BADH (EC 1.2.1.8) seven unitags were identified. Most of them (six) were upregulated (UR) in the first comparison (I) that refers to the bulk of tolerant accessions under stress *versus (vs)* non stressed tolerant control (Table 1). From these UR unitags, only one (SD186519) was also UR in the comparison II (sensible bulks under stress *vs* the sensible control; Table 1). This unitag was aligned (BlastN) with accession gb A275267.1 and TC139975 (SOGI, *Sacharum officinarum* Gene Index, release 3). The first EST refers to a cDNA showing a perfect match while the second regarded a transcript similar to a BADH (UniRef100_Q6BD86) with a single mismatch. Two other UR unitags in the tolerant bulk (SD161066 and SD158219) showed contrasting expression (DR) as compared with the susceptible bulk (comparison II; Table 1). The SD158219 unitag aligned with the same TC139975 of SOGI database, in the 3'UTR region regarding the BADH cDNA of maize (gb BT067636.1). Alignments of tags in the 3'UTR region are expected when using this methodology that is based on cDNAs originated from the vicinity of the poli-A tail of RNAs.

Comparison		I		II		III		IV	
Libraries		DTS/DTC		DSS/DSC		DTS/DSS		DTC/DSC	
Tag	Annotation	FC	Reg	FC	Reg	FC	Reg	FC	Reg
SD186519	BADH	2.2	UR	1.6	UR	1.80	UR	1.33	ns
SD161066	BADH	1.3	UR	-1.4	DR	2.81	UR	1.58	UR
SD158219	BADH	6.4	UR	-5.3	DR	6.36	UR	-5.32	DR
SD160278	BADH	2.0	UR	-1.4	ns	3.44	UR	1.27	ns
SD167799	BADH	2.1	UR	-1.7	ns	1.57	ns	-2.20	DR
SD167796	BADH	3.4	UR	1.1	ns	2.04	ns	-1.51	ns
SD7041	BADH	1.2	ns	-1.3	ns	2.69	UR	1.64	ns
SD68048	P5CS	3.3	UR	4.7	UR	2.0	ns	2.8	UR
SD130985	P5CS	5.8	UR	-1.1	ns	1.2	ns	-5.2	ns
SD154736	P5CR	1.3	ns	-1.1	ns	1.3	ns	-1.1	ns
SD175871	P5CR	1.2	ns	-1.7	ns	1.3	ns	-1.6	ns
SD50849	MIPS	1.3	UR	-2.9	DR	2.22	UR	-1.71	DR
SD50847	MIPS	1.1	ns	-2.3	DR	-1.03	ns	-2.50	DR
SD134872	MIPS	1.1	ns	-3.2	DR	#	ns	-3.41	ns
SD61158	TPS	2.4	UR	1.8	ns	3.62	UR	2.80	UR
SD146286	TPS	2.5	ns	2.8	UR	1.28	ns	1.41	ns
SD267553	TPS	-1.3	ns	-2.7	ns	-1.76	ns	-3.73	DR
SD6994	TPP	6.3	UR	-1.8	DR	7.37	UR	-1.50	ns
SD25600	TPP	2.4	UR	#	ns	2.38	UR	#	ns
SD190162	TPP	7.1	UR	1.5	ns	8.50	UR	1.76	ns

Alt Tra: Alternative transcript version; Chr: chromosome; put: putative; exp.: expressed; Align: Alignment Length; Mis: Mismatch; Orient: Orientation. BADH (Betaine aldehyde dehydrogenase); P5CS (Delta(1)-pyrroline-5-carboxylate synthetase); P5CR (Delta(1)-pyrroline-5-carboxylate reductase); MIPS (Myo-inositol 1-phosphate synthase); TPS (Trehalose-6-phosphate synthase); TPP (Trehalose-phosphatase protein)

Table 1. Comparison of sugarcane SuperSAGE libraries showing tags annotated as osmoprotectant-relative, the respective fold change, and regulation of the tags ($p \leq 0.05$).

On the other hand, the SD161066 unitag aligned with two mismatches in the TC24905 of the PAVIGI (*Panicum virgatum* Gene Index, release 1). This TC presented an annotation against a partially homologue (85 %) *Zea mays* sequence of betaine aldehyde dehydrogenase.

All the unitags were aligned against the *Sorghum bicolor* genome available at the Phytozome site (http://www.phytozome.net/) and the respective cDNAs. From seven BADH unitags three mapped on the genome (SD161066 at chromosome 7; SD160278 and SD7041 both at chromosome 6) (see the loci at the Table 2). As mentioned by Ming *et al*. (1998), the levels and patterns of chromosome structural rearrangement in *Saccharum* and *Sorghum* based in their close relationship, high degree of colinearity, and cross-hybridization of DNA probes, all impel use of the small genome of *Sorghum* to guide molecular mapping and positional cloning in *Saccharum*.

For each identified locus a single transcript was identified in *Sorghum* (Table 2). Further, as shown in Table 2, the unitag SD7041 presented a perfect BlastN alignment (score 52) of +/- (plus/minus) type against the transcript Sb06g019200.1, the same cDNA that aligned to another unitag in the +/+ sense (in this last case presenting some mismatches). A detailed analysis of the +/- alignment revealed its positioning in a complementary 3'UTR region in

Tag ID	Annot	Locus/Alt Tra	Phytozome Annotation	Chr	Identity (%)	Align bp	Mis	Tag start	Tag end	Subject start	Subject end	Orient.	E-value	Score
SD160278	BADH	Sb06g019200.1	aldehyde dehydrogenase, put., exp.	6	96	26	1	1	26	212	237	+/+	3E-05	44
SD161066	BADH	Sb07g020650.1	aldehyde dehydrogenase, put., exp.	7	96	26	1	1	26	1014	1039	+/+	3E-05	44
SD7041	BADH	Sb06g019200.1	aldehyde dehydrogenase, put., exp.	6	100	26	0	1	26	845	820	+/-	1E-07	52
SD130985	P5CS	Sb09g022290.1	amino acid kinase, put., exp.	9	100	22	0	1	22	2457	2478	+/+	3E-05	44
SD130985	P5CS	Sb09g022290.2	amino acid kinase, put., exp.	9	100	22	0	1	22	2263	2284	+/+	3E-05	44
SD68048	P5CS	Sb03g039820.1	amino acid kinase, put., exp.	3	96	26	1	1	26	2394	2419	+/+	3E-05	44
SD154736	P5CR	Sb03g045690.1	pyrroline-5-carboxylate reductase, put., exp.	3	100	26	0	1	26	671	696	+/+	1E-07	52
SD134872	MIPS	Sb01g044290.1	inositol-3-phosphate synthase, put., exp.	1	100	26	0	1	26	1194	1219	+/+	1E-07	52
SD134872	MIPS	Sb01g044290.2	inositol-3-phosphate synthase, put., exp.	1	100	26	0	1	26	1194	1219	+/+	1E-07	52
SD134872	MIPS	Sb01g044290.3	inositol-3-phosphate synthase, put., exp.	1	100	26	0	1	26	652	677	+/+	1E-07	52
SD50847	MIPS	Sb01g044290.1	inositol-3-phosphate synthase, put., exp.	1	100	26	0	1	26	1890	1915	+/+	1E-07	52
SD50847	MIPS	Sb01g044290.2	inositol-3-phosphate synthase, put., exp.	1	100	26	0	1	26	1848	1873	+/+	1E-07	52
SD50847	MIPS	Sb01g044290.3	inositol-3-phosphate synthase, put., exp.	1	100	26	0	1	26	1348	1373	+/+	1E-07	52
SD50849	MIPS	Sb01g044290.1	inositol-3-phosphate synthase, put., exp.	1	96	26	1	1	26	1890	1915	+/+	3E-05	44
SD50849	MIPS	Sb01g044290.2	inositol-3-phosphate synthase, put., exp.	1	96	26	1	1	26	1848	1873	+/+	3E-05	44
SD50849	MIPS	Sb01g044290.3	inositol-3-phosphate synthase, put., exp.	1	96	26	1	1	26	1348	1373	+/+	3E-05	44
SD146286	TPS	Sb04g035560.1	trehalose-6-phosphate synthase, put., exp.	4	96	26	1	1	26	2880	2905	+/+	3E-05	44
SD61158	TPS	Sb04g035560.1	trehalose-6-phosphate synthase, put., exp.	4	96	25	1	1	25	3066	3090	+/+	1E-04	42
SD190162	TPP	Sb07g020270.1	trehalose-6-phosphate synthase, put., exp.	7	96	26	1	1	26	2909	2934	+/+	3E-05	44
SD25600	TPP	Sb07g020270.1	trehalose-6-phosphate synthase, put., exp.	7	96	26	1	1	26	3006	3031	+/+	3E-05	44

Alt Tra: Alternative transcript version; Chr: chromosome; put: putative; exp.: expressed; Align: Alignment Length; Mis: Mismatch; Orient: Orientation. BADH (Betaine aldehyde dehydrogenase); P5CS (Delta(1)-pyrroline-5-carboxylate synthetase); P5CR (Delta(1)-pyrroline-5-carboxylate reductase); MIPS (Myo-inositol 1-phosphate synthase); TPS (Trehalose-6-phosphate synthase); TPP (Trehalose-phosphatase protein).

Table 2. BlastN results of SuperSAGE osmoprotectants-related tags from sugarcane roots under hydric deficif against cDNAs of *Sorghum bicolor* (Phytozome database).

the reverse strand as compared with the genome and the transcript, suggesting a putative NAT (natural antisense transcript). NATs are naturally occurring RNA transcripts that are complementary to other endogenous RNA transcripts. They may regard Cis-natural antisense transcripts (cis-NATs) when transcribed at the same genomic loci of other genes, but from the opposite direction, while trans-NATs are transcribed from different genomic *loci* (Lavorgna *et al.*, 2004). Such antisense transcripts do not compose an uniform group, but present some features in common, with emphasis on the complementarity to the sense genic sequences that may (or not) codify proteins (Faghihi & Wahlestedt, 2009). NATs have been reported in different types of expression assays, including SAGE (Quéré *et al.*, 2004), LongSAGE (Obermeier *et al.*, 2009) and SuperSAGE (Molina *et al.*, 2008).

An annotation against the Phytozome regarding the BADH unitags identified them as aldehyde dehydrogenase, a protein family that includes BADH. Considering their absolute frequency observed (from six to 95 tags per million – tpm - in the tolerant bulk), the sensitivity of the SuperSAGE methodology in detecting rare transcripts could be verified in posterior assays.

For most UR unitags (four out of six) in the tolerant bulk (Table 1), the amount of BADH tags after the drought tolerant stresses (DTS) was significantly higher (comparison III) than that observed for the drought sensible stressed (DSS) bulk. In the absence of stress (comparison IV), both bulks, that are genetically diverse, presented variable expression, depending on the unitag. Still, a single unitag (SD7041) presented no significant expression differences after stress in both comparisons (I and III).

5.2 Delta(1)-pyrroline-5-carboxylate synthetase (P5CS)

With respect to P5CS (EC 2.7.2.11) two unitags were induced in the comparison I (stressed tolerant *vs* tolerant control), while in the comparison II (sensible bulk) a unitag (SD68048) appeared induced (Table 1). For this unitag the observed difference among both bulks under stress was not significant at the studied level ($p \leq 0.05$; comparison III), revealing similar amounts in both bulks 24 hours after drought stress. The same unitag (SD68048) was also UR in the comparison IV (both controls without stress), being significantly most represented than in the tolerant bulk (Table 1).

In turn, the SD130985 unitag was UR in the comparison I (tolerant bulk stressed *vs* control), presenting a higher FC than that estimated for the unitag SD68048 (5.8 *vs* 3.3). Both unitags aligned to the 3'UTR region of the associated ESTs with a single mismatch (data not shown). Considering the alignment against the *Sorghum* genome, both tags mapped, with SD68048 in the chromosome 3 (associated with a transcript) while SD130985 mapped in the chromosome 9 in a region corresponding to two alternative transcripts with different sizes regarding their UTR portions, with no consequences to the CDS and the final protein. The absolute frequencies of these unitags in the sugarcane transcriptome via SuperSAGE varied from three to 19 tpm (tags per million) considering their presence in the tolerant bulk.

5.3 Delta(1)-pyrroline-5-carboxylate reductase (P5CR)

For P5CR (EC 1.5.1.2) two unitags have been observed, but these presented no significant variation in the analyzed SuperSAGE comparisons, being therefore not commented here. After mapping both unitags against the *Sorghum* genome, only the SD154736 tag mapped in the chromosome 3 (Table 2) in a CDS region of an identified transcript.

5.4 Myo-inositol 1-phosphate synthase (MIPS)

Concerning MIPS (EC 5.5.1.4), from three annotated unitags only one (SD50849) was overexpressed (UR) in the comparison I, while all three unitags were repressed (DR) in the comparison II (Table 1). All three unitags mapped in the chromosome 1 of *Sorghum* in the *locus* Sb01g044290, with three predicted alternative transcripts. Two of the tags presented perfect alignments (score 52) with the referred *locus*, one of them (SD134872) aligned in the CDS of the three predicted transcripts, while the other (SD50847) aligned in the region covering the transition from CDS to the first four bases of the 3'UTR. The remaining unitag (SD50849) presented a mismatch with the *Sorghum* genome and also with the respective transcript (Table 2). Interestingly, this unitag represents a possible single base polymorphism (A/G substitution) compared to unitag SD50847. Regarding this polymorphism, sequencing errors are not probable, especially considering the tag frequency. Both unitags were the most frequent in the SuperSAGE libraries, varying from 37 to 59 tpm, while the unitag SD134872 presented less than 3 tpm. This potential SNP and its relation to the differential expression in the tolerant (comparison I) is worth additional efforts for its validation in the future.

5.5 Trehalose-6-phosphate synthase (TPS)

Three unitags have been annotated for TPS (EC 2.4.1.15) one of them UR in the comparison I (SD61158) the second UR in comparison II (SD146286) and the third (SD267553) not varying significantly among the different compared conditions (Table 1). All three unitags mapped against the *Sorghum* genome in the chromosomes 4 and 9 (Table 2). Considering the matching region of chromosome 4, a single *Sorghum* transcript was associated (Sb04g035560.1) similar to a putative uncharacterized protein. Compared to this transcript, the SD146286 unitag presented a substitution (G/A) in a CDS region, while the unitag SD61158 presented two G/A substitutions. From the three unitags, SD61158 was the most expressed, varying from 16 to 39 tpm, while the other two tags were less frequent (< 4 tpm). The alignment against chromosome 9 revealed two mismatches as compared with the unitag SD267553.

5.6 Trehalose-phosphatase protein (TPP)

For trehalose-phosphatase (EC 3.1.3.12) three unitags were UR in the comparison I (Table 1), while one of them (SD6994) was also DR in the comparison II. The mentioned unitag and the SD190162 unitag presented the highest FCs (6.3 and 7.1) for comparison I (Table 1) with the related ESTs sharing 91 % identity in 230 aligned bases. From all three unitags, SD6994 was the most expressed (13 to 83 tpm), followed by SD190162 (3 to 20 tpm) and by SD25600 (< 3 tpm). All three unitags mapped in the sorghum genome, in the chromosome 7, aligning with the transcript Sb07g020270.1 (Table 2) annotated as a putative trehalose-6-phosphate synthase.
The most expressed unitag was SD6994 aligned perfectly with a *Sorghum* transcript at the 3'UTR portion of the sequence. Besides, the unitag SD25600 is similar to the SD6994, with a single A/C substitution. The third unitag (SD190162) also aligned in the 3'UTR of the same *sorghum* transcript in a more distant position in comparison to the 3' end than SD6994 presenting a single mismatch to the *Sorghum* transcript (T in *Sorghum* and C in the tag). If the alignment was perfect, one could argument that it was the consequence of an incomplete digestion by the *Nla*III enzyme. However, in the present work a double digestion was carried out, avoiding this error source.

6. Concluding remarks

The present review highlights how scarce information about sugarcane osmoprotectants are at physiological, genomic and transcriptomic levels. Many crops lack the ability to efficiently synthesize some types of osmoprotectants that are naturally accumulated by stress-tolerant plants. Our SuperSAGE data revealed that all procured osmoprotectants categories are present and expressed in sugarcane. However, most of them are discretely expressed in roots after 24 hours of drought stress and also considering the same tissue in non-stressed controls. These discrete expression and their fold changes, detected by SuperSAGE, would probably remain undetected using other transcriptomics approaches, justifying the scarce previous informations about this protein group in sugarcane. Some identified candidates may have an osmoprotectant role in the initial response against drought in this crop and deserve additional evaluations. Hence, as shown by different research approaches in plants lacking osmoprotectants, their transgenic expression represented dramatic differences in the tolerance and survival to abiotic stresses including drought, salinity and freezing, what may be the case of sugarcane. The present chapter brings the first overview of the sugarcane transcriptome under drought with a combination of the high-throughput transcriptome profiling SuperSAGE technology coupled with a next-generation sequencing platform. This approach allowed the identification of some potential target osmoprotectants candidates in the drought stress response. Validation procedures as well as transient expression assays are planned for future works, aiming to collaborate with breeding and biotechnological approaches for benefit of the sugarcane culture, especially facing the scenario of future climate change.

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5

Effect of UV Light on Secondary Metabolite Biosynthesis in Plant Cell Cultures Elicited with Cyclodextrins and Methyl Jasmonate

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1. Introduction

All the chemical reactions that occur in the cells of a living organism are called metabolism. By these reactions, a large number of organic compounds, including sugars, amino acids, fatty acids, nucleotides and their polymers derived, that is, polysaccharides, proteins, lipids, RNA, DNA, etc ... are produced. These processes are essential and common to all organisms and are known as primary metabolism and related compounds are known as primary metabolites. In the case of plants, in addition to the primary metabolic pathways, other metabolic pathways are activated under certain situations and the compounds produced are called secondary metabolites. The role of secondary metabolites may seem irrelevant, but the truth is that the plant spends a great deal of energy in their synthesis and they have remained in the plant kingdom up to date. This is because that wide variety and high diversity of secondary metabolites have apparently evolved as a means for plants to interact with the environment and for the development of resistance against both abiotic and biotic stress. In fact, secondary metabolites are useful to protect plants against herbivores (insects and vertebrates), mammals, bacteria, fungi, viruses and even other competing plants. In addition, some plants use secondary metabolites to attract pollinators and seed dispersers, as signals for communication between plants and symbiotic microorganisms or for protection against UV light and other physical stress (Wink 2003, 2008).

Daucus carota L. (*Umbelliferae*) is a biennial herb, whose fruits (common name: wild carrot fruits) have been used in traditional Chinese medicine for the treatment of ancylostomiasis, dropsy, chronic kidney disease and bladder afflictions (Pant & Manandhar, 2007) due to a wide range of reported pharmacological effects, including antibacterial (Rossi et al., 2007), antifungal (Tavares et al., 2008), antihelminthic, hepatoprotective (Bishayee et al., 1995) and cytotoxic activities (Yang et al., 2008; Fu et al., 2009). Carrot roots contain a variety of carotenoids and anthocyanins that are responsible for the typical colour of the root. In addition, this vegetable also produces phenolic compounds such as scopoletin, *p*-hydroxy benzoic acid and the isocoumarin, 6-methoxymellein, all major components of the phytoalexin complex (Mercier et al., 2000). These compounds are induced in carrot by fungal infection, heavy metals or UV light (Marinelli et al., 1994), and therefore they are involved in plant defence responses. Recently, Sabater-Jara et al., (2008) have described the production of sterols in different cell cultures including *D. carota.*

Plant sterols, also called phytosterols, are isoprenoid-derived lipids that play essential roles in plant growth and development since they are integral components of the plant cell membranes and are responsible for its permeability and fluidity (Posé et al., 2009). In addition, phytosterols play an important role in cellular processes as precursors for brassinosteroids biosynthesis. They are also components of a wide variety of secondary metabolites such as the glycoalkaloids, cardenolides and saponins. Moreover, phytosterols have important pharmacological activities, including cholesterol-lowering, antitumor effects against lung, stomach, ovary and estrogen-dependent human breast cancer (Woyengo et al., 2009) and recently, they have reported to exert anti-atherosclerotic, anti-inflammatory and anti-oxidative activities in animals (Delgado-Zamarreño et al., 2009). The beneficial health effects of phytosterols have led to search potential strategies for enhancement these compounds from other natural sources. In this sense, the use of plant cell cultures has been developed as a promising alternative, especially when the production of bioactive compounds is difficult or unprofitable, or when it involves serious damage to the environment. In this way, campesterol, stigmasterol, β-sitosterol and fucosterol (Fig. 1), being major phytosterols found in plants, have been recently produced using plant cell systems (Sabater-Jara et al., 2010a,b; Bonfill et al., 2011; Lee et al., 2004 and Herchi et al., 2009).

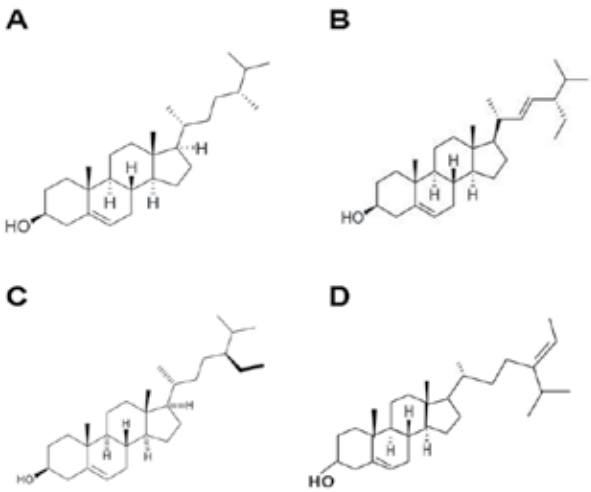

Fig. 1. Structures of Campesterol(A), Stigmasterol (B), β-Sitosterol (C), and Fucosterol (D).

Catharanthus roseus (Madagascar periwinkle) is a perennial tropical plant belonging to the family *Apocynaceae* that produces more than 130 alkaloids (van der Heijden et al., 2004). The importance of this plant relies in its ability to synthesize a wide range of terpenoid indole alkaloids as part of its secondary metabolism. These compounds have vital roles as mediators of ecological interactions, and are very important for plant survival. They are involved in the defense against competitors, herbivores and pathogens, in attracting pollinators or symbionts and in the adaptation to both biotic and abiotic stress conditions. Although they are constitutive compounds, their levels may be enhanced by several factors.
Some terpenoid indole alkaloids have a high added value because of their broad spectrum of pharmacological applications. Special attention has focused on the production of the anti-hypertensive monomeric terpenoid indole alkaloids serpentine and ajmalicine (Fig. 2A), which are used to combat heart arrhythmias and to improve the blood circulation in

the brain (Asada & Shuler, 1989). The dimeric terpenoid indole alkaloids, 3',4'-anhydrovinblastine, vincristine, and vinblastine have powerful effects as anticancer drugs (Zhou et al., 2009). These dimeric terpenoid indole alkaloids are synthesized from vindoline and catharanthine (Fig. 2B). Catharanthine can be chemically coupled with vindoline to form the clinically important anticancer drug, vinblastine and so this could provide a novel and efficient way to produce vinblastine commercially. Vindoline is abundant in *C. roseus* plants, and catharanthine can be produced by *C. roseus* cell or hairy root cultures (Zhao et al., 2001), so a combination of both pathways could lead to a very high vinblastine production. Therefore, the production of catharanthine by various *C. roseus* cell types in culture and its mechanism of biosynthesis has been one of most extensively explored areas of plant cell or hairy root cultures in recent years. In this sense, the application of biotic or abiotic stimuli has been one of the most effective strategies for improving the productivity of terpenoid indole alkaloids from *C. roseus* cell cultures (Zhao et al., 2000).

Vitis vinifera produces stilbenes, which are a small group of compounds characterized by a 1,2-diphenylethylene backbone, derived from the phenylpropanoid pathway. Most plant stilbenes have phytoalexin activity and are derivatives of the monomeric unit *trans*-resveratrol (3,5,4´-trihydroxystilbene, Fig. 2C) although other structures are found in other plant families. In grape berries, stilbenes are synthesized under natural environmental conditions (Jeandet et al., 1991; Versari et al., 2001). The *cis*- and *trans*-isomers of resveratrol are mainly accumulated in the exocarp (skin) during all stages of development and are almost totally absent from pericarp (flesh). Monomers and oligomers of resveratrol are also constitutively present in the lignified organs of grapevine such as stems and roots (Jeandet et al., 2002). Therefore, both pre-existing high contents of stilbenes in plants and those synthesized after microbial attack are part of both constitutive and inducible defence responses. In addition to the well-known function of stilbenes as phytoalexins, these compounds may also be involved as chemical signals in allelopathy (Seigler et al., 2006), or in response to oxidative stress generated by UV irradiation (He et al., 2008; Privat et al., 2002; Teguo et al., 1998). The formation of stilbenes (namely viniferins in Vitis) is therefore considered to be a part of the general defense mechanisms since they also display strong antifungal and antimicrobial activities (Bru et al., 2006; Pezet et al., 2004; Morales et al., 1998). In fact, *trans*-resveratrol, is found in both grapevine tissue and berries, and in cell cultures as the result of both abiotic and biotic stress (Pezet et al., 2003, 2004; Cantos et al., 2003).

Since *trans*-resveratrol was postulated to be involved in the health benefits associated with a moderate consumption of red wine (Siemann & Creasy, 1992), it is one of the most extensively studied natural products.

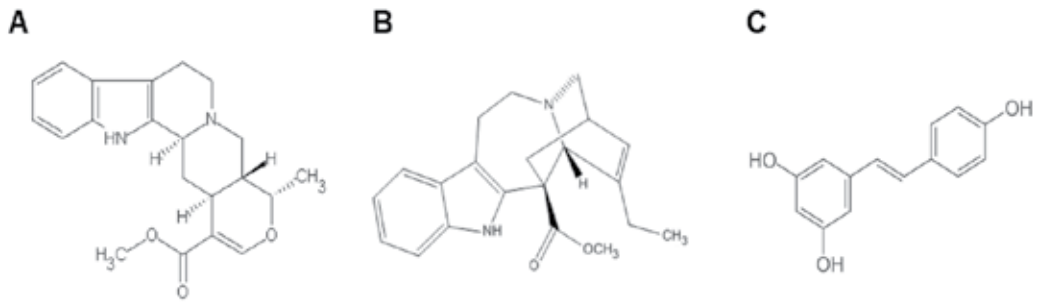

Fig. 2. Structures of ajmalicine (A), catharanthine (B) and *trans*-resveratrol (C).

Over the last 15 years, stilbenes, especially *trans*-resveratrol has received considerable interest, due to their biological activities and possible pharmacological applications. Hundreds of studies have reported the beneficial effects of *trans*-resveratrol on neurological (Okawara et al., 2007) and cardiovascular systems (Bradamante et al., 2004). One of the most striking biological activities of *trans*-resveratrol investigated during recent years has been its anticancer activity and it has been seen to prevent carcinogenesis in the stages of tumour initiation, promotion and progression (Pervaiz, 2003; Pezzuto, 2008). More results provide interesting insights into the effect of this compound on the lifespan of yeast, worms and flies, suggesting that *trans*-resveratrol could be regarded as a potential antiaging agent in treating age-related human diseases (De la Lastra & Villegas, 2005). In addition, effects described in mice subjected to a high-calorie diet (Baur et al., 2006) point to new approaches for treating not only age-related diseases but also obesity-related disorders (Kaeberlein & Rabinovitch, 2006). For these reasons, the wide ranging of pharmacological and clinical potential applications of *trans*-resveratrol and other stilbenes have been recently reviewed (Pezzuto, 2008; Shakibaei et al., 2009; Espín et al., 2007). That is why new strategies based on the use of *V. vinifera* cell cultures have been used to increase the level of *trans*-resveratrol production.

The close relationship between plant secondary metabolism and defence response is widely recognized. Plants not only respond to attack of pathogens, insects and herbivores or to other biotic and abiotic stresses but also to small molecules of different origin, called elicitors that trigger the same response in the plant as the pathogen or organism itself. When introduced in a living cell in small concentrations, elicitors are capable of re-directing the metabolism, leading to increased production of particular secondary metabolites. Then, elicitors are useful tools for improving the production of plant valuable secondary metabolites. In general, elicitors are classified on the basis of their origin and molecular structure. Biotic elicitors are derived from the pathogen or from the plant and can have a defined composition, when their molecular structures are known, or have a complex composition when they comprise several different molecular classes making impossible to define a unique chemical identity. On the other hand, abiotic elicitors have not a biological origin and are grouped in physical and chemicals factors (Vasconsuelo & Boland, 2007).

A wide array of external stimuli are capable of triggering changes in the plant cell, leading to a cascade of reactions that ultimately result in the formation and accumulation of secondary metabolites, which help plants to overcome the stress factors. Amongst these, ultraviolet (UV) light which is a minor part of the solar spectrum, represents an important ecological factor that influences the organisms and ecosystems, and it is related to the occurrence of some adaptive changes in organisms throughout the development of life on Earth.

UV radiation is divided into three regions: UV-C (wavelengths below 280 nm), UV-B (280-315 nm) and UV-A (315-400 nm). UV-C is the most damaging, but it is almost completely absorbed by the stratosphere. By contrast, UV-B radiation is only partially absorbed by the stratospheric ozone layer, and UV-A is not absorbed at all. Therefore, a fraction of UV-B and all UV-A reaches the earth's surface, where they cause various biological effects. Moreover, the effectiveness of biological responses to UV radiation increases with decreasing wavelength, so these responses are normally dominated by the UV-B. However, in recent years UV-A action spectra have also been considered.

Accumulation of UV-absorbing compounds, mainly those of phenolic nature, is a typical defense mechanism of plants to increased UV radiation, and is the most common response produced by vascular plants (Searles et al., 2001). Derivatives of hydroxycinnamic acid are UV-absorbing compounds which have received less attention, probably because they have been considered as constitutive, rather than inducible, protective barrier, against UV-B radiation (Bornman et al., 1997). However, they absorb UV-B more effectively than flavonoids, the other UV-absorbing compounds, whose absorption peaks are shifted to the UV-A radiation. As a result, derivatives of hydroxycinnamic acid may provide greater attenuation of UV-B radiation than flavonoids (Sheahan, 1996).

In plant cell cultures, UV light acts as an abiotic factor which stimulates the biosynthesis of secondary metabolites (Broeckling et al., 2005). Thus, it has been shown that UV-B light induces both the formation of dimeric terpenoid indole alkaloids and *tryptophan decarboxylase and strictosidine synthase* mRNA accumulation in *C. roseus* (Ouwerkek et al., 1999). Ramani & Jayabaskaran (2008) also observed the enhanced production of catharanthine and vindoline from *C. roseus* cell cultures, when cells were irradiated with UV-B for 5 min. In a similar way, Gläβgen et al., (1998) studied the effect of continuous irradiation with UV-containing white light (315-420 nm) on the anthocyanin content of *D. carota* cell cultures after 7 days of culture, and observed that the total anthocyanin concentration was strongly enhanced by the UV treatment.

The effect of UV irradiation on stilbene content in grapevine cell cultures is little known and most of the research related with UV light has been directed at enhancing the stilbene content of grape berries (Adrian et al., 2000; Versari et al., 2001; Cantos et al., 2003), leaves (Langcake & Pryce 1977; Pezet et al., 2003) and callus tissue (Keller et al., 2000; Keskin & Kunter 2008, 2010). In addition, when Keller et al., (2000) studied stilbene accumulation in callus of grapevine irradiated with UV light, they found that only actively growing callus was capable of producing stilbenes (including *trans*-resveratrol), whereas old callus had lost this ability. This response was similar to that found in ripening grape berries, which gradually lose their potential for synthesizing stilbenes as they approach maturity.

On the other hand, special attention has been paid to the use of chemical compounds such as β-cyclodextrins (CDs, Fig. 3) which are cyclic oligosaccharides consisting of seven α-D-glucopiranose residues linked by α (1→4) glucosidic bonds formed by the enzymatic modification of starch. These compounds chemically resemble the alkyl-derived pectic oligosaccharides naturally released from the cell walls during fungal attack (Bru et al., 2006), thus they have been used to increase both the biosynthesis of *trans*-resveratrol and its secretion to the extracellular medium in *V. vinifera* cell cultures (Morales et al., 1998; Bru & Pedreño, 2003; Bru et al., 2006). Recently, Zamboni et al., (2009) reported that CDs trigger a signal transduction cascade which activates different families of transcription factors in grapevine cells, inducing a halt in cell division, reinforcement of the cell wall and the biosynthesis of *trans*-resveratrol and defence-related proteins. The method based on the use of CDs (Bru & Pedreño, 2003) differs from those that use other elicitors (Liswidowati et al., 1991) not only in the high levels of *trans*-resveratrol produced but also in the extraction process of this compound. Thus, in traditional elicitation methods, *trans*-resveratrol is extracted from elicited cells gives low yields, whereas in this new process, *trans*-resveratrol is secreted as it is produced by cells and recovered directly from the spent media with no biomass destruction. In addition, the high levels of *trans*-resveratrol accumulated in the culture medium were seen to have no toxic effect on the cell lines, allowing successful subcultures. This innovative elicitation process is mainly based on the CD characteristics.

They have a hydrophilic external surface and hydrophobic central cavity that can trap hydrophobic compounds, including *trans*-resveratrol. This hydrophobic cavity forms inclusion complexes, which in the case of CDs with *trans*-resveratrol, is of the 1:1 type, altering its physicochemical behaviour and making it a highly water-soluble compound (Morales et al., 1998).

This procedure has successful been applied to the production of phytosterols. Thus, Sabater-Jara et al., (2008) have developed a method based on the use of CDs to enhance phytosterol production by using *D. carota* cell cultures since these cyclic oligosaccharides are able to induce a cascade of cellular events that gives rise to the accumulation of phytosterols.

Methyl jasmonate (MJ, Fig. 3) is considered a key molecule in the signal transduction pathway involved in the induction of the biosynthesis of secondary metabolites which take part in plant defence reactions (Gundlach et al., 1992; Creelman & Mullet, 1997; Staswick et al., 1998; Chung et al., 2003). In this way, the application of MJ alone or in combination with CDs triggers the accumulation of secondary metabolites in *Solanaceae* cell cultures, e.g. capsidiol and solavetivone in *Capsicum annuum* (Ma, 2008; Sabater-Jara et al., 2010b). Lee-Parsons et al., (2004) showed that *C. roseus* cell cultures respond to MJ by increasing extracellular accumulation of ajmalicine whose production was dependent on MJ dose and elicitation time. Almagro et al., (2010) demonstrated that the maximum level of ajmalicine produced by cells and secreted to the media was reached when cell suspensions were incubated in the presence of MJ and CDs, production being around 2.2-fold higher than when cells were treated only with CDs.

Moreover, Tassoni et al., (2005) showed that MJ was highly effective in stimulating endogenous *trans*-resveratrol accumulation, as well as promoting its release into the extracellular medium of *V. vinifera cv* Barbera cell cultures. In this case, the endogenous *trans*-resveratrol accumulation was around 24 µg/g dry weight (DW) and the *trans*-resveratrol secreted to the medium was over 8 µg/g DW. In a similar way, Belhadj et al., (2008) described the production of 0.6 mg *trans*-resveratrol/g DW in *V. vinifera* cv Gamay elicited with MJ in a culture medium with the sugar concentration increased. However, the most significant success in increasing *trans*-resveratrol content in grapevine cell cultures has been reached using CDs alone or in combination with MJ (Pedreño et al., 2009). Lijavetzki et al., (2008) analysed the effects of MJ, CDs and a combination of both on extracellular *trans*-resveratrol production and the expression of stilbene biosynthetic genes in grapevine cell cultures. MJ and CDs significantly but transiently induced the expression of stilbene

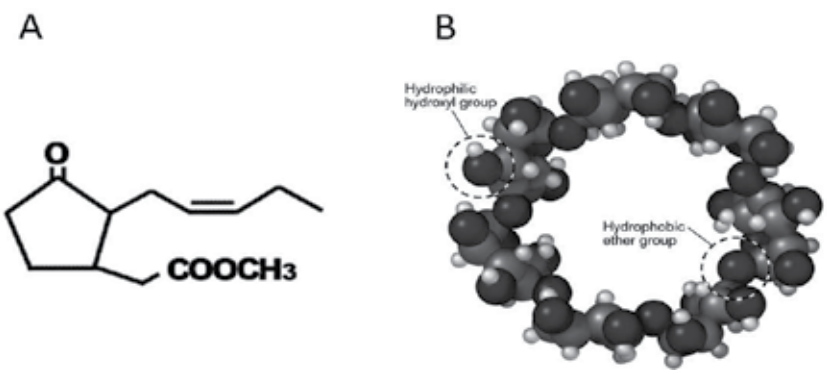

Fig. 3. Structures of methyl jasmonate (A) and cyclodextrins (B).

biosynthetic genes when used independently to treat grapevine cells. Such expression correlated with *trans*-resveratrol production in CD-treated cells but not in MJ-treated cells. In the combined treatment involving CDs and MJ, *trans*-resveratrol production which is secreted to the spent medium, reached a maximum value (360 mg/g DW) that was correlated with the maximum expression levels of stilbene biosynthetic genes, demonstrating the synergistic effect of the combination of MJ with CDs (Lijaveztky et al., 2008).
Based on these evidences, the main objective of this chapter is to show the effect of UV light on the production of secondary metabolites in *C. roseus, V. vinifera* and *D. carota* cell cultures elicited with CDs and MJ, alone or in combination.

2. Effect of UV light exposure, CDs and MJ on the production of *trans*-resveratrol

The production of *trans*-resveratrol in cell cultures of *Vitis sp* has been analyzed by several groups (Kiselev 2011 and see therein). Analysis of *trans*-resveratrol production in untreated *Vitis* cell cultures (Teguo et al., 1996; Morales et al., 1998; Krisa et al., 1999; Tassoni et al., 2005) revealed a low level of *trans*-resveratrol accumulation, less than 0.01% DW or 2-3 mg/l. Therefore, various strategies such as the use of biotic and abiotic elicitors, addition of biosynthesis precursors and genetic transformation have been considered to improve the production of *trans*-resveratrol.
Fig. 4 shows the level of *trans*-resveratrol (6.72 ± 1.03 mg/g FW) when Monastrell cell cultures were incubated with 50 mM CDs and 100 µM MJ, and how this value was higher than when cell cultures were treated only with CDs (3.15 ± 0.35 mg/g FW). However, very low amounts of *trans*-resveratrol were detected in the spent medium when grapevine cell cultures were elicited only with MJ, and no *trans*-resveratrol was detected in cell cultures treated with ethanol, the solvent in which MJ is delivered to the culture (data not shown). In a similar way, Krisa et al. (1999) described that the amount of total stilbenes secreted to the culture medium was negligible in both MJ and control cultures of three *V. vinifera* cultivars. These authors reported that piceid (glucosylated form of resveratrol) accumulation inside the grape cells of Cavernet-Sauvignon cultivar was notably induced when 25 µM MJ was added to the induction medium on day 6 (6.3 ± 0.2 mg piceid/g DW). Very low levels of piceid in cells (9.75 ± 1.17 µg/g DW) both in the presence of 25 µM MJ as when 25µM MJ and 50 mM CDs were jointly used as inducers (82.7 ± 10.9 µg/g DW, Lijavetzki et al., 2008). However, under the last conditions described above, the accumulation of *trans*-resveratrol in the extracellular medium was 212.6 ± 12.6 mg /g DW that is, 10.6 ± 0.6 mg/g FW (Pedreño et al., 2009).
On the other hand, Kiselev et al. (2007) reported a high *trans*-resveratrol production in *V. amurensis* callus cultures transformed with the *rol*B gene of *Agrobacterium rhizogenes* (31.5 mg *trans*-resveratrol/g DW) which was 6.7 times lower than those obtained in the culture medium under conditions described above using CDs and MJ jointly.
UV-C light has been described as a physical inductor of stilbene biosynthesis in *Vitis sp*. As mentioned above, there are no reports on *trans*-resveratrol production in grapevine cell cultures elicited with UV-C light and most of the research related with UV-C light has been directed at enhancing the stilbene content of grape berries (Adrian et al., 2000; Versari et al., 2001; Cantos et al., 2003; González-Barrio et al., 2006), leaves (Douillet-Breuil et al., 1999; Pezet et al., 2003) and callus tissue (Keller et al., 2000; Keskin & Kunter 2008). Keller et al.,

(2000) found that only actively growing calli of grapevine cv Cabernet-Sauvignon irradiated with UV-C light were capable of producing stilbenes, whereas old calli had lost this ability. Similar results were described by Keskin & Kunter (2008) working with Cabernet-Sauvignon callus cultures irradiated with UV-C light. They found that the effect of UV-C light on *trans*-resveratrol production was dependent on callus age since the highest *trans*-resveratrol production was found in 12 day old calli (62.66 ± 0.40 µg *trans*-resveratrol/g FW) in comparison with those values obtained in 15 day old calli (18.12 ± 0.10 µg *trans*-resveratrol/g FW) at the same irradiation time (15 min). In our experiments, Monastrell cell cultures treated with and without MJ and exposed to different UV-C light exposure times produced a negligible extracellular amount of *trans*-resveratrol and browning cell cultures (data not shown). The results obtained by Keskin & Kunter (2008) could explain the low

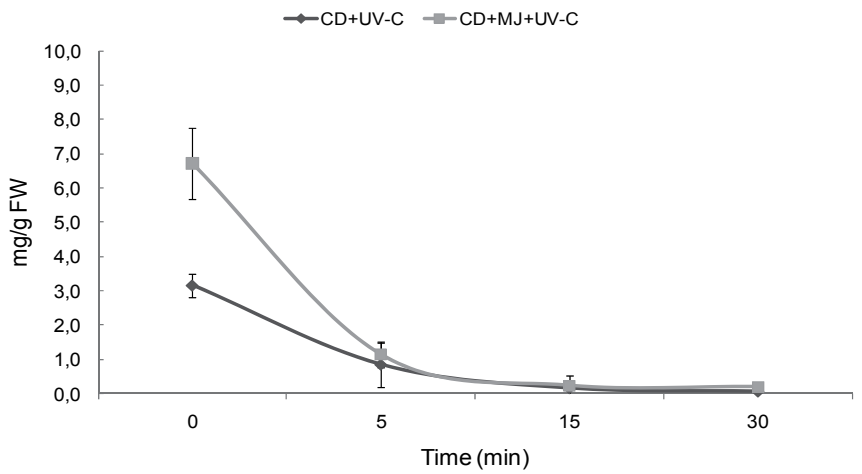

Fig. 4. Effect of UV-C light exposure on the production of *trans*-resveratrol in Monastrell cell cultures elicited in presence of CDs individually or in combination with MJ. Elicitation experiments were performed in triplicate using 14 day old *V. vinifera* cv Monastrell cell cultures. At zero time, 4 g of fresh weight (FW) of washed cells were transferred into 100 ml flasks and suspended in 20 ml of Gamborg B_5 medium supplemented as described Bru et al., (2006), and in the presence of CDs alone or in combination with MJ. After that, cell cultures were maintained in a rotary shaker during 96 h at 25 °C in darkness. Control treatments without elicitors were always run in parallel (data not shown). In the other cases, elicitation was carried out under UV light at different exposure times in the presence of CDs alone or in combination with MJ. For this, flasks were opened in a laminar flow hood and exposed to UV-C light (254 nm, 10µW/cm^2) at an irradiation distance of 15 cm. During this time and after irradiation, flasks were kept in continuous agitation for 96 h. After elicitation, cells were separated from the culture medium under a gentle vacuum and the spent medium of *V. vinifera* cell cultures was used for quantifying the *trans*-resveratrol. For this, aliquots of the spent medium of *V. vinifera* were diluted with water and methanol to a final concentration of 80% methanol (v/v). 20 µl of diluted and filtered samples were analysed in a HPLC-DAD as described Bru et al., (2006). *trans*-Resveratrol was identified (at 304 nm) and quantified by comparison with commercial *trans*-resveratrol of >99% purity. Values are given as the mean ± SD of three replicates.

trans-resveratrol levels found when Monastrell cell cultures were exposed to UV-C light (15 min) and elicited with CDs separately (0.16 ± 0.21 mg *trans*-resveratrol/g FW), and in combination with MJ (0.24 ± 0.29 mg *trans*-resveratrol/g FW), because these elicitation experiments were performed using 12-14 day old grapevine cell cultures which are just entering in their stationary phase.

Moreover, as shown in Fig. 4, Monastrell cell cultures treated with CDs and MJ, followed by short or long exposures to UV-C light, showed lower *trans*-resveratrol levels (5 min, 1.14 ± 0.36 and 30 min, 0.20 ± 0.29 mg *trans*-resveratrol/g FW) than UV-unexposed cells (6.72 ± 1.03 mg *trans*-resveratrol/g FW), so that UV-C light exposure was clearly detrimental to *trans*-resveratrol production. In fact, prolonged exposure to UV-C light (between 15 and 30 min, Fig.4) or even 120 min (data not shown) caused a drastic reduction in *trans*-resveratrol accumulation although no cell browning was observed.

However, when Monastrell cell cultures were jointly elicited with CDs and MJ and exposed to UV-A light (Fig. 5), the maximal level of *trans*-resveratrol was found at long exposures (30 min, 8.26 ± 0.48 mg/g FW, 90 min, 7.20 ± 1.14 mg/g FW) although no significant differences were found between CD+MJ-treated cells exposed to UV-A light at these long times and unexposed cells treated with the same chemicals elicitors (6.72 ± 1.03 mg/g FW). By the contrary, at short UV-A light exposures, a drop in the production of *trans*-resveratrol was observed and this decrease was more drastic when cells were elicited with CDs and MJ jointly (15 min, 3.18 ± 0.62 mg/g FW) than in CD-treated cells (2.20 ± 0.15 mg/g FW) in comparison with unexposed-cells (Fig. 5). In addition, when grapevine cell cultures were elicited with CDs and exposed to UV-A during 30 min, a slight increase in the production of *trans*-resveratrol (4.50 ± 0.30 mg/g FW) was detected in comparison with unexposed CD-treated cells (3.15 ± 0.35 mg/g FW).

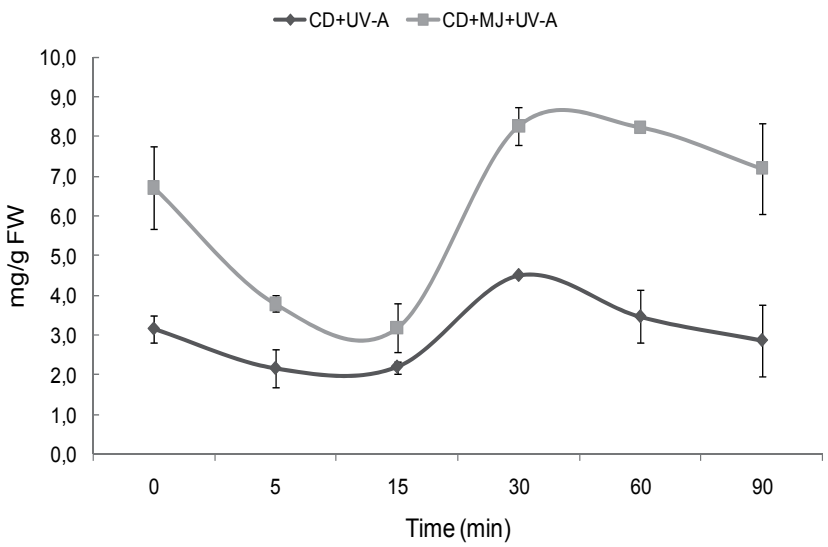

Fig. 5. Effect of the exposure of Monastrell cell cultures to UV-A light (360 nm, 10μW/cm²) in presence of CDs individually or in combination with MJ in cells elicited for 96 h. Elicitation experiments and analysis of *trans*-resveratrol in the culture medium were performed as described in the legend of the Fig.4. Values are given as the mean ± SD of three replicates. Solid lines represent mg *trans*-resveratrol/g FW.

All these results suggested that long UV-A light exposures (30-60 min) only increased slightly the levels of *trans*-resveratrol when cell cultures were elicited with CDs, and did not enhance *trans*-resveratrol levels when MJ was also present, so it seems that there is an antagonistic effect between MJ and UV-A light since short UV-A light irradiation decreased drastically the production of *trans*-resveratrol when cells were elicited in the presence of MJ. In addition, when grapevine cell cultures elicited with or without MJ in the absence of CDs, and exposed to UV-A light for 15 and 30 min, neither *trans*-resveratrol nor cell browning was detected (data not shown).
The results suggested that the effect of UV light on *trans*-resveratrol production was dependent not only on exposition time (short or long) and UV light type (C or A) but also on the presence of one or two chemical elicitors (CDs and/or MJ).

3. Effect of UV light exposure, CDs and MJ on the production of indole alkaloids

Fig. 6. shows the effect of the exposure of *C. roseus* cell cultures to UV-C light in the presence of 50 mM CDs and 100 µM MJ, separately or in combination. As can be observed, the extracellular accumulation of ajmalicine is dependent on UV-C light time exposure since both short and long UV-C light exposures increased ajmalicine levels in all treatments. However, the maximal levels of ajmalicine were reached when *C. roseus* cell cultures were exposed at UV-C light during 30 min and these levels of production decreased as UV-C exposures increased. In fact, long UV-C light irradiation (60-90 min, Fig. 6) or even more (120 min, Almagro et al., 2010) caused a reduction in ajmalicine production in comparison with 30 min UV-C treatment (Fig. 6). However, 15 min UV-C irradiation was equivalent to long exposures (60-90 min) since no significant differences in ajmalicine production were found. In addition, short and long UV-C exposures had no stimulatory effect when *C. roseus* cell cultures were elicited only with MJ (data not shown). Similarly, control cells treated with short and long UV-C light showed similar low levels of ajmalicine to those unexposed control cells (data not shown). All these results suggested, besides the additive effect observed on ajmalicine accumulation provoked by the joint presence of CDs and MJ, that there was a synergism between these elicitors and UV-C light, and an antagonism between MJ and UV light (short and long exposures) when these elicitors are used to stimulate cells in the absence of CDs (data not shown). This fact may be due to the induction of some sort of stress that does not involve an increase in the production of ajmalicine, while UV-C light exposure may enhance the ajmalicine production but the elicitation in the presence of CDs is needed to provoke any enhancement. Zhao et al., (2001) also observed a synergistic effect on indole alkaloid accumulation in *C. roseus* cell cultures elicited with fungal preparations and chemicals. Among them, the combination of tetramethyl ammonium bromide and *Aspergillum niger* mycelial homogenate gave a maximal ajmalicine production of 0.84 ± 0.05 mg/g DW and an improved catharanthine accumulation of 0.57 ± 0.04 mg/g DW, values that are below those obtained in the best production conditions described above.
Peebles et al., (2009) demonstrated that the octadecanoid pathway, which is involved in the biosynthesis of jasmonates does not actively control the production of indole alkaloids under normal or UV-B stress conditions in *C. roseus* hairy roots. Their results also suggested that the role of the octadecanoid pathway in the abiotic or biotic stress response may differ, depending on the stress or culture type. In *C. roseus* cell cultures, the octadecanoid pathway was active when cells were exposed to biotic compounds (e.g. partially purified yeast

extracts, Menke et al., 1999). If we consider that CDs act in a similar way to fungal elicitors because of they chemically resemble to the alkyl-derived pectic oligosaccharides naturally released from the cell walls during fungal attack (Bru et al. 2006), results described by Peebles et al., (2009) could be agreed with the antagonistic effect observed in our experiments when only MJ and UV light (short and long exposures) were used to elicit *C. roseus* cell cultures.

As regards the production of catharanthine, its level increased when cells were treated with CDs separately or in combination with MJ and exposed to UV-C light both short and long exposure times (Fig. 6). However, the maximal level of catharanthine was observed when cells were exposed 30 min to UV-C irradiation and elicited both with CDs and CDs plus MJ, and no significant differences were found in the rest of experiments under UV light exposure (Fig. 6). There are not reports about how the exposition to UV-C affect to indole alkaloid production and only the effect of UV-B has been tested. In fact, Ramani & Jayabaskaran (2008) observed the enhanced production of catharanthine and vindoline from *C. roseus* cell cultures, in where increased their levels 3 and 12 fold, respectively when cells were irradiated with UV-B for 5 min. Although we do not test the effect of UV-B, these results are in accordance with our results since different UV-C exposition times increased the production of catharanthine.

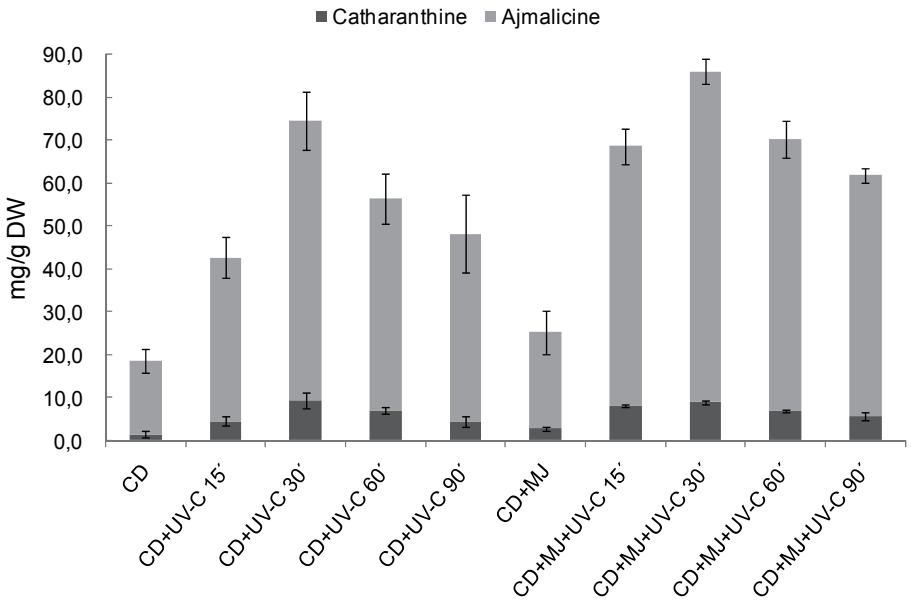

Fig. 6. Effect of different UV-C light exposure times on extracellular accumulation of ajmalicine and catharanthine in cell cultures of *C. roseus* elicited with CDs alone or in combination with MJ.

C. roseus cv First Kiss Apricot cell cultures were obtained as described Almagro et al., (2010). Elicitation experiments were performed in triplicate using 10 day old *C. roseus* cell cultures. At zero time, cell cultures were elicited in the presence of CDs alone or in combination with MJ and they were maintained at 25°C in a rotary shaker at 110 rpm in darkness. UV light treatments were carried out as described in the legend of Fig. 4. Results were evaluated 96 h after treatments. Ajmalicine and catharanthine were extracted and analysed as described Almagro et al., (2010). Values are given as the mean ± SD of three replicates.

In relation to the effect of UV-A, cell cultures treated with or without MJ and exposed to UV-A light were not able to produce indole alkaloids nor accumulate them in the culture medium (data not shown). However, when *C. roseus* cell cultures were elicited in the presence of CDs and exposed to UV-A light for 15 and 30 min, the production of ajmalicine increased in relation to non-exposed cells (data not shown) and this increase was similar to that observed in cell cultures elicited, in the same conditions, and irradiated with UV-C light (Fig. 6).

The combination of these three elicitors synergistically increased more the level of indole alkaloids than when each single or two elicitors are used. The reasons why this combination of three elicitors promoted best effects on indole alkaloid production are still not known. Because each chemical elicitor (CDs and/or MJ) or UV light stimulates indole alkaloid accumulation by different pathways, the mechanism for every treatment may be result of combining them since it depends on the interactions between physiological effects caused by the three elicitors.

4. Effect of UV light exposure, CDs and MJ on the production of phytosterols

Fig. 7 shows the effect of different UV-C light exposure times on phytosterol production in *D. carota* cell cultures elicited in the presence of CDs alone or in combination with MJ. As can be observed in this figure, the production of these four phytosterols was greater when cell cultures were elicited in the presence of CDs alone than in those combined with MJ. In addition, control cells elicited with or without MJ and exposed to UV-C light for different exposure times (15, 30 and 60 min) did not produce any of these phytosterols (data not shown). When cell cultures were elicited only with CDs and exposed to UV-C irradiation, total production levels of phytosterols were kept identical to that of unexposed cells and these levels only decrease when the UV light exposure is prolonged (60 min). However, when the cells were elicited with CDs and MJ, a slight enhancement in the total content of phytosterols was observed as the UV-C light exposure times increased (Fig. 7). These results suggested that the production of phytosterols is sustained during UV exposure time through the joint presence of the three elicitors. In these conditions, production levels of fucosterol were lower than those of β-sitosterol and these, in turn, lower than those of stigmasterol. This low level of fucosterol can be explained by knowing its biosynthetic pathway. All of them are biosynthesized via the mevalonate-dependent isoprenoid pathway. After the synthesis of squalene catalyzed by squalene synthase and its epoxidation catalyzed by squalene epoxidase, 2,3-oxidosqualene is mainly cyclized to cycloartenol which requires cycloartenol synthase (Boutté & Grebe, 2009). Following conversion of 2,3-oxidosqualene to cycloartenol, the first cycle structure providing the basic sterol skeleton, the pathway is essentially linear until reaching 24-methylene lophenol. After formation of this compound, there is a bifurcation to either 24-methyl sterols, which include campesterol and its derivatives, the brassinosteroids, or 24-ethyl sterols, which include the structural sterols fucosterol, β-sitosterol and stigmasterol (Clouse, 2002; Posé et al., 2009).

The effect of different UV-A light exposure times on phytosterol production in *D. carota* cell cultures elicited in the presence of CDs alone or in combination with MJ is shown in Fig. 8. Similarly to results described above, when *D. carota* cell cultures were elicited with CDs and exposed to different UV-A irradiation times, total phytosterol content remained

identical to that of unexposed cells and its level decreased only when the exposition to UV-A was prolonged (Fig. 8). However, the addition of the third elicitor (UV-A) to cells elicited with CDs plus MJ provoked a biphasic response in the production of phytosterols reaching a maximal level when cell cultures were exposed 30 min to UV-A light. Also, the levels of fucosterol were lower than their derivatives, β-sitosterol and stigmasterol. It is also worth noting that the production of β-sitosterol was identical to that of campesterol (Fig. 8).

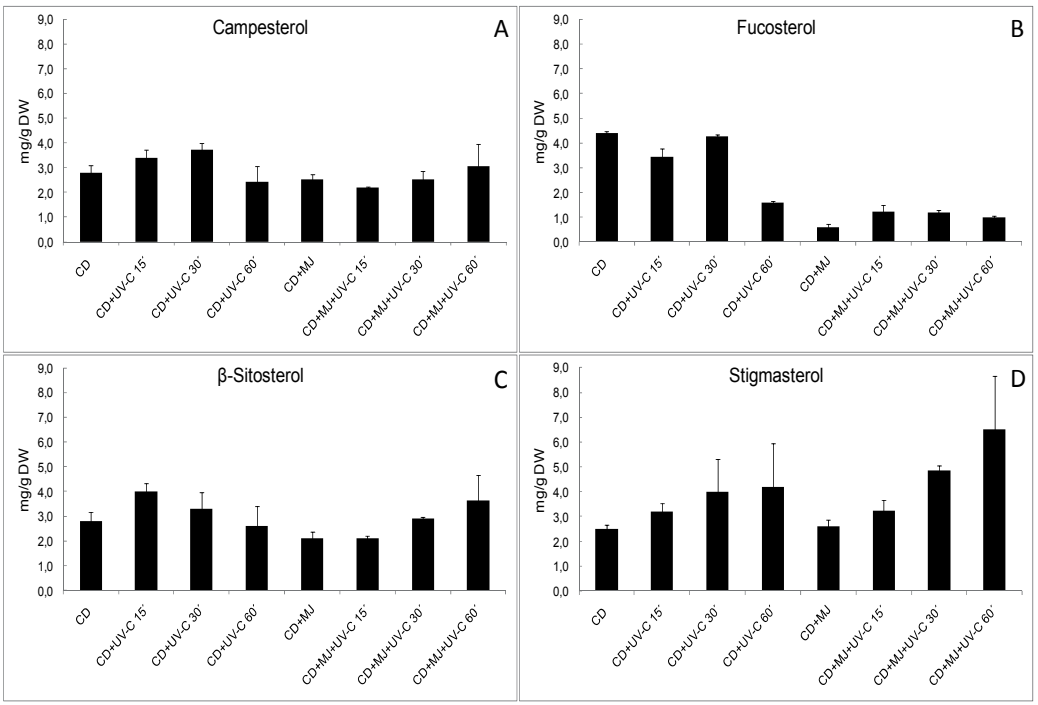

Fig. 7. Effect of different UV-C light exposure times on extracellular accumulation of phytosterols in cell cultures of *D. carota* elicited with CDs alone or in combination with MJ. *D. carota* calli were established in our laboratory in 2005 from root explants and they have been maintained at light at 25 ºC in 250 ml flasks containing 100 ml of Murashige & Skoog medium supplemented as described Sabater-Jara et al., (2008). *D. carota* cell cultures were initiated by inoculating friable callus pieces into 250 ml flasks containing 100 ml of the same medium without agar and were maintained at 25ºC under a 16-h light/8-h dark photoperiod at 25°C in a rotary shaker at 110 rpm. Elicitation experiments were performed in triplicate using 10 day old *D. carota* as described in the legend to the Fig. 4. Results were evaluated 96 h after treatments. Extraction, analysis and identification of different phytosterols in the culture medium were carried out as described Sabater-Jara et al., (2010b). Values are given as the mean ± SD of three replicates. Bars represent mg of different phytosterols/g DW.

As regards the biosynthesis of major components of the phytoalexin complex described in *Daucus*, the isocoumarin, 6-methoxymellein was detected in those cell cultures jointly elicited with CDs plus MJ both UV-A irradiated and non-irradiated, and its level decreased as UV-A

exposure time increased (data not shown). Similarly, the accumulation of UV-absorbing compounds, mainly those of phenolic nature, that is, phenylpropanoid derivatives, were observed. In fact, eugenol and isoeugenol were identified both CD- and CD plus MJ-treated cells. The content of these phenols decreased when CD-treated cells were exposed to UV-A light. By the contrary, the levels of these compounds increased in cell cultures elicited with CDs plus MJ and exposed to a short UV-A irradiation (15 min), and they returned to decrease when UV-A exposure time is prolonged (60 min) (data not shown).

Gläβgen et al., (1998) reported that the biosynthesis and accumulation of anthocyanins in carrot cell cultures was strongly enhanced by continuous irradiation with UV-containing white light (315-420 nm) and that was preceded by the corresponding induction of the enzymes activities of the phenylpropanoid and flavonoid pathways. These authors also showed that the treatment with UV-A light and fungal elicitors resulted in a rapid induction of the phenylpropanoid pathway, whereas the inducing effect of UV-A light on the anthocyanin content, on chalcone synthase and on the enzymes catalyzing the final steps of anthocyanins biosynthesis was suppressed. Their results indicated a coordinated regulation of the enzymes involved in anthocyanins biosynthesis, an independent inducibility of the phenylpropanoid pathway, and a hierarchy of the different effectors, as shown by the dominating role of the fungal elicitor signal over the UV stimulus.

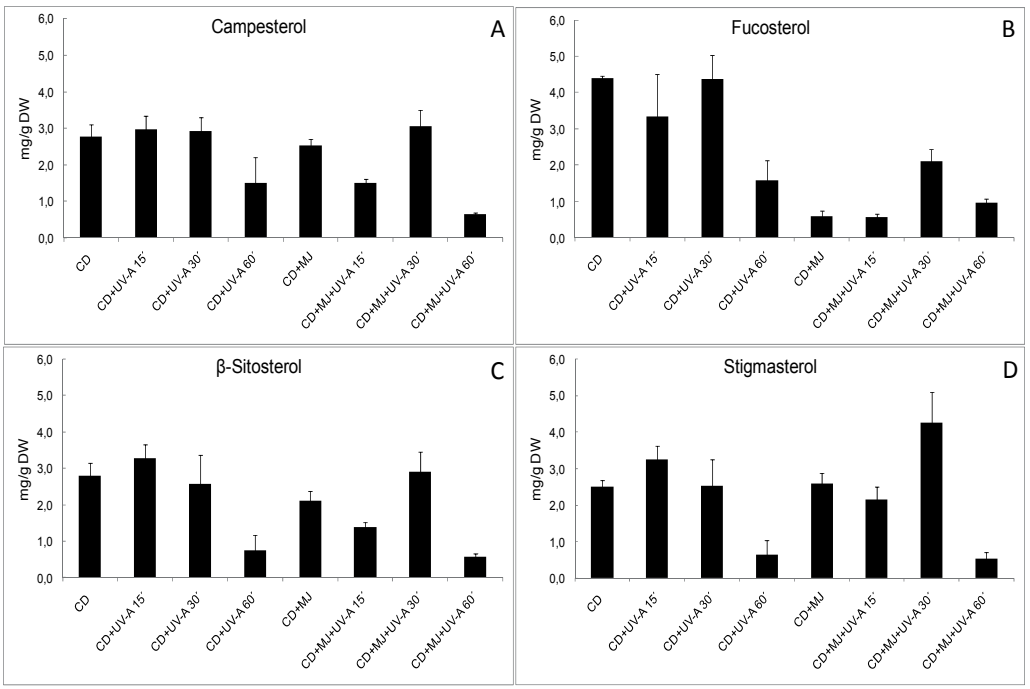

Fig. 8. Effect of different UV-A light (360 nm, 10µW/cm²) exposure times on extracellular accumulation of phytosterols in cell cultures of *D. carota* elicited with CDs alone or in combination with MJ. Elicitation experiments, extraction, analysis and identification of different phytosterols in the culture medium were performed as described in the legend of the Fig. 7. Values are given as the mean ± SD of three replicates. Bars represent mg of different phytosterols/g DW.

Therefore, it is possible to think that, in the simultaneous treatment of carrot cells with UV-A light and elicitors, the chemical elicitor signal was dominant over the UV light signal, resulting in the UV induction or inhibition of different biosynthetic pathways which leads to phenylpropanoid or flavonoid derivatives.

5. Conclusion

The application of biotic or abiotic stimuli has been one of the most effective strategies for improving the productivity of different useful secondary metabolites from plant cell cultures (Vasconsuelo & Boland, 2007; Zhao et al., 2005). The most frequently used elicitors are of fungal and yeast origin, jasmonic acid and MJ, chitosan, metal ions and UV light.

More recently, special attention has been paid to the use of CDs as true elicitors, which act inducing defence responses, which include pathogenesis-related proteins and phytoalexin synthesis, especially the stilbenes like *trans*-resveratrol in *Vitis sp* (Morales et al., 1998; Bru et al., 2006; Zamboni et al., 2009; Martinez-Esteso et al. 2009), the accumulation of sesquiterpenes and phytosterols as well as pathogenesis-related proteins in *Capsicum sp* (Sabater-Jara et al., 2010b; Sabater-Jara et al., 2011), the increase of ajmalicine and catharanthine in *C. roseus* (Almagro et al., 2010) and the enhancement of the production of silymarin in *Silybum marianum* (L.) Gaernt cell cultures (Belchí-Navarro et al., 2011). The success in the production of secondary metabolites with the use of CDs lies in the properties of these compounds since they act not only as elicitors but also forming inclusion complexes (clathrates) with stilbenes (Morales et al., 1998), sesquiterpenes (Sabater-Jara et al., 2010b), indole alkaloids (Almagro et al, 2010) and silymarins (Belchí-Navarro et al., 2011), favouring the substantial accumulation of these metabolites and also preventing the toxic and/or inhibitory effect of their extracellular accumulation on cells cultured in suspension.

Most of the publications concerning secondary metabolite production by means of plant cell cultures reported that the elicitation with MJ increased the accumulation of secondary metabolites (Zhao et al., 2005; Sánchez-Sampedro et al., 2005; Belchí-Navarro et al., 2011; Almagro et al., 2010; Lee-Parsons et al., 2004; Repka et al., 2004; Szabo et al., 1999; Tassoni et al., 2005; Yukimune et al., 1996; Mandujano-Chavez et al., 2000; Sabater-Jara et al., 2010a,b; Wang & Wu, 2005). In fact, the addition of a second elicitor, for instance MJ to cell cultures elicited with CDs increased significantly the production of *trans*-resveratrol, indole alkaloids and phytosterols in Monastrell grapevine, *C. roseus* and *D. carota* cell cultures, respectively. However, the effect of UV light on the production of these secondary metabolites is dependent not only on the exposition time (short or long) and UV light type (C or A), but also on the presence of one (CDs) or two chemical elicitors (CDs and MJ). Thus, the addition of a third elicitor (UV light) in Monastrell grapevine cell cultures, elicited with CDs separately or in combination with MJ, decreased (UV-C light, Fig. 4) or did not increase significantly the production of *trans*-resveratrol (UV-A light, Fig. 5). By the contrary, to achieve high ajmalicine production levels using a productive *C. roseus* cell line, the best operating conditions were elicitation using a combination of CDs and MJ and a cell exposition to UV light (A or C) during 30 min although this enhancement was also observed at all irradiation times tested (Fig. 6). Moreover, the production of phytosterols depended on exposition time (short or long) and UV light type (C or A) since the exposition of *D. carota* cell cultures to UV-C light did not increase phytosterol extracellular accumulation in CD- or CD plus MJ-treated cells (Fig. 7) whereas in cells elicited with CDs and MJ and exposed to UV-A during 30 min, an increase in the phytosterol production was observed (Fig. 8). The

mechanism by which the elicitor signal leads to suppression or activation of metabolite biosynthesis has yet to be investigated. All effects observed after application of the elicitors alone or in combination with UV light may be regulated at the level of both protein and mRNA of crucial enzymes whose activities increased or decreased. Further experiments are needed in order to elucidate the mechanism by which the joint action of three different elicitors in some cases, improves the production of secondary metabolites while in others, no effect is observed.

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6

Drought Tolerance and Stress Hormones: From Model Organisms to Forage Crops

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1. Introduction

Among environmental factors, water availability is probably the most limiting for crop quality and productivity, comprising economical output and human food supply (Roche et al., 2009). Water deficit is a multidimensional stress affecting plants at various levels of their organization (Yordanov et al., 2000). Thus, the effects of stress are often manifested at morpho-physiological, biochemical and molecular level, such as inhibition of growth (Bahrani et al., 2010), accumulation of compatible organic solutes (Sánchez-Díaz et al., 2008; DaCosta and Huang 2009), changes in phytohormones endogenous contents (Perales et al., 2005; Seki et al., 2007; Huang, 2008; Dobra et al., 2010), modifications in expression of stress responsive-genes (Xiong and Yang 2003; Yamaguchi-Shinozaki and Shinozaki, 2005; Huang et al., 2008), among others. Some of these responses are directly triggered by the changing water status of the tissues while others are brought about by plant hormones (Chaves et al., 2003). In this sense, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) are involved in a complex signal-transduction network that coordinates growth and development with plant responses to the environment (Agrawal et al., 2002; Jiang and Zhang 2002; Fujita et al., 2006; Szalai et al., 2010).

The aim of this chapter is to present the results of an actual progression of water stress tolerance, its associated hormones and the crosstalk between them in *Panicum virgatum* (switchgrass), a member of the Poaceae family intensively studied as a source of lignocellulosic biomass to produce renewable energy. In recent years, important research efforts have been focused on improving the yields of crop species under water stress. Advances in functional genomics have been a major contribution to both the study and manipulation of abiotic stress in cereals as well as in forage species. These have been possible, in part, because of the increasing success in methods of grass genetic manipulation which have facilitated basic and applied research. The top four agricultural commodities by quantity are grass crops (sugarcane, maize, rice, wheat). Cow's milk, the sole animal product in the top 10 agricultural commodities by quantity, for the most part comes from animals fed by grasses. Primary production from agriculture, therefore, assumes an important role in the transition to increasingly sustainable food and industrial production

methods. Grass crops are centrally important targets for biotechnological improvement for food and fuel production. In particular the exploitation of a currently untapped resource of grass biomass (primarily lignocellulosic cell walls) is of high interest for sustainable fuel production. Basic and applied research on the sequencing of the rice, sugarcane, maize and sorghum genome have provided an invaluable resource to infer gene localization in other grasses that have not been sequenced yet. In the mid term, increases in drought tolerance could be introgressed from tolerant genotypes using a marker-assisted breeding approach. The Poaceae is the fourth largest plant family in the world -with over 10000 species distributed widely across the earth- and has an extensive synteny among the genomes of its members. Hence, what we learn about one member of the family can enhance our understanding of the entire group and contribute to the improvement of grass crops in meeting the challenges of attaining a sustainable agriculture for feeding the world's population and for developing renewable supplies of fuel and industrial products (Baven et al., 2010).

2. Phytohormones and drought stress

As sessile organisms, plants are only able to survive by their ability to build up fast and highly adapted responses to diverse environmental stresses, *e.g.*, drought, high salinity, and low temperature. Perception of these stress signals often results in the production of a huge arsenal of chemical compounds, among which a variety of hormones to adapt and respond to environmental challenges are included. Some of these compounds, such as the phytohormones are in a prominent position, playing important regulatory roles in plant physiology (Wasternack and Hause 2002; Chen et al., 2006; Browse, 2009a) affecting both developmental processes and responses to a wide range of abiotic and biotic stresses.

The key role of ABA, JA and SA as primary signals in the regulation of plant defense has been well established (Bari and Jones 2009; Pieterse et al., 2009). These hormones generate a signal transduction network that leads to a cascade of events responsible for the physiological adaptation of the plant to stress. It should be noted that the degree of drought tolerance varies with developmental stages in most plant species (El-Far and Allan 1995; Reddy et al., 2004; Rassaa et al., 2008). Experiments conducted to identify highly drought sensitive growth stages of sunflower showed that maximum reduction in yield occurred when drought was imposed during flowering (Karaata, 1991). In addition, drought during the vegetative phase of sunflower plants affects both final biological and economic yields (Agele 2003; Turhan and Baser 2004). In maize water deficit in the late developmental stage tends to reduce kernel size rather than number (Saini and Westgate, 2000; Boyer and Westgate, 2004). Similarly, Barney et al. (2009) evaluated fitness under stressful growing conditions to characterize the agronomic and ecological traits related to environmental tolerance of switchgrass and found that drought treatments (-4.0 and -11.0 MPa) reduced tiller length and number, leaf area, and biomass production by up to 80%.

The final outcome of stress response indicates that there is no single response pattern that is highly correlated with yield under all drought environments.

2.1 Abscisic acid (ABA)

ABA is well known hormone for its regulatory role in integrating environmental adversity with the developmental programs of plants (Chow and McCourt 2004; Christmann et al., 2005). Thus, it affects a wide range of processes at different developmental stages such as

embryo and seed development, acquisition of desiccation tolerance and dormancy, flowering and organogenesis (Finkelstein et al., 2002; Barrero et al., 2005; De Smet et al., 2006; Liang et al., 2007). ABA also promotes plant growth under non stressful condition and has shown to be essential for vegetative growth in several organs (Sharp et al., 2000; Spollen et al., 2000; Cheng et al., 2002).

Continuous synthesis, transport and degradation dynamically maintain ABA levels in plant cells. Therefore, plants control their developmental programs and stresses responses by modulating endogenous ABA levels (Schwartz et al., 2003).

The molecular basis of ABA biosynthesis and catabolism were established by genetic and biochemical approaches (Seki, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). Based on these studies it has become clear that ABA is synthesized from zeazanthin, a C_{40} carotenoid. The conversion of zeaxanthin to xanthoxin, which is the C15 intermediates, is catalyzed in plastids by distinct enzyme: zeaxanthin epoxidase (ZEP) (Agrawal et al., 2001; Xiong et al., 2002), neoxanthin synthase (North et al., 2007), an unidentified epoxycarotenoid isomerase, and 9-cis-epoxycarotenoid dioxygenase (NCED) (Schwartz et al., 1997; Qin and Zeevaart 1999; Iuchi et al., 2001). In cytosol, the oxidation of xanthoxin produces abscisic aldehyde, which can be converted to ABA by aldehyde oxidase 3 (AAO3) (Seo et al., 2000).

Catabolism of ABA can occur through different pathways, the nature of which often depends on the species, their developmental stage or tissue. There are at least two pathways for ABA catabolism, an oxidative pathway and conjugation (Kushiro et al., 2004; Nambara and Marion-Poll 2005). The most common oxidative pathway is initiated by oxidation of the 8'-hydroxy ABA (8'-OH ABA), which can reversibly cyclize to phaseic acid (PA) (Zaharia et al., 2005). This compound can then be reduced to the major product dihydrophaseic acid (DPA), with minor amounts of epi- dihydrophaseic acid (epi-DPA). The minor oxidation pathway includes the formation of 7'-hydroxy ABA (7'-OH ABA) and 9'-hydroxy ABA (9'-OH ABA). The latter can cyclize reversibly to neophaseic acid (neoPA) (Zhou et al., 2004). In addition, ABA and hydroxy ABA may be conjugated with glucose, thereby forming corresponding glucose esters at C-1 (ABA-GE) or glycosides at C-1′ or C-4′ (Zeevaart 1999; Oritani and Kiyota 2003).

ABA action is one of the most studied topics in abiotic stress response research (Hirayama and Shinozaki 2007; Wasilewska et al., 2008). An increase in ABA content in response to water-deficit stress may arise from an increase in ABA biosynthesis and/ or a decrease in ABA breakdown (reviewed by Cutler and Krochko, 1999; Zeevaart, 1999). In *Arabidopsis thaliana* seedlings, Huang et al. (2008) showed that drought enhanced both ABA biosynthesis and catabolism, resulting in an increase in ABA and catabolites. Likewise, drought-treated plants of *Laurus azorica* (Seub) showed an increase in leaf ABA concentrations respect to that of the control (Sánchez-Díaz et al., 2008). On the other hand, exogenous application of ABA enhances the tolerance of plants or plant cells to drought (Lu et al., 2009). In relation to endogenous ABA, different reports showed that drought tolerant cultivars have more ABA than susceptible ones (Perales et al., 2005; Veselov et al., 2008; Thameur et al., 2011). Nevertheless, the direct relation between stress tolerance and increased ABA contents does not always exist.

In addition to the well established model of *Arabidopsis*, increments in endogenous ABA level under water stress are also reported in cereals and forage crops. For instance, increment in ABA contents under water stress in diverse developmental stages was reported in maize (Xin et al., 1997; Wang et al., 2008; Nyysar 2005), sorghum (Kannangara et al.,

1983), wheat (Iqbal et al., 2010; Raziuddin et al., 2010), festuca (Abernethy and McManus 1998), barley (Thameur et al., 2010) and alfalfa (Han et al., 2008).

Plants of wheat and maize, representatives of C3 and C4 plants, respectively, were subjected to mild (−0.4MPa), moderate (−0.8MPa) and high (−1.5MPa) water stress levels induced by PEG-6000 for 7 days under controlled conditions. No significant change occurred in ABA content in roots and leaves of both species at mild stress level. Moderate stress resulted in higher accumulation of ABA in roots and leaves of maize as compared to wheat roots and leaves. At high stress level, ABA content increased in maize whereas wheat did not show any significant change. The differences were more pronounced between the leaves of the two species. These findings suggest a differential sensitivity of C3 and C4 plants to water stress. Higher ABA content in maize may also impose greater stomatal restrictions on these species to reduce water loss more effectively compared with wheat having lower ABA content (Nayysar, 2005).

In maize seedlings, Wang et al. (2008) assesed the inhibitory effect of ABA on the grain growth and reported that, at early stages, the endogenous ABA contents increased dramatically in leaves after 24 h of exposure to water stress, and then it remained high till the end. On the other hand, ABA content in seeds of wheat plants subjected to water deficit during grain filling showed variations. Water status parameters, ABA levels in flag leaf and grains, and grain yield were investigated in two drought tolerant (i.e. cv. MV Emese and cv. Plainsman V) and two drought-sensitive (i.e. cvs. GK E´let and Cappelle Desprez) wheat genotypes. In flag leaves, endogenous ABA levels increased significantly after the suspension of irrigation in all genotypes and remained high during anthesis; afterwards, it decreased markedly. In grains, ABA increased significantly in all genotypes exposed to water stress at 9 days post anthesis (DPA). Tolerant cultivars had higher ABA levels at 9 DPA and then it decreased rapidly toward maturity. By contrast, in sensitive cultivars ABA levels remained high until the end of grain filling period, which affected more negatively the grain yield of sensitive cultivars (Guoth et al., 2009).

Water stress effect and ABA levels were studied in sorghum cv. CSH8. A gradient of water stress was created among sorghum plants with a line-source sprinkler irrigation system and it was observed that leaf ABA levels increased with decreasing irrigation. ABA was very sensitive to stress, ranging over the irrigation gradient from 50 to 800 ng g^{-1} DW in the well irrigated and water stressed plants, respectively. This study shows that ABA synthesis in leaves begins with a water potential of -1.3 MPa. This threshold has been observed in several species in a variety of conditions. The increase in ABA levels also correlated with a marked decrease in plant height and leaf senescence (Kannangara et al., 1983).

In plants of *Festuca arundinacea* cv. Grasslands Roa drought was imposed through water deprivation. An increase in leaf ABA levels from a range of 5–30 ng g^{-1} FW in leaf tissue from water sufficient plants (control) to up to 200 ng g^{-1} FW in leaf tissue of stressed plant was observed. ABA concentration was correlated with soil moisture content and leaf water potential. The accumulation of ABA occurred after the soil moisture content had dropped below approx. 8% in pots of treatment. The maximum rate of ABA accumulation occurred between water potential values of -1.5 and -2.5 MPa. Under these conditions, leaf elongation ceased and there was an increase in proline levels (Abernethy et al., 1998).

In barley, the differences in responses among five genotypes (i.e. Ardahoui, Pakistan, Rihane, Manel ad Roho) were evaluated. Water stress induced a reduction in relative water content, as well as an increase in proline content and endogenous ABA in all genotypes. Drought tolerant cv. Ardhaoui had the highest increase in endogenous ABA (5-fold) after

water deprivation, while intermediate values were obtained in cvs. Rihane, Pakistan and Manel (Thameur et al., 2010).

In alfalfa cvs. Longdong (strong drought-resistance) and BL-02-329 (weak drought-resistance) ABA contents were evaluated. Under water stress, the ABA content increased in leaves. In response to severe drought stress, the drought- resistant cv. Longdong adjusted better to growth rate reduction to ensure surviving and avoid water deficit damage (Han et al., 2008).

In addition, exogenous ABA was demonstrated to increase drought tolerance in some forage crops. For example, Shaoyun et al. (2009) studied the effect of exogenous ABA added on plant of bermudagrass cv. TifEagle. They evaluated the protective effect of ABA based on relative water content and found that every ABA treatments (e.g. 19, 38 and 57 μM) significantly decreased the mortality rate in drought conditions compared to control. Treatment of 19 μM ABA showed the best protection against injury.

The increase in ABA endogenous level under drought induces the stomatal closure. This fact constitutes one of the first external symptoms of water deficit, and is recorded as the increase of stomatal resistance or the decrease of its inverse (stomatal conductance). Stomatal closure take place to minimize the water loss by transpiration, and ABA plays a fundamental role in this process. Thus, stomatal resistance is used as a reference to compare the intensity of water deficit in different species and growth conditions (Medrano et al., 2002). Guard cells continuously sense information from the surrounding environment, biotic and abiotic, as well as long distant signals coming from the roots. Stomatal closing under drought is a response to increasing levels of endogenous ABA synthesized in the roots as a result of water deprivation in the soil (Kim et al., 2010). Hence, decreasing of stomatal conductance under water stress is a wide-ranging response in plants. For instance, in kidney beam stomatal conductance diminishes rapidly after two days of drought, but it recovers the levels of well watered plants after two days of re-watering (Miyashita et al., 2005). In *Brachiaria decumbens* and *brizantha*, stomatal conductance significantly decreased after six days of water deprivation (Carmona et al., 2003).

Another symptom of water deficit is the reduction in cell turgency, which in turn, limits cell expansion and growth. Drought tolerance of grasses is associated closely with their morphological and physiological traits, with varying degrees of reduction of them among the species. For example, water stress decreases plant height in most grass species (Pennypacker et al., 1990; Jiang et al., 1995; Berg and Zeng 2006). On the contrary, this stress generally had no effects on the root: shoot ratio of the grasses.

Bahrani et al. (2010) found that water stress constrained the total water use in ten forage species through a reduction in plant height, leaf water potential, leaf area and dry weight of roots. In corn, 160 lines (pure lines and hybrids) were evaluated in their tolerance to drought; one of the first detected symptoms was a reduction in plant height, with values ranging from 60 to 75 % (Dass et al., 2001). Similar effect was found in genotypes of wheat, where plant height showed a significant reduction under water stress (Shirazi et al., 2010).

In our laboratory we investigated the response of *Panicum virgatum* cv. Greenville to water stress. Plants of 55 days old were grown in a growth chamber; water was withheld at the same time as stomatal conductance was monitored. After water withholding, a consistent drop in the conductance was detected (drought treatment) and plants were re-watered to evaluate their recovering after 12 and 24 h. Plant height, stomatal conductance, content of stress related hormones (ABA, JA, SA) were evaluated. Water stress negatively affected the

plant height and, after watering was restored (i.e. 24 h after re-watering) plant growth reached the control height (Fig. 1.B). In addition, the stomatal resistance drastically increased during the stress period and it gradually decreased to the control level at 24 h of re-watering (Fig. 1.C).

After five days under stress, endogenous ABA content increased 4.5 fold compared to the control (Fig. 1.A). After 12 h of rehydration ABA content decreased to 1.5 fold the control and, after 24 h, ABA content in treated and control plants were similar. This increment in ABA content under stress is associated with the increase of stomatal resistance. Once plants recoved, both ABA content and stomatal resistance decreased to the control level. These results are in agreement with reports from other plant species as we discussed earlier.

The first steps of ABA sensing and signaling during stomatal closure under drought is related to the localization of ABA receptors in the guard cells. Two of these ABA receptors reside inside the cell but a third was found on the cell surface (Liu et al., 2007). Therefore, plant cell could sense both extracellular and intracellular ABA concentrations. Under drought, an increasing in stomata closure occurs because of an increasing in the pH of sap. This fact suggests that extracellular ABA is sensed by guard cells via receptors on the plasma membrane (Schachtman and Goodger, 2008). In the last decade, hydrogen peroxide (H_2O_2) and nitric oxide (NO) have also been involved in the ABA-induced stomatal closure (Assmann 2003; Desikan et al., 2004; Bright et al., 2006).

It is well documented that, in response to biotic and abiotic stimuli, there is an increment in the reactive oxygen species (ROS). ROS are short-lived molecules produced through diverse cellular mechanisms in different cell compartments, e.g. chloroplast, peroxisomes, mitochondria (Cho et al., 2009). This overproduction of ROS is highly controlled by a versatile oxidative system that establishes the redox balance inside the cell. On the other side, increase of ROS under stress conditions act as a signal of warning that activates responses of acclimation and/ or defense. Particularly, it activates specific pathways where H_2O_2 is involved as a second messenger. ROS signaling is connected to ABA, flux of Ca^{+2} and sugars, and it is possible that they participate both up and downstream of pathways dependent of ABA in drought conditions (Kwak et al., 2006). In *Panicum virgatum*, ROS has been related to ABA signaling during germination (Sarath et al., 2007). Inhibition of germination imposed by ABA apparently requires both ROS and NO as intermediates in its action, where ROS produced by membrane-bound NADPH-oxidases responsive to ABA. In switchgrass seeds, externally supplied hydrogen peroxide restrain ABA-imposed inhibition of germination. Apart from this study on germination, no other report has involved ABA and ROS in switchgrass responses.

At molecular level, many transcription factors (TFs), such as dehydration-responsive element binding protein 1 (DREB1)/C-repeat binding factor (CBF), DREB2 and ABA-responsive element (ABRE) binding protein (AREB)/ABRE binding factor (ABF) can be used to improve stress tolerance to abiotic stresses in various grasses. ABA is involved in transcriptional regulations of numerous drought responsive genes (Zhang et al., 2006). Some drought-inducible genes may be regulated by both the ABA-independent and the ABA-dependent regulatory systems. For example, the promoter of a drought-, high salinity-, and cold- inducible gene, RD29A/COR78/LTI7, contains two major cis-acting elements (ABRE) and DRE/ C-RepeaT (CRT), both of which are involved in stress-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005).

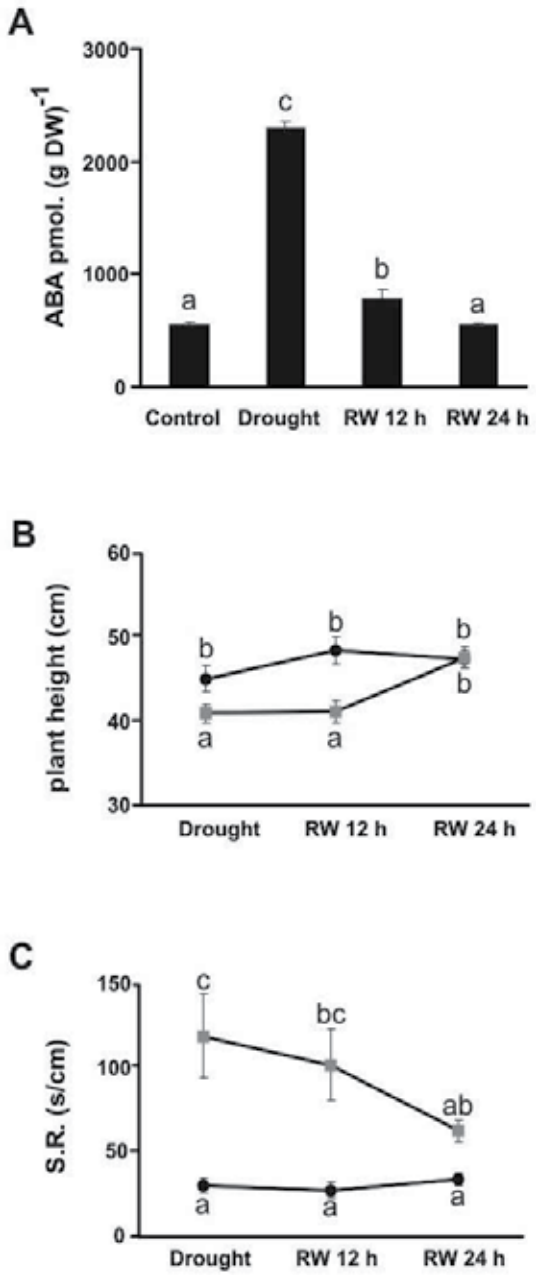

Fig. 1. **A.** Content of ABA in leaves of Panicum virgatum cv. Greenville grown under drought (Drought) and after 12 and 24 h of re-watering (RW 12 h and RW 24 h). Data are means and SEs of three replicates, $P \leq 0.05$. **B.** Plant height C. Stomatal resistance (S.R.). Measurements were made with porometer Delta-T on the abaxial side of leaves. Black circle: control conditions. Gray square: Drought, RW 12 h and RW 24 h of re-watering. Data are means of twenty-four replicates with SEs. Values with the same letter are not significantly different, $P \leq 0.05$.

2.2 Salicylic acid (SA)

SA is an endogenous regulator of growth involved in a broad range of physiologic and metabolic responses in plants (Hayat, 2010). During the last years, SA has been intensively studied as a signal molecule mediating local and systemic defense responses against pathogens. Currently, it has been reported that this compound plays also a role in plants responses to abiotic stresses, such as drought, low and high temperatures, heavy metals, and osmotic stress (Janda et al., 1999; Rao and Davis 1999; Molina et al., 2002, Nemeth et al., 2002; Munne-Bosch and Peñuelas 2003; Shi et al., 2008; Rivas-San Vicente and Plasencia 2011). SA was also shown to influence a number of physiological processes, including seed germination, seedling growth, fruit ripening, flowering, ion uptake and transport, photosynthesis rate, stomata conductance, biogenesis of chloroplast (Fariduddin et al., 2003; Khodary 2004; Hayat et al., 2005; Shakirova 2007).

There are two main routes for SA biosynthesis in plants (Shah 2003). Earlier studies suggested that SA is synthesized from phenylalanine via cinnamic acid. The decarboxylation of the side chain of cinnamic acid may generate benzoic acid, which may then undergo hydroxylation at the C-2 position forming SA (Yalpani et al., 1993 ; Ribnicky et al., 1998). The other pathway for the SA biosynthesis involves a 2-hydroxylation of cinnamic acid to o-coumaric which is then decarboxylated to salycilic acid (Alibert and Ranjeva 1971; 1972). Recent studies in *Arabidopsis* plants showed that there is another main route for SA biosynthesis taking place in the chloroplast, where SA is synthesized from chorismate via isochorismate (Wildermuth 2006; Mustafa et al., 2009). SA may be conjugated with a variety of molecules either by glycosylation or by esterification (Popova et al., 1997), and may also be metabolized to 2,3 dihydrobenzoic acid or 2,5 dihydrobenzoic acid (Billek and Schmook, 1977).

Recent results show that most abiotic stresses altered *in planta* SA endogenous contents, which also point to its involvement in stress signaling (Horváth et al., 2007). For example, endogenous SA increased in roots of barley plants under water stress. In addition, when plants were treated with SA before stress, the damaging effect of water deficit on the cell membrane in the leaves decreased, and an increase in ABA content was observed. Also, the proline level increased only in the wild species of *Hordeum spontaneum*. These results suggest that ABA and proline may contribute to the development of the antistress reactions, induced by SA (Bandurska and Stroinski, 2005). Previously, Munne- Bosch and Peñuelas (2003) reported that in *Phillyrea angustifolia* L. plants exposed to drought the SA level increased progressively to as much as 5-fold, and showed a strong negative correlation with the relative water content. During recovery, SA levels decreased, but remained slightly higher than those observed before drought. SA levels were positively correlated with those of tocopherol -also known as vitamin E acetate- during drought, but not during recovery. This result also indicates the possible role of endogenous SA in the induction of a protective mechanism during water stress.

Application of exogenous SA improves the plant performance under water, as reported by several authors. Low concentrations of exogenous SA provided tolerance against the damaging effects of drought in tomato and bean plants, whereas, higher concentrations did not show the same positive results (Senaratna et al., 2000). Enhanced tolerance to drought and dry matter accumulation was also observed in plants of wheat raised from grains soaked in acetyl salicylic acid aqueous solution (Hamada 1998; Hamada and Al-Hakimi 2001). Wheat seedlings subjected to drought and treated with SA exhibited higher moisture content and dry matter accumulation, carboxylase activity of Rubisco, SOD and total

chlorophyll content compared to untreated control. The SA treatment also provided a considerable protection to the enzyme nitrate reductase thereby maintaining the level of diverse proteins in leaves (Singh and Usha, 2003). In addition, the treatment of water stressed *Licopersicum esculentum* plants with SA low concentrations significantly enhances the photosynthetic parameters, membrane stability index, leaf water potential, activities of the enzymes nitrate reductase and carbonic anhydrase; thus improving tolerance to drought (Hayat et al., 2008). SA is also involved in the promotion of drought-induced leaf senescence in *Salvia officinalis* plants grown under drought in Mediterranean filed conditions (Abreu and Munne-Bosch 2008). In addition, SA applied exogenously was effective in providing resistance to the plants against the excessive water stress in cell suspensions from the fully turgid leaves of *Sporobcdus stapfianus* (Ghasempour et al., 2001).

Exogenous application of SA and glycin-betaine (GB, a compatible osmotic solute) enhanced the yield of sunflower hybrids under different degrees of water stress. Under stress, diameter of the head (inflorescence), number of achene and seed oil content was reduced. However, applications of SA and GB improved these parameters (Hussain, 2008).

In plants exposed to abiotic stress (e.g. salinity and drought), the accumulation of ROS, such as superoxide radicals (O^{2-}), hydroxyl (OH^-), and H_2O_2 is induced. The increasing ROS levels in plants produce oxidative stress of lipids, proteins and nucleic acids, which, in turn, alter the redox homeostasis (Smirnoff, 1993). SA increases the activity of the oxidative enzymatic system as is the case of CAT and SOD. In plants of *B. juncea,* exogenous application of SA increased CAT and SOD activity. In the same line of evidence, Kadioglu et al. (2010)

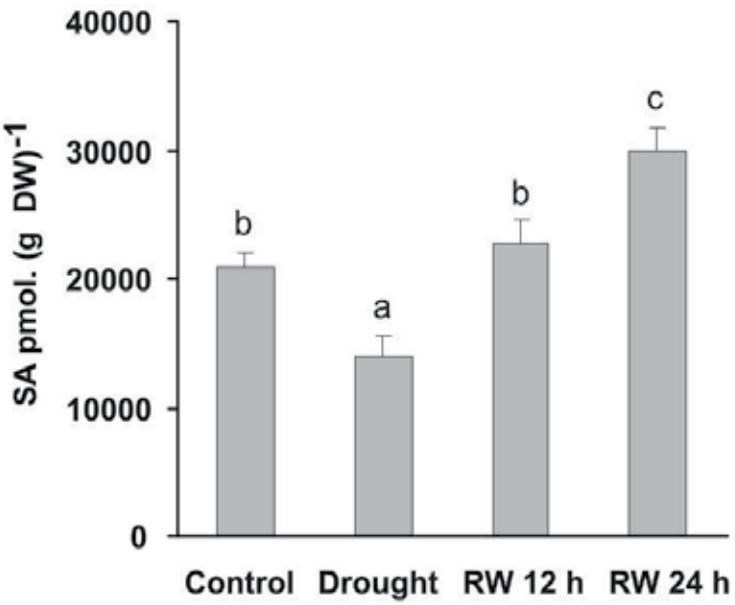

Fig. 2. Content of SA in leaves of *Panicum virgatum* cv. Greenville grown under drought (Drought) and after 12 and 24 h of re-watering (RW 12 h and RW 24 h). Data are means of three replicates with SEs. Values with the same letter are not significantly different at $P \leq 0.05$.

reported that exogenous application of SA induced the activity of antioxidant enzymes at the same time that alleviates the water stress damage in the long run in plants of *Ctenanthe setosa*. In seedlings of wheat under water stress and supplemented with SA (1 mM), ABA (0,5 mM), Ca^{2+} (5 mM) and H_2O_2 (0,05 mM), the activity of SOD, CAT, ascorbate peroxidase (APX), and NADPH oxidase (Agarwal, 2005) was induced.

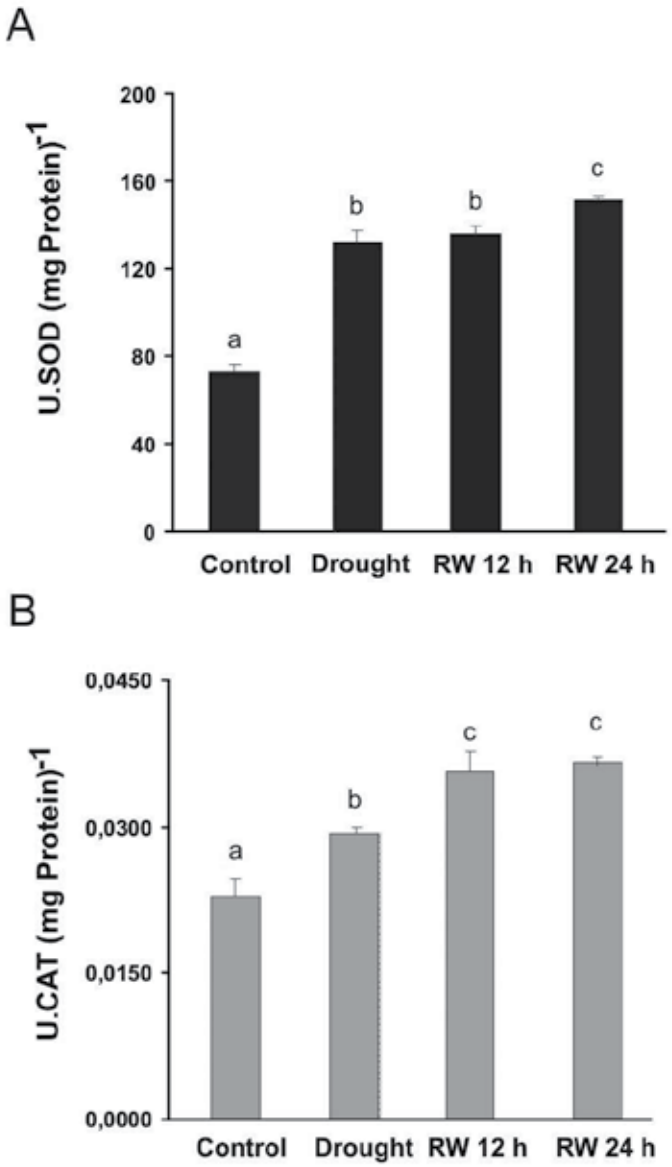

Fig. 3. **A.** Activity of superoxide dismutase (SOD) and **B.** catalase (CAT), on leaves of *Panicum virgatum* cv. Greenville grown under drought (Drought) and after 12 and 24 h of re-watering (RW 12 h and RW 24 h). Data are means of four replicates with SEs. Values with the same letter are not significantly different at $P \leq 0.05$.

In *P. virgatum,* we found that endogenous SA contents decreased considerably during a moderate water stress treatment and after 24 h of rehydration the endogenous contents increased significantly ($p \leq 0.05$, Figure 2). This decrease in SA is accompanied with important peak of ABA content (four-fold increase) during the stress treatment (Figure 1.C). It has been proposed that an antagonistic interaction between these two hormones in response to water stress naturally occurs in several species, probably as a result of sharing common intermediaries in the signaling cascade (Yasuda et al., 2008). In addition, the increase in SA content corresponds with a raise in SOD and CAT activities after plants were rehydrated (Figure 3 A and B).

Despite of SA participation in abiotic stress responses, its role is ambiguous. The stress tolerance imparted by SA appears to be dosis-dependent, since deficiency or very high SA contents increase the susceptibility. Hence, the role of SA under a certain level of moderate or severe stress might be different. It could possibly be a result of the interaction between ROS and SA down-stream signals, where redox regulations play a key role (Yuan and Lin, 2008).

2.3 Jasmonic acid (JA)

JA, and its cyclic precursors and derivatives constitute a family of bioactive oxylipins that regulate plant development and responses to environmental cues (Turner et al., 2002; Devoto and Turner, 2003). This family of compounds is form by 12-oxophytodienoic acid (OPDA), methyl jasmonate (Me-JA), JA hydroxylated (11-OH-JA and 12-OH-JA), JA conjugated to some amino acids such as leucine (JA-leucine) and isoleucine (JA-Ile) as well as the glucoside and sulfate of 12-OH-JA (12-*O*-Glc-JA, 12-HSO_4-JA), and collectively receive the name of jasmonates (JAs). These molecules are involved in a variety of processes related to plant development and survival, including direct and indirect defense responses (*e.g.*, defense against insects and necrotrophic pathogens), secondary metabolism, reproductive processes (*e.g.*, pollen maturation and anther dehiscence, ovule development), and fruit development, among others (Seo et al., 2001; Wasternack and Hause, 2002; Arimura et al., 2005; Liechti and Farmer, 2006; Wasternack, 2007). In addition, it is known that JA-related responses are directly associated with a reset downstream of gene expression in the biosynthesis pathway (Thines et al., 2007).

Vick and Zimmerman (1983) were the first authors to demonstrate the steps of the JA biosynthesis, and recently it was reviewed by Wasternack and Kombrink (2010). JA biosynthesis and signaling pathway have been extensively studied, mainly in dicots such as *Arabidopsis* and tomato, and to a lesser extent in some monocots (Kazan and Manners, 2008).

JAs are produced from α-linolenic acid (α-LeA; C18:3) or hexadecatrienoic acid (C16:3) released from plastidial galactolipids by phospholipases. Following the oxidation of α-LeA by lipoxygenase (LOX) to 13(S)-hydroperoxyoctadecatrienoic acid (13(S)-HPOT), the first committed step of JA biosynthesis is conversion of the LOX product to the allene oxide 12,13(S)-epoxyoctadecatrienoic acid (12,13(S)-EOT) by allene oxide synthase (AOS). This unstable allylic epoxide can be enzymatically cyclized by allene oxide cyclase (AOC) to optically pure cis-(+)-12-oxophytodienoic acid (9S,13S)-OPDA), which is the last product of the plastid-localized part of the JA biosynthesis pathway. Translocation of OPDA into peroxisomes, where the subsequent part of the JA biosynthesis pathway occurs, is mediated by the ABC transporter COMATOSE and/or an ion-trapping mechanism (Theodoulou et al., 2005). The OPDA reduction is catalyzed by a peroxisomal OPDA reductase (OPR) to

produced 3-oxo-2(2[Z]-pentenyl) cyclopentane-1-octanoic acid (OPC-8:0). Then, three cycles of β-oxidation catalyzed by acyl-CoA oxidase (ACX), multifunctional protein (MFP), and L-3-ketoacyl-CoA thiolase (KAT) lead to jasmonoyl-CoA, from which a yet unknown thioesterase releases (+)-7-iso-JA ((3R,7S)-JA) that equilibrates to the more stable (-)-JA ((3R,7R)-JA).

The participation of JA in response to abiotic stress, such as drought and salinity, has been reported in several species. For instance, the treatment of barley leaves with sorbitol or mannitol (compatibles solutes to simulate water stress) increased JAs endogenous contents, followed by synthesis of jasmonate-induced proteins (JIPs, Lehmann et al., 1995). Other study showed that sorbitol treatment enhanced octadecanoids and JAs content, and this threshold was necessary and sufficient to initiate JA-responsive gene expression (Kramell et al., 2000). In addition, under water stress, endogenous JA content increased in maize root cells (Xin et al., 1997) and this compound was able to elicit betaine accumulation in pear leaves (Gao et al., 2004). Pedranzani et al. (2003) showed that tomato cultivars differing in salt tolerance differed in basal JA content. Steady-state amounts of JA and related compounds were higher in salt-tolerant cv. Pera compared to the salt-sensitive cv. Hellfrucht frühstamm. Moreover, studies in contrasting environments showed different basal JAs contents and patterns of response to water stress in two populations of *Pinus pinaster* Ait., perhaps as an adaptation to diverse ecological conditions (Pedranzani et al., 2007).

Studies performed in our laboratory with *Panicum virgatum* showed that, during the drought treatments, JA levels did not increase significantly compared to the control level. However, after watering was restored, contents of JA consistently increased and overcome the control (Figure 4).

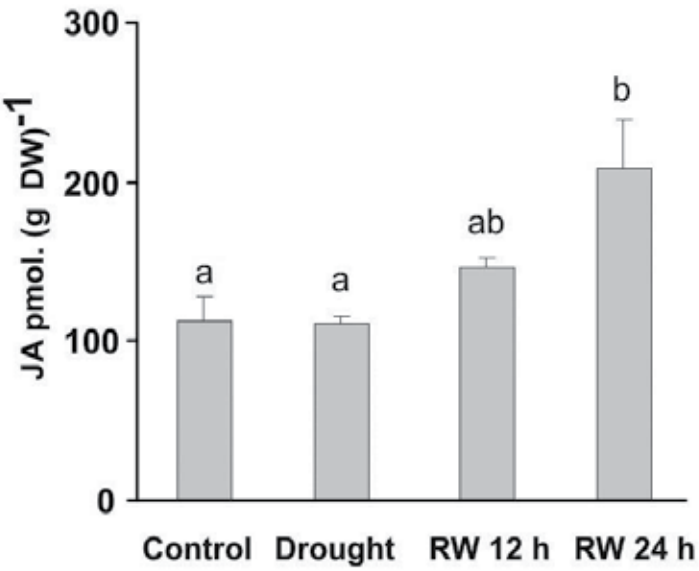

Fig. 4. Content of JA in leaves of *Panicum virgatum* cv. Greenville grown under drought (Drought) and after 12 and 24 h of re-watering (RW 12 h and RW 24 h). Data are means of three replicates with SEs. Values with the same letter are not significantly different at $P \leq 0.05$

Exogenous application of JA or Me-JA increased antioxidative ability of plants under water stress (Wang, 1999; Bandurska et al., 2003). Along the same line, other studies also showed that JAs play an important role in signaling drought-induced antioxidant responses, including ascorbate metabolism (Li et al., 1998; Ai et al., 2008). For instance, in shoots and roots of maize seedlings treated with Paraquat, an herbicide, and exogenous concentrations of Me-JA (50 and 100 µM) the expression of genes corresponding to the anti-oxidative defense system was detected. Certainly, Me-JA promoted increased production of several anti-oxidative enzymes, including glutathione reductase, guaiacol peroxidase and ascorbate peroxidase, and it has been suggested that this increase may be due to up-regulation of genes controlling the synthesis of these enzymes, or by activation of diverse constitutive genes (Norastehnia and Asghari, 2006).

3. ABA, SA, JA and cross talk between each other

To survive under various biotic and abiotic stresses, plants have developed complex mechanisms to perceive external signals, allowing them optimal response to the environment. ABA, SA, JA, and ethylene (ET) regulate protective responses of plants against both abiotic and biotic stresses via synergistic and antagonistic actions, which are referred to as signaling crosstalk (Fujita et al., 2006). Furthermore, ROS generation has been proposed as a pivotal process that is shared between abiotic and biotic responses (Apel and Hirt, 2004; Torres and Dangl, 2005).

ABA has been extensively involved in responses to various abiotic stresses (*e.g.*, drought, salinity, low temperature) and, on the other side, SA, JA and ET play a key role in responses to biotic stress upon pathogen infection. Several studies have indicated that plant responses to environmental stresses have some effects on their response to pathogens. In many cases, ABA acts as a negative regulator of disease resistance (Narusaka et al., 2004). For instance, the ABA-deficient tomato mutant *sitiens* has increased resistance to pathogens and application of exogenous ABA restored the susceptibility of *sitiens* mutants. The *sitiens* mutant has greater SA-mediated responses, suggesting that high ABA concentrations inhibit the SA-dependent defense response in tomato (Fujita et al., 2006). It has also been reported that ABA treatment suppresses the induction of SAR in *Arabidopsis*. The use of several mutants in combination with chemicals that inhibit and/or stimulate SA revealed that ABA suppressed the SAR induction by inhibiting the pathway both upstream and downstream of SA, independently of the JA/ET mediated signaling pathway. These data strongly suggest that an antagonistic crosstalk might occur at multiple steps between the SA-mediated signaling of SAR induction and the ABA-mediated signaling of environmental stress responses (Yasuda et al., 2008). This antagonistic interaction between ABA mediated abiotic stress signaling and disease resistance might simply suggest that plants developed strategies to simultaneously producing proteins that are involved in abiotic stress and disease resistance (Anderson et al., 2004). Since pathogen infection requires relatively humid conditions, a simultaneous exposure of plants to drought and necrotrophic pathogens attack is actually rare in nature. In fact, high humidity and temperature weaken the plant resistance to pathogen attack. Thus, the view that the ABA-mediated abiotic stress signaling potentially takes precedence over biotic stress signaling (Anderson et al., 2004) supports the notion that water stress threatens plant survival more significant than pathogen infection does (Fujita et al., 2006).

Likewise, a positive interaction between SA and ABA might occur in abiotic stress. The roles playing by free SA, conjugated SA, and ABA in thermo-tolerance induced by heat acclimation (38°C) were investigated. To evaluate their potential functions, three inhibitors of synthesis or activity were infiltrated into pea leaves prior to heat acclimation treatment. The results showed that the burst of free SA in response to heat acclimation could be attributed to the conversion of SA 2-*O*-D-glucose, the main conjugated form of SA, to free SA. Inhibition of ABA biosynthesis also resulted in a defect in the free SA peak during heat acclimation. Overall, these results suggest that exogenous SA and ABA may lead to the enhancement of thermo-tolerance (Liu et al., 2006).

Our study in *Panicum virgatum* adds evidence to ABA/SA association. Results in our laboratory show that under drought, the content of endogenous SA is lower than that of the control. However, after 12 h of re-watering SA content reach the control value, and after 24 h the contents are significantly higher than that of the control ($p \leq 0.05$, Figure 2). On the other hand, an opposite trend is described in ABA (Figure 1.C), showing that when ABA reach its maximum, SA content is minimum. By the time that ABA recovers the control value, SA content significantly increases over the well-watered control.

Interaction between ABA and JA has been reported in salt stress response. Moons et al. (1997) compared the effects of exogenous ABA and JA in the rice seedlings response to salt stress. In view of the proposed roles for JA and Me-JA in plants exposed to water-limiting stresses, changes in endogenous jasmonates -in particular MeJA content- were compared with the well established increase in endogenous ABA in plants subjected to salt stress. Salt shock (150 mM NaCl) induced a rapid increase in ABA content in roots of 10-day-old seedlings, reaching a maximum at 8 h of stress and decreasing to near control values after 12 h. On the contrary, Me-JA concentrations, showed a delayed and gradual increase of approximately 4-fold after 12 h of stress. This accumulation occurred when ABA levels were decreasing. In the same study, eight stress- induced proteins were compared for their ABA and/or JA response. In addition, the effect of JA, ABA, and salt stress on the transcript levels of three genes encoding pathogenic related proteins, a salt stress-responsive protein, and a group three LEA protein were analyzed. ABA and JA were found to exert antagonistic effects on the transcript and/or protein accumulation of two classes of salt stress-responsive genes.

In addition, in *Arabidopsis* it has been proposed that both ABA and JA participate in the responses to moderate drought (30% field capacity). Nevertheless, ABA and JA would be involved in different stages of the response, driving an acclimation process during growth through an extensive genetic reprogramming to finally reach a new homeostasis (Harb et al., 2010). These authors suggest that, during early stages of moderate drought, endogenous JA in combination with high ABA level is enough to stimulate the preparatory response needed for drought acclimation (e.g. stomatal closure and cell wall modification). JA is probably not required at high concentration under drought stress, and an increase in its concentration might negatively affect plant response to growth. Under moderate drought treatment, the response of *Arabidopsis* mutants *coi1* and *jin1* (both JA-insensitive) were found to be significantly resistant (or insensitive to drought stress). Compared to the wild type, biomass accumulation under drought did not differ from the well-watered control. These results are in agreement with studies showing that in *coi1* mutant the JA-mediated inhibition of seedling and root growth is suppressed (Xie et al., 1998). Harb et al. (2010)

suggest that the reduced growth in response to drought stress, as a developmental program for acclimation, is not switched on in the absence of JA signal perception. Thus, the down-regulation of JA biosynthesis to minimize the inhibitory effect of JA on plant growth as well as signaling pathways under prolonged drought can establish new homeostasis during the acclimation process.

The crosstalk between JA and ABA might occur as they utilize a similar cascade of events to stimulate some responses (Harb et al., 2010; Fujita et al., 2006). Recent studies have revealed several molecules, including transcription factors and kinases, as promising candidates for common players that are involved in this crosstalk. The convergence points in JA and ABA stress signaling occurs, in part, by sharing some transcription factors. Transcription factor AtMYC2 plays a role in multiple hormone signaling pathways. Genetic analysis of the jasmonate-insensitive jin1 mutant revealed that JIN1 is allelic to AtMYC2, which was first identified as a transcriptional activator that is involved in the ABA mediated drought stress signaling pathway (Abe et al., 2003). The dehydration-inducible RD22 gene (involved in response to salt stress and response to desiccation) respond to both AtMYC2 and the R2R3MYB-type transcription factor. RD26 expression is induced by JA, hydrogen peroxide and pathogen infections, as well as by drought, high salinity and ABA treatment (Fujita et al., 2004; Harb et al., 2010; Fujita et al., 2010). In addition, protein phosphorylation and dephosphorylation significantly influence both the regulation of physiological morphology and gene expression associated with basic cellular activities in JA-dependent root growth and in AtMYC2 gene expression. The gene expression and kinase activity of OsMPK5 is also induced by ABA, various abiotic stresses and pathogen infection (Xiong et al., 2003).

Participation of ABA and JAs in stomatal closing was studied in *Arabidopsis* wild type and mutants, ABA-insensitive (*ost1-2*), and Me-JA-insensitive mutants (*jar1-1*), in order to examine a crosstalk between ABA and Me-JA signal transduction. In that study, cytoplasmic pH changes and ROS production in response to ABA or Me-JA were used to assess the respective roles of these genes in ABA or Me-JA signaling pathways, leading to stomatal closure. The modulation of Ca^{2+} mediates the response, and it appears to be a common effect of ABA and Me-JA. The primary actions of ABA and Me-JA at the plasma membrane level appear to be different: while Me-JA targets the Ca^{2+} channels, ABA activates effectors in the plasma membrane (i.e. phospholipase C, D). However, both signal transduction pathways converge at level of intracellular Ca^{2+}. The regulation of intracellular Ca^{2+} level, indeed, has a much greater dependence of Me-JA action than that of ABA (Blatt et al., 1993; McAinsh et al., 1995; Suhita et al., 2004).

Similar interaction between ABA and JA signaling pathways has been observed in seed germination in *Arabidopsis*. In this case, seed germination of the JA-resistant1 (*jar1*) and JA-insensitive4 (*jin4*) mutants were more sensitive to ABA than its wild type (Staswick et al., 1992; Berger et al., 1996).

Evidence of antagonistic interactions of ABA/JA was also found at the level of gene expression in *Arabidopsis* (Balbi and Devoto, 2007). Wild type and *coi1* plants were wounded or treated with Me-JA, and changes in the expression of 8200 genes were examined using microarrays. A survey of the genes that were repressed by Me-JA identified many genes that have been implicated in ABA and drought stress response. These include the ATHB-12 transcription factor, the bZIP-transcription factor ABF3, COR47 and LEA D113. The nitrate transporter NTP2 and three members of the aquaporin family of transporters were also repressed by Me-JA in a COI1-independent manner. These findings reinforce the role of JA

in osmotic homeostasis and are complementary to the study of Armengaud et al. (2004). This author shows that transcript levels for the JA biosynthetic enzymes (i.e. lipoxygenase, allene oxide synthase, and allene oxide cyclase) as well as JA responsive genes (i.e. genes involved in storage of amino acids –VSP-, glucosinolate production -CYP79-, polyamine biosynthesis -ADC2-,and defense -PDF1.2) strongly increase during potassium starvation and quickly decreased after potassium resupply. These finding highlight the role of JA in nutrient signaling and stress management through a variety of physiological processes such as nutrient storage, recycling, and reallocation.

In our work, the experiments with *Panicum virgatum* show that endogenous JA content is not affected by a moderate water deficit, but such contents increase significantly after 24 h of re-watering. This trend is similar to the response observed in *Arabidopsis* during early stages of water and salt stress, where the contents of JA remain constant under drought and gradually recover after re-watering (Moons et al., 1997; Harb et al., 2010). Conversely, there is an increment in ABA levels under a moderate stress that corresponds with an increase in SOD and CAT activities (Figure 5). At the same time, the SA contents decreased, resembling an antagonistic interaction ABA/SA. After re-watering, ABA contents decreases at the same time as SA and JA endogenous contents display an increase. This last trend is accompanied by a rising in SOD and CAT activity during 24 h of plant recovering. Thus, recovered plants

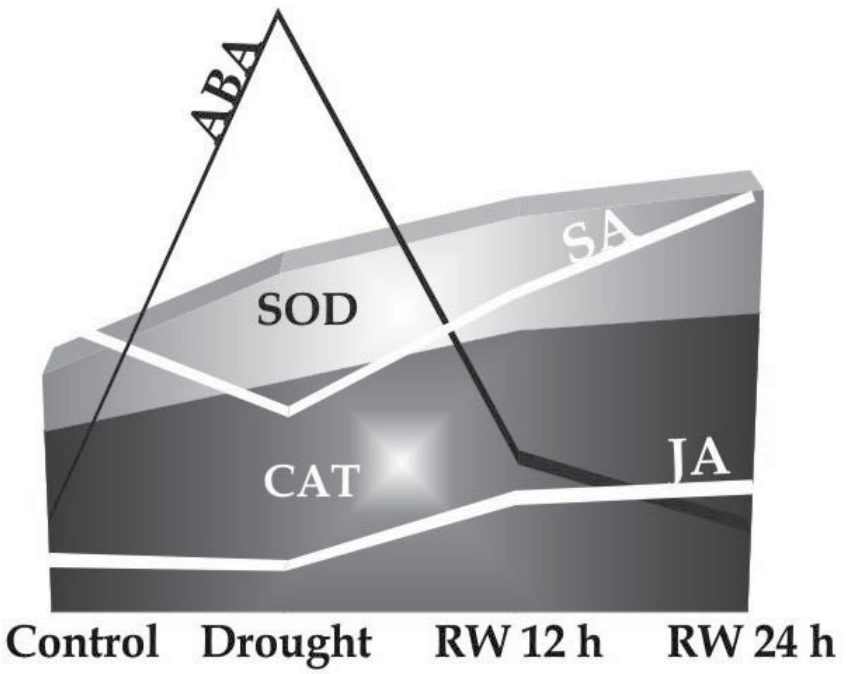

Fig. 5. Model of hormonal response of *Panicum virgatum* cv. Greenville grown under drought (Drought) and after 12 and 24 h of re-watering (RW 12 h and RW 24 h). Abscisic acid(ABA), salicylic acid (SA), jasmonic acid (JA), catalase (CAT), superoxide dismutase (SOD).

reached a new homeostasis status where SA-JA-ABA balance is different from the well-watered control. Humidity produced by re-watering after a water stress could trigger a defense response to pathogen facing a potential attack. Or, it could reorganize the endogenous levels of plant hormones to reach a new homeostasis in acclimation to new environmental conditions (Fujita et al., 2006). Overall, this new hormonal status suggests the interplay among SA-JA-ABA in water stress responses in *P. virgatum*.

4. Conclusions and perspectives

Forage crops, which are grown to be utilized by grazing or harvesting as a whole crop, are essential for the successful operation of animal production systems. This fact is more relevant for ruminants which heavily depend upon forages for their health and for a cost-effective and sustainable production . While forages are an economical source of nutrients for animal production, they also help conserve the soil integrity, water supply and air quality (Chaudhry, 2008). In the last years, forage species have been widely studied for non-forage purposes—especially for bioenergy. Grasses are a source of lignocellulosic biomass to generate biofuels and they belong to a group of plant species considered as second generation crops. Nowadays, second generation of biofuels have gained relevance since they do not directly compete with human nutrition, unlike first generation of biofuel crops. The incorporation of forage species to the production of bioenergy is expected to expand the amount of biofuel that can be produced sustainably by using biomass of non-food crops such as swithgrass, whole crop maize, miscanthus and cereals that bear little grain, among others (Inderwildi and King, 2009). However, one of the major concerns about these crops is the environmental impact. It is likely that the expansion of crops for bioenergy utilization occurs with greater intensity in natural ecosystems, often characterized by their fragility in soil stability and water content. Global climate change intensifies these challenges as current crops are poorly adapted to more uncertain and extreme climatic conditions. In this context, the study of plant responses to water deficit as a strategy for the optimization in the use of water is of remarkable importance to increase production without further damage to the environment. In this chapter, we presented our contribution to this topic through the study of drought tolerance in *Panicum virgatum*, a member of the Poaceae family intensively studied as a source of lignocellulosic biomass to produce renewable energy. The Poaceae, a family with numerous species important to human nutrition, shares an extensive similarity among its members; hence, the comprehension of the bases of water stress tolerance in *Panicum virgatum* will improve our understanding of the entire group. Providing food and energy in conditions that maintain the sustainability of resources is a challenge that must be addressed. Faced with a global energy crisis and the steadily growing world population, forage crops are a suitable alternative to meet current and future demands of food and energy.

The biological significance of crosstalk between signaling pathways operating under stress conditions as well as the mechanism that underlie this crosstalk are still unclear. At present, these pathways have become better resolved due to the development of new tools that allow for the exploration of the physiological, genetic, and biochemical foundation of such processes. The genomic, proteomic and metabolomic approach is now widely used in model plants and, to a lesser extent, in crop and forage plants. The growing interest in forage crops has promoted its study at the molecular level, making it promising to research the improvement of these species. To date, the complete genome sequences of four grass species

(i.e. maize, sorghum, rice and brachypodium) representing the three most economically important grass subfamilies have been analyzed. In the same line, the first pooid grass, *Brachypodium distachyon* (*Brachypodium*), has recently been sequenced completely and proposed as a new model that can contribute to grass crop improvement (Bevan et al., 2010). This knowledge can be directly applied to accelerate the domestication of wild grasses (e.g. Switchgrass and Miscanthus) that are promising biomass crops. Genomics and functional genomics resources are centrally important for this research as they also directly facilitate biotechnological and genetic improvement through plant breeding. This information along with a system-level approach will significantly increase our knowledge of grass biology in order to understand how biotic and abiotic environments influence crop yield. In the near future, the combination of these new technologies will help to unravel the complex interactions between plant hormones in forage crops.

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7

Iron Stress in Citrus

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1. Introduction

Iron is an essential element for plant growth and development, since it is fundamental for the proper functioning of numerous metabolic and enzymatic processes. Calcareous soils with restricted iron availability for plants are commonly found in the Mediterranean basin where citrus are the major fruit crop. The genus *Citrus* and related rootstocks species are considered to be susceptible to iron chlorosis. Iron deficiency tolerance is determined by the rootstock so citrus trees display differences in susceptibility according to the rootstock they have been grafted on.
Field trials have been carried out to select chlorosis-tolerant genotypes. Evaluation of growth and yield parameters may not be sufficient to rank citrus rootstocks according to their tolerance to iron chlorosis, and in addition, field trials take a long time to obtain results. Greenhouse screening tests are easier to implement than field trials in order to evaluate the tolerance. Several physiological parameters are measured in order to test the tolerance to iron deficiency. Nevertheless new screening techniques are needed to identify chlorosis-tolerant genotypes which can be used in breeding programs.

2. Iron chlorosis in plants

The term "essential mineral nutrient" was initially proposed by Arnon & Stout (1939) and applies to all elements necessary for to develop life and reproduction of plants. A plant can complete its life cycle if it is supplied in sufficient quantity and every one of the minerals that are essential. There are two aspects to consider a nutrient as essential. The first one is that the requirement on the element must be specified and can not be replaced by another, and the second one is that the elements must have a direct influence on the metabolism of plants.
The Fe is involved in important processes as photosynthesis, respiration, metabolism of proteins, fixation and nitrogen assimilation and nitrate reduction (Romera & Guardia, 1991). It is also a cofactor for enzymes (such as cytochrome oxidase, catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7) that catalyze biochemical reactions only, making it essential role in the growth and development of plants (Abbey, 1992; Larbi et al. 2006; Molassiotis et al. 2006; Agustí, 2003).
Of the two oxidation states that iron may occur in the soil: ferric (Fe3 +) and ferrous (Fe2 +), it is accepted that the plant takes preferably the latter, for this plant is forced to reduce the predominant form of iron in aerobic soils (Fe3 +). This process is performed by a reductase enzyme located in the plasma membrane of the root (Bienfait 1985; Römheld 1987) . This enzyme reaches its maximum activity at pH between 4 and 5 (Schmidt & Bartels 1997). On

the other hand, extreme temperatures (significantly above or below 25 º C), pH values greater than 7.5 and the presence of heavy metals affects their activity (Lucena 2000).
Fe is involved in such important processes as photosynthesis, respiration, metabolism of proteins, fixation and nitrogen assimilation and nitrate reduction (Romera & Guard 1991). It is also a cofactor for enzymes (such as cytochrome oxidase, catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7) that catalyze biochemical reactions only, making it essential role in the growth and development of plants (Abbey 1992; Larbi et al. 2006; Molassiotis et al. 2006; Agustí 2003).
Chlorosis means lack of chlorophyll in a plant organ, resulting in a loss of green color. Chlorosis can be caused by both the supply deficit of essential elements for plant growth (Fe, manganese (Mn), magnesium (Mg), zinc (Zn), nitrogen (N), etc), water stress or pests, fungi, bacteria or viruses. Iron deficiency, also called iron chlorosis, is one of the most important abiotic stresses. The causes of iron deficiency are numerous and vary, highlighting the availability of iron and bicarbonate ion concentration in the middle of development and other factors. Iron deficiency is usually caused by an insufficient concentration of it in the soil, but the existence of several factors makes affect the solubility and mobility of the Fe. These factors may be a high pH insolubilice some compounds, there limestone and other components, redox potential, interaction between Fe and other nutrients, moisture, organic amendment, salinity, extreme temperatures, etc. (Tagliavini & Rombola 2001). The low availability of iron is increased in soils with the presence of high bicarbonate levels that make the pH is around 8 (Marchner & Römheld 1995), this pH the concentration of Fe quite low and therefore difficult to iron nutrition of the plant (Lucena et al. 2006a). The problem of iron chlorosis is widespread because the limestone soils occupy approximately 30% of the land surface (Chen et al. 1982). It is estimated that between 20 and 50% of fruit growing in Mediterranean areas deficient in iron (Jaeger et al. 2000).
Some higher plants, in the absence of Fe have developed a number of mechanisms to increase the availability of Fe in the soil solution. Strategy I plants increase the Fe-making by the excretion of reducing substances of low molecular weight, through the extrusion of protons by roots acidifying the rhizosphere, solubilizing nutrients not available in an alkaline medium (Jones 2000) and increasing the activity of a reductase associated with the plasma membrane of the root responsible for the reduction of Fe3 +. Other, as the formation of root hairs and transfer cells in the root occur before or simultaneously with the physiological responses, which suggests that structural alterations may be a prerequisite for the functioning of mechanisms for efficient Fe (Landsberg 1986).
In higher plants Fe is predominantly found in chloroplasts, thus iron deficiency affects almost exclusively to the chloroplast, while the other cellular organelles that contain iron, such as peroxisomes, endoplasmic reticulum, mitochondria, etc.. seems to remain unchanged (Platt-Aloia et al., 1983).
Most of the mobilized Fe in plants is a phosphoprotein in the form of iron, the fitoferritina. The chloroplasts can contain up to 80% iron plant as fitoferritina (Tiffin, 1972).
The effect of Fe deficiency results in decreased concentrations of photosynthetic pigments and other components of the thylakoid membrane (Morales et al., 1991, 1994). Iron-deficient plants have less chlorophyll per unit chloroplast, but the number of these does not decrease per unit leaf. However, reducing the amount of chlorophyll is accompanied by alteration of the structure and functions of the chloroplast, reducing both the number and degree of stacking of the thylakoid membranes (Spiller & Terry, 1980, Terry & Abadía, 1986; Terry & Zayed, 1995; Soldatini et al., 2000).

The reduction of the thylakoid membrane is accompanied by decreased concentrations of photosynthetic pigments (chlorophylls a and b and carotenoids) in the leaves of affected plants (Morales et al., 1990, 1994). The loss of leaf pigments sheet does not imply that diminish their ability to capture light energy (Terry & Zayed, 1995) due in part to the increase in the relationship leaf carotene / chlorophyll concentrations decline with leaf carotene deficiency Fe to a lesser degree than the chlorophylls (Terry, 1980, Morales et al., 1990, 1994, 2000). The characteristic yellow chlorotic leaves is a consequence of the imbalance between the contents of chlorophyll and carotenoids (Abbey, 1992, Terry & Zayed, 1995).

Iron is also involved in chlorophyll synthesis (Miller et al., 1984) in different crops subject to conditions of deficiency, has shown an increase in the ratio of chlorophyll a: chlorophyll b (Abbey et al. 1989; Monge et al., 1987, Nishio et al., 1985). One explanation for this increase is that under conditions of iron deficiency in the field and there is full sunlight preferential photodestruction of chlorophyll b (Díez-Altar, 1959). Fe deficiency also increases the activity of chlorophyllase, which is involved in the degradation of chlorophyll in citrus fruits (Fernandez-Lopez et al., 1991).

Finally, iron is a constituent of many electron transporters (Terry & Abadía, 1986; Abbey, 1992, Terry & Zayed, 1995; Soldatini et al. 2000), so that iron deficiency is also reduced photosynthetic electron transport. These facts lead to a reduction in photosynthetic capacity of the plant that results in decreased levels of sugars, starch, certain amino acids and accumulation of others, thus altering the spectrum of proteins (Terry & Abadía, 1986; Abbey, 1992, Terry & Zayed, 1995) and enrichment in unsaturated lipids (Terry & Abadía, 1986; Abbey, 1992, Terry & Zayed, 1995), which alters the physiological functioning of the plant. The reduction in plant growth may be related to decreased photosynthetic capacity of chlorotic leaves. Both vegetative growth and production decrease with iron chlorosis (Hurley et al., 1986). The low capacity of the plant to translocate iron from old leaves, is manifested by the yellowing of young leaves , except their nerves remain green. These outbreaks are becoming less vigorous and its leaves, small, can fall prematurely, starting with the most apical (Agustí, 2003). One can also induce severe chlorosis reduced stem growth by inhibiting the formation of new leaves (Loue, 1993). Reducing the number and final size of the fruit, as well as total soluble solids content of the juice are also consequences that result from a deficiency of Fe (Agustí, 2003).

3. Effects of iron chlorosis in citrus

Many agricultural crops in arid and semiarid regions suffer chlorosis. Among the plants most affected are the citrus, planted in chalky soils often show signs of severe iron deficiency (Wallace, 1986). Iron chlorosis affects many biochemical, morphological and physiological parameters, and therefore their growth and development of plants (Larbi et al., 2006, Molassiotis et al., 2006, Agustí, 2003).

Iron deficiency in citrus tends to be observed during the months of winter and spring. And internervial yellowing of the leaves of young shoots are manifested, due to the inability of the plant to translocate iron from old leaves. The loss of pigmentation is caused by decreased chlorophyll content in chloroplasts (Marschner, 1995). This negatively affects the rate of photosynthesis and, therefore, the development of biomass (Abbey et al., 2004). The young shoots are becoming less vigorous and its leaves, small, can fall prematurely, starting with the most apical (Agustí, 2003).

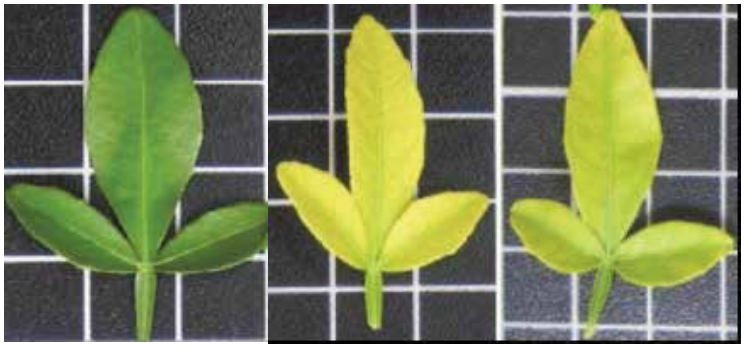

Fig. 1. Control and chlorotic leaves of citrange Carrizo.

In the case of a more serious deficiency, loss of pigmentation may also affect adult leaves, and the younger end completely yellow and devoid of chlorophyll. The fruits are also affected by severe iron deficiency. It reduces the number of fruits and their growth shortly after the set and these become yellow unexpected; Also decreases the concentration of juice soluble solids (Amorós, 1995; Agustí, 2003).

The treatment for iron chlorosis can be started before it appears, preventing the causes that lead. The best preventive practices include avoiding certain combinations plantations growing on soils unsuitable or use of tolerant cultivars. Once detected the symptoms of iron chlorosis trees can be treated with corrective methods to increase the availability of iron (Wallace, 1991), avoiding a loss of production (Pestana et al., 2001b). In cases of late diagnosis, will not be able to avoid the loss of annual production, but could be enhanced vegetative growth for the next harvest (Abbey et al., 2004). It has been described different types of treatments to correct iron chlorosis. Most important are foliar application, soil application and trunk injections. Foliar applications are very fast acting and can be less expensive than soil-applied treatments (Pestana et al., 2003), but have disadvantages of short-lived effects and cause phytotoxicity in certain cases (Wallace et al. 1984; Mortvedt, 1986), as well as cause the appearance of burns, defoliation, the greening in spots and no effect on the leaves that develop after treatment (Legaz et al., 1992, García et al., 1998; Fernández & Ebert, 2005). Thus foliar treatments are effective only in situations of symptoms of mild to moderate chlorosis (Rombolá et al., 2000). Moreover, Pestana et al. (2001a) indicated that foliar application of FeSO4 in citrus avoid losses in production and quality of fruits caused by it. The same authors found that several foliar applications are able to relieve iron chlorosis in field trials. The application of ferrous sulfate was more effective in increasing the size and quality of the fruits that the Fe (III)-chelate. Pestana et al. (2001b) also indicated that the addition of sulphuric acid produced a small increase in the concentration of chlorophyll and Fe in the leaves, without causing any effect on the size and quality of fruit. The same authors concluded that foliar application can reduce production losses and quality of fruits caused by iron deficiency.

Injections into the trunk and branches consist of introducing iron compounds either in solid or in solution, by drilling into the wood. This type of treatment is recommended for mature trees in which has a big trunk diameter. Both the solid and the liquid injection under high pressure have the advantage of having a great effect and great persistence of treatment, usually two or more years (Hurley et al., 1986, Yoshikawa, 1988). The major drawback to injections of solid type is the need for a large number of holes around the trunk, which

sometimes leads to problems of gumming (Heras et al., 1976). The main disadvantages of high pressure fluid injections are its high cost, because we have to move to field a pressure generating equipment with an injection machine (Yoshikawa, 1988).
Land application of iron fertilizer is the most widely used practice, and is done once the chlorosis appears in the crop (Pestana et al., 2003). The iron is supplied to the soil as chelates. Chelates are organic substances containing Fe in stable molecules. The iron remains assimilable by plants and doesn´t suffer the insolubilization reactions of this type of soils. The most commonly used is chelated Fe (III)-EDDHA and is often added to irrigation water (Papastylianou et al., 1993). Zude et al. (1999) showed that application of chelated iron in the soil produces a greening of Fe-deficient leaves of citrus. The same authors also indicated that both the chlorophyll content and photosynthesis increase after the application of Fe chelates.

4. Screening citrus rootstocks to iron chlorosis

Citrus trees grown today are composed of two parts, the rootstock and the variety, the second grafted onto it first, so that together combine the best features possible. In the screening citrus for iron chlorosis, the selection of plant material has to be done primarily, being the rootstock the determinant of tolerance to chlorosis. The performance of citrus rootstocks with iron chlorosis is very variable. Thus, although development is not limited by low levels, elevated content in active lime limit their use. The sensitivity of a rootstocks to elevated levels of iron chlorosis has many symptoms, on which also affected by other factors such as soil rich in calcium carbonate, calcium, moisture, pH and the grafted variety (Agustí, 2003).
The Swingle citrumelo is very sensitive to iron chlorosis. The rootstocks P. *trifoliata*, Carrizo citrange and sweet orange are susceptible to lime-induced chlorosis, while the sour orange and Cleopatra mandarin are tolerant to this deficiency (Hamza et al., 1986, Castle, 1987; Treeby & Uren, 1993; Pestana et al., 2005). Cleopatra mandarin are tolerant to iron chlorosis, but grows slowly in the field. Besides the production of fruit is not very high and although the fruit is of good quality, is smaller than that produced on other rootstocks.
There is a citrus rootstock breeding program taking place in the IVIA (Valencia, Spain) with the objective of the search for new citrus rootstocks well adapted to calcareous soils. In this regard, a new hybrid is now available in Spanish citrus nurseries: Forner-Alcaide 5 (FA 5) (Cleopatra mandarin x P. *trifoliata*). This rootstock has been evaluated from an agronomic point of view in calcareous soils (Gonzalez-Mas et al., 2009; Llosá et al., 2009) and it is more tolerant than Carrizo citrange, and trees 'Navelina' grafted in FA 5 produce 40% more than the trees grafted on Carrizo citrange, with the smaller fruit but equal quality to those of trees grafted on Carrizo (Forner et al., 2003, Forner-Giner et al., 2003).
There is no a perfect rootstock. All citrus rootstocks, that are currently used in the world, have some limitations. Over the years, every citrus producing area has been selected those rootstocks best suited to their conditions. However, there are still many areas for which there is no pattern can solve all their problems properly. Currently, there is large number of studies aimed at looking for a methodology for assessing the tolerance to iron chlorosis of citrus rootstocks (Castle, 2010; Gonzalez-Mas et al., 2009; Llosá et al., 2009). So far, that assessment could only be done reliably in field tests. The fruit breeding rootstocks represents the most cost-effective and sustainable solution to the problem of iron deficiency (Cianzio, 1995; Wirén, 2004).

Fig. 2. Differences between a tolerant (left) and susceptible (right) citrus rootstock

5. Molecular mechanisms underlying iron deficiency in citrus

The molecular components that are involved in the Fe deficiency response have been poorly identified and only in isolated cases. Mechanisms by which plants adapt to Fe deficiency have been frequently described in grasses (Strategy II) and other plants (strategy I) (Moog & Brueggemann, 1994). Strategy I (which is the one followed by *Citrus*) relies on increasing iron solubility by inducing membrane-bound Fe(III)-chelate reductases that reduce Fe(III) to Fe(II), which is more soluble and is subsequently taken up by specific transporters (Chaney et al. 1972).

In citrus, little is known about the behaviour at a molecular level of of the Fe(III)-chelate reductase in response to Fe starvation. Fe(III)-chelate reductase activity is much more increased in roots of *Citrus junos* (tolerant to iron chlorosis) than in *Poncirus trifoliate* (susceptible to iron chlorosis). Ling et al. (2002) founded that increase of Fe(III)-chelate reductase activity was about twenty fold whereas *P. trifoliate* was stimulated to increase only about threefold at the same time and under the same conditions. The result suggest that the increase in the enzyme activity under Fe stress was an important reason for the tolerance of the *Citrus junos* rootstock to Fe deficiency.

It has been reported a correlation between the development of Fe(III)-chelate reductase activity, acidification of the rhizosphere, and the accumulation of organic acids as citrate and malate in roots of *Capsicum annum* L. (Landsberg, 1986). In citrus, Fe(III)-chelate reductase activity and rhizosphere acidification have been reported to be induced in roots of several rootstocks as *Citrus volkameriana*, *Citrus taiwanica* and *Citrus junos* by Fe-shortage (Chouliaras et al. 2004). An increase in citric acid content in parallel with an aconitase activity decrease have also been reported in roots and fruit vesicles and calli of C*itrus Lemon* under Fe deprivation (Shlizerman et al., 2007). Aconitase catalyses the conversion of citrate into isocitrate, requiring Fe for its activity. Cytosolic aconitase represents a regulatory link between Fe homeostasis and organic acid metabolism. Under Fe shortage the enzyme loses

its enzymatic activity and binds to RNA of genes involved in Fe homeostasis, altering their expression (Hentze & Kuh 1996). In Fe-sufficient conditions, during the first half of fruit development, the decrease in the mitochondrial aconitase activity could play a role in the citric acid accumulation in the vacuole of the sac cells of the fruit (Sadka et al. 2000). During the second half of fruit development, the activity of the cytosolic aconitase increases, playing a role in acid decline. Shlizerman et al. (2007) studied the relation between the aconitase activity and the citric acid content that takes place after Fe deprivation. Cytosolic aconitase is more susceptible than mitochondrial one to Fe shortage. They demonstrate that only cytosolic activity was affected by Fe limitation whereas mitochondrial one was not. The reduction in the activity of the cytosolyc aconitase results in a decrease of the citrate catabolism, what account for the increase in pulp acidity of citrus fruit detected in trees grown in Fe deficiency. The authors observed that the application of Fe treatments during fruit maturation can reduce the acid content of the fruit juice what is an important practical question as in many scions as sweet oranges or clementines, high acid contents reduces the quality of the mature fruits delaying the harvest. However no data exist about this acid content decline is due to an activation of the cytosolic aconitase activity in the fruits.

Organic acid accumulation has in addition been interpreted in terms of the pH-stat theory (Sakano, 1998). The pH of the root cytosol and vacuole increases a consequence of the apoplast acidification that occurs in response to Fe deficiency (Espen et al., 2000). This argument is supported by the fact that Fe deficiency stress correlates with and increase in Phospoenolpyruvate carboxylase (PEPC) activity, resulting in an increased non-photosynthetic carbon fixation and a net carbon fluxes toward organic acid production (De Nisi & Zocchi, 2000).

The adaptative changes in strategy I plants include morphological changes in the root of Fe.deficient plants. Such morphological changes have been reported for other plants as Arabidopsis, *Ficus* or sunflower (Römheld & Marschner, 1981, Rosenfield et al., 1991, Schmidt et al. 2000). Typical morphological changes included additional cell division in the rhizodermis layer and enhanced formation of root hairs. In citrus, there is currently no

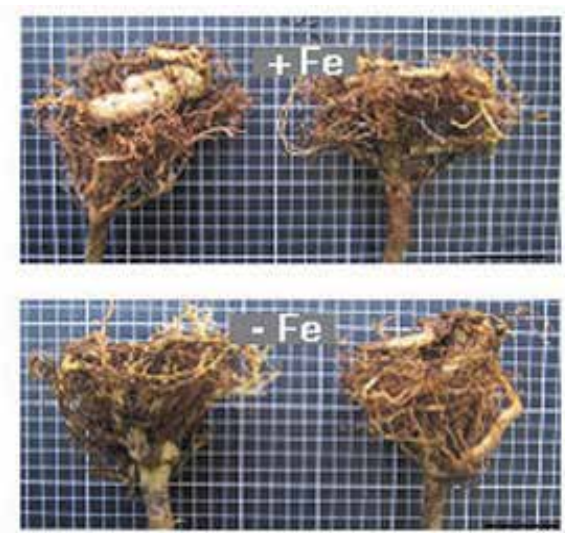

Fig. 3. Root morphology of Fe-sufficient and Fe-deficient *Poncirus trifoliata* plants

report about morphological changes in the root caused by iron deficiency. In the susceptible rootstock *Poncirus trifoliata*, Forner-Giner et al. (unpublished data) have not observed morphological changes in roots due to iron starvation (Fig 1). Changes occurring in citrus roots of susceptible plants are mainly at a molecular level. Forner-Giner et al. (2010), in a genomic survey, found that within the genes that were differentially expressed in the plant in response to iron deficiency, a large group had to do with cell wall (Table 1).

Gene ID	Best *Arabidopsis* BLAST hit	Cell Wall Component
C31502B08	Calmodulin-regulated Ca(2+)-pump	pectin
C05811H06	polygalacturonase (pectinase)	pectin
C05140C08	SYR1, Syntaxin Related Protein 1, also known as PEN1	cuticle
C05133B06	endo-xyloglucan transferase	xyloglucan
C19009B12	xyloglucan endotransglucosylase	xyloglucan

Table 1. Cell wall related gene that are overspressed in response to Fe-deficiency

By specific staining for each component, they demonstrated that the cell wall becomes thinner in *Poncirus* plants under iron starvation and they also highlight that pectin and xyloglucan component changed in these plants. As it is shown in figure 2, ruthenium red staining (specific for pectin) revealed the thinning of the cell wall in the Fe deficient plants.

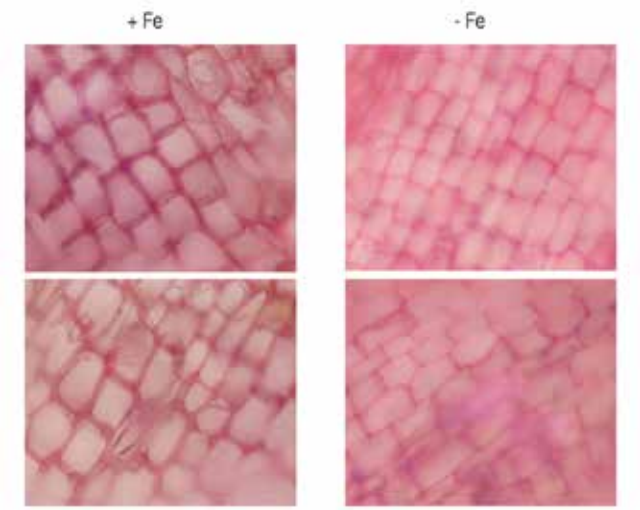

Fig. 4. Ruthenium red staining in roots of *Poncirus trifoliate* treated with (control) or without Fe-EDDHA (iron deficiency)

Moreover, calcofluor (a dye specific for various beta-D-glucans including Xyloglucans) stained the cell wall of the roots of more intensely than those of control plants (fig. 3).

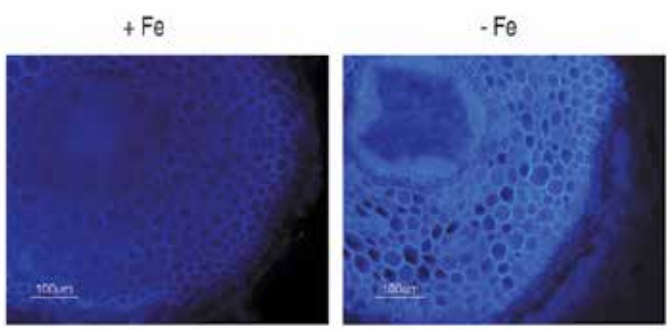

Fig. 5. Calcofluor staining in roots of *Poncirus trifoliate* treated with (control) or without Fe-EDDHA (iron deficiency)

The composition of the cell wall and the cross-linking of the different polymers that form it, most probably determine the wall mechanical properties. So that, one may hypothesize that this changes may occur because the plasticity of the cell wall there must be probably important for plant acclimation to iron deficiency (as well as to other environmental conditions). However, whether this is a mechanism to improve adaptation to iron shortage or it is a final consequence of the stress produced by the iron deficiency is still unknown and it will have to be elucidated in the future.

As for other plants, some authors have found an important decrease in soluble and ionically-bound-to-cell-wall peroxidase activities in citrus in response to Fe-deficiency (Chouliaras et al. 2004, Forner-Giner et al. 2010). As to ionically-bound-to-cell-wall peroxidases are implied in lignin metabolisms and several cell wall modifications are taking place in response to iron deficiency, one may infer that this component is being also altered during the response process. However no changes in lignin accumulation could be

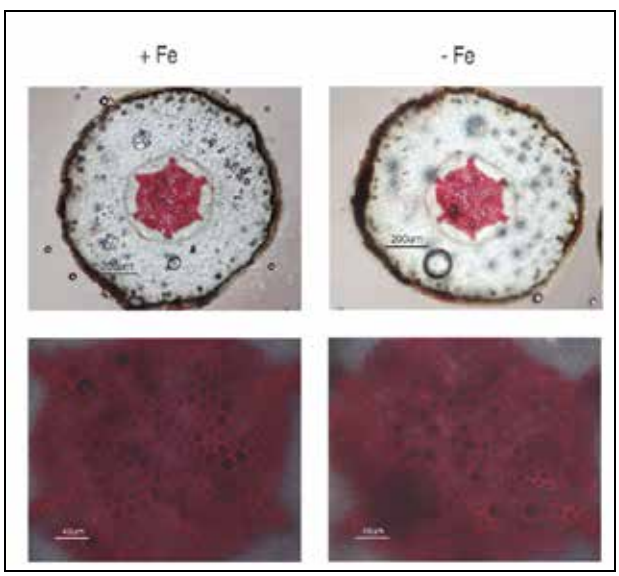

Fig. 6. Phoroglucinol staining in roots of *Poncirus trifoliate* treated with (control) or without Fe-EDDHA (iron deficiency)

appreciated (fig 4) when root sections of control and Fe-deficient plants of *P. trifoliate* were stained with phoroglucinol (a specific dye that stains lignified tissue red and leaves everything else unstained) and visualised under the microscopy (Forner-Giner & Ancillo, unpublished).

Peroxidase activity is also responsible of the conversion of H_2O_2 into water by oxidizing various hydrogen donor molecules such as phenolic compounds and auxin metabolites (Dunand et al. 2007). As in other plants, in citrus, the Fe-related decline of peroxidase activity coincide with a significant decline in catalase activity (Chouliaras et al. 2004, Forner-Giner et al. 2010), which is also involved in H_2O_2 metabolism. In maize, the decrease in these enzymatic activities entails an increase in the concentration of H_2O_2 and superoxide anion radical (O^{2-}) that causes oxidative stress in Fe- deficient plants (Tewari et al. 2005). No direct evidences of the H_2O_2 increase have been published in citrus, but Forner-Giner et al. (2010) reported the induction, in response to Fe-deficiency, of a NADPH thioredoxin reductase C which is involved in reduction of H_2O_2. All together, these results suggest that the reduction in the level of catalase and peroxidase activities in iron-deficient citrus plants produce an increase of H_2O_2 and other ROS that could lead to oxidative stress and, as reported for other plants (Perez-Ruiz et al., 2006) the induction of NADPH thioredoxin reductase C may be part of the response of the plant to prevent damage by the oxidative stress caused by iron deficiency. Chouliaras et al. (2004) founded that peroxidase and catalase activities were higher in the case *Citrus taiwanica* (a tolerant rootstock) rather than in *Citrus volkameriana* (a non-tolerant one). They propose that the ability of plants to synthesize more peroxidase and catalase could be a feature associated with tolerance to Fe deficiency.

6. Future research and conclusion

Iron is an essential nutrient playing critical roles in life-sustaining processes. Due to its ability to gain and lose electrons, iron works as a cofactor for enzymes involved in a wide variety of oxidation-reduction reactions (as photosynthesis, respiration, hormone synthesis, and DNA synthesis, etc). This essential role made of iron an absolutely required nutrient, and its deficiency causes iron chlorosis which seriously constraints the normal development of the plant. Iron chlorosis is a widespread problem especially for regions where the bioavailability of iron in soil is low. Usual remediation strategies consist of amending iron to soil, which is an expensive practice. Thus genetically improved chlorosis resistant rootstocks and new cultivar/rootstock combinations offer the best solution to iron chlorosis and is one of the most important lines of investigation needed to prevent this nutritional problem nowadays. However tolerant rootstocks are difficult to develop when not available and mean a long-term approach. Therefore, there is a need for new methods to diagnose and correct this nutritional disorder.

The use of microarray techniques revealed changes in gene expression level due to Fe deficiency and has allowed insights into the transcriptional regulation of some functions. These studies have extended our knowledge of citrus response to iron deficiency. These experiments have identified candidate genes and processes for further experimentation to increase our understanding of citrus response to iron deficiency stress.

It is likely that more extensive microarray analysis, coupled with a suitable annotation of the citrus genome, which has recently been completely sequenced, will prove invaluable in future studies of iron-stress responses in plants. Thus, while the use of functional genomic approaches to study iron-stress responses in plants already has yielded important

information, the continued development of resources promises to yield many more insights in the future. Information gained from studies of this type may allow the development of citrus plants that are capable of growth on soils that are iron-deficient.

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8

Response, Tolerance and Adaptation to Abiotic Stress of Olive, Grapevine and Chestnut in the Mediterranean Region: Role of Abscisic Acid, Nitric Oxide and MicroRNAs

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1. Introduction

Hot, dry summers and mild to cool, wet winters are the characters of the Mediterranean climate. Drought, extreme temperatures and extreme irradiation (UVs) often concomitantly - in some cases also together with salinity, significantly affect the growth, yield and quality of the Mediterranean crops.

Olive (*Olea europaea* L), grapevine (*Vitis vinifera* L) and sweet chestnut (*Castanea sativa*) are the most important woody crops in the Mediterranean among others. The olive tree and vineyard are familiar features of the Mediterranean landscape. In some mountain regions, these features are accompanied by the orchards of chestnut. Olive oil and wine are important products in that region. In some regions, such as Italy, Turkey, Spain, Portugal, and Greece, chestnut is one of the most important fruit products as well. Olive oil, grape and wine are a traditional icon of the Mediterranean diet. Enjoying the plentiful indigenous plant products, especially wine, olive oil and chestnut, is part of the Mediterranean civilization.

Olive oil is the main source of fat in the Mediterranean diet and one of those basic ingredients essential to life in the Mediterranean. It may also protect against heart disease, stroke, and certain cancers. The vine and wine are among the most important symbols of

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societies that have emerged around the shores of the Mediterranean (Stanislawski, 1970). In most Mediterranean countries such as Portugal, France, Italy, Greece and Spain—wine is more than just a beverage; it is an integral part of meals and an essential aspect of social gatherings.

The European chestnut species *C. sativa* has been cultivated in the Mediterranean region for both fruit and timber for dozens of centuries. Sweet chestnut provided staple food with nutritious and health properties for people in the Mediterranean for centuries especially in the mountains and used to be called the 'bread-tree' (Avanzato, 2009). Chestnuts are delicious and healthy foods, containing many highly valuable carbohydrates and phytochemicals, and no cholesterol and low fat. It is an ingredient in many traditional recipes.

Plant abiotic stresses and response of plants to these stresses have been extensively studied. In this chapter, we have summarized the recent advance in the response, tolerance and adaptation of these Mediterranean woody crops to the environmental stresses especially drought and extreme temperatures imposed by the typical Mediterranean climate, and the underlying mechanism. At molecular level, plants share some common pathways involved in different abiotic stress responses. Different forms of abiotic stresses may lead to similar responses in plants during the stress; likewise, different kinds of stresses have also been found to trigger responses in similar sets signalling molecules. The perception of stresses and the consequent adaptation by plants include physiological, molecular and biochemical changes in plants which largely depend on factors such as severity of stress, plant developmental stage and their genotype (Agarwal & Zhu, 2005). After the perception of a signal by plants, immediately there will be generated secondary signals which are normally nonprotein molecules, including membrane ion (K^+ and Ca^{2+}) flux, inositol phosphates (IPs), reactive oxygen species (ROS), and nitric oxide (NO). Each of these can activate plant mitogen-activated protein kinase (MAPK) and Ca^{2+}-dependant protein kinase (CDPK) and activation of protein phosphatases. These early events lead to hormone accumulation, particularly abscisic acid (ABA), salicylic acid (SA) and brassinosteroid hormonal, the synthesis of heat shock proteins, activation of antioxidant enzymes and synthesis of low molecular antioxidants and compatible solutes and membrane lipid peroxidation, followed by changes in transpiration, gas exchange, respiration, and growth, resulting in stress adaptation. MicroRNAs (miRNAs) also participate in stress adaption response in plants. In this chapter we concentrate in the role of ABA, NO and miRNAs in the abiotic stress response and adaptation. Finally, the progress in genetic modification targeting improved abiotic stress tolerance of these plant species is reviewed.

2. Morpho-anatomical, physiological, and biochemical response and adaption

2.1 Olive capacity to withstand arid environments

Olive is a perennial, long-lived, evergreen tree of subtropical origin (Bongi & Palliotti, 1994) that, in the Mediterranean, flowers in mid-to-late spring. This adaptation allows olive to escape the deleterious effects of cold on flowering and fruit set but serves to increase reliance on a range of avoidance and tolerance mechanisms that maintain internal water status and metabolic activity during the hot, dry summers (Connor, 2005).

Olive tree is well known to be very resistant to drought (Bacelar et al., 2009; Bacelar et al., 2006; Connor, 2005; Fernández & Moreno, 1999; Giorio et al., 1999; Tognetti et al., 2004). Furthermore, it has been postulated that the minimum water requirement for olive is 200

mm year^{-1} (Bongi & Palliotti, 1994). Abd-El-Rahman et al. (1966) measured the water content of olive leaves at saturation, finding a value, 1.59 g water g^{-1} dry weight, extremely low compared with other species growing in the same environment (5.77 g water g^{-1} dry weight for fig, 5.85 g water g^{-1} dry weight for grape). There are many mechanisms by which it resists to more or less extended drought periods but some differences among olive cultivars have been observed concerning their ability for adaptation and production under drought conditions (Bacelar et al., 2004; Bosabalidis & Kofidis, 2002; Chartzoulakis et al., 1999b).

Olive culture has prospered under rainfed conditions in Mediterranean environments because the tree is capable of acceptable yield while subjected to the characteristic prolonged summer water shortage. Olive achieves this result with physiological, biochemical and morpho-anatomical responses that reduce water loss and maintain water uptake at high plant water status as drought commences (drought avoidance), and with others that tolerate dehydration at low plant water status as the drought deepens (drought tolerance) (Connor & Ferreres, 2005).

Olive leaves are well designed to control water loss. Morphological characteristics allow minimum radiation load and maximum heat exchange while the physiological responses of stomata to leaf water status and atmospheric humidity provide effective control of transpiration (Fernández et al., 1997; Loreto & Sharkey, 1990). Leaves minimise radiation load by small size, a dominantly vertical display (Mariscal et al., 2000) that is further aided by paraheliotropic movement under water stress (Natali et al., 1999) (Fig. 1A), a dense packing of the mesophyll layers (Bongi et al., 1987) and high reflectivity by a thick cuticle and epicuticular wax layers (Leon & Bukovac, 1978) (Fig. 1B). This combination of morphological features restricts temperature increase in leaves with small latent heat exchange when transpiration is restricted by stomatal closure.

Stomata are small and dense and occur only on the abaxial surface, under dense layers of peltate trichomes (or peltate scales) (Fig. 1C). The peltate trichomes reflect the sunlight and reduce the transpiration of the leaves.

An interesting characteristic in the anatomy of olive leaf is the presence of a complicated, dense network of filiform sclereids that are of idioblast nature (Karabourniotis et al., 1994) (Fig. 1D). This entangled network follows two major distribution patterns: the "subepidermal layer" consisting of the "T"-shaped sclereids extending between the adaxial epidermis and the palisade layer, and the branched the ˙polymorphic sclereids that transverse the spongy mesophyll layers, producing a chaotic pattern. Sclereids act like synthetic optical fibres and, besides other functions, may contribute to the improvement of the light microenvironment within the mesophyll of the thick and compact sclerophyllous olive leaves (Karabourniotis et al., 1994).

It has been reported that olive leaves formed under water stress are more able to control transpiration, being smaller and thicker and having more dense and smaller stomata (Bosabalidis & Kofidis, 2002; Chartzoulakis et al., 1999b). However, Lo Gullo and Salleo (1988) observed that despite all this protection against water loss, leaves of the wild olive tree (*O. oleaster*) underwent a substantial water loss under conditions of water stress.

A drought avoidance response not displayed by olive is the development of a deep rooting system (Bongi & Palliotti, 1994). However, the extensive root system of olive tree seems to be designed for absorbing the water of the light and intermittent rainfall usual in its habitat (Fernández & Moreno, 1999). Most of the main roots grow more or less in parallel to the soil surface, and the highest root density is found close to the trunk, although the volume explored by the roots can easily extend beyond the canopy projection (Fernández & Moreno,

1999). This rooting habit is probably the result of sensitivity to hypoxia and may allow for efficient water absorption (Bongi & Palliotti, 1994; Fernández & Moreno, 1999). A high portion of the root is of small diameter, which also favours the absorption capacity. Absorption by olive roots is also enhanced by high potential gradients between roots and soil caused by osmotic adjustment (Fernández & Moreno, 1999).

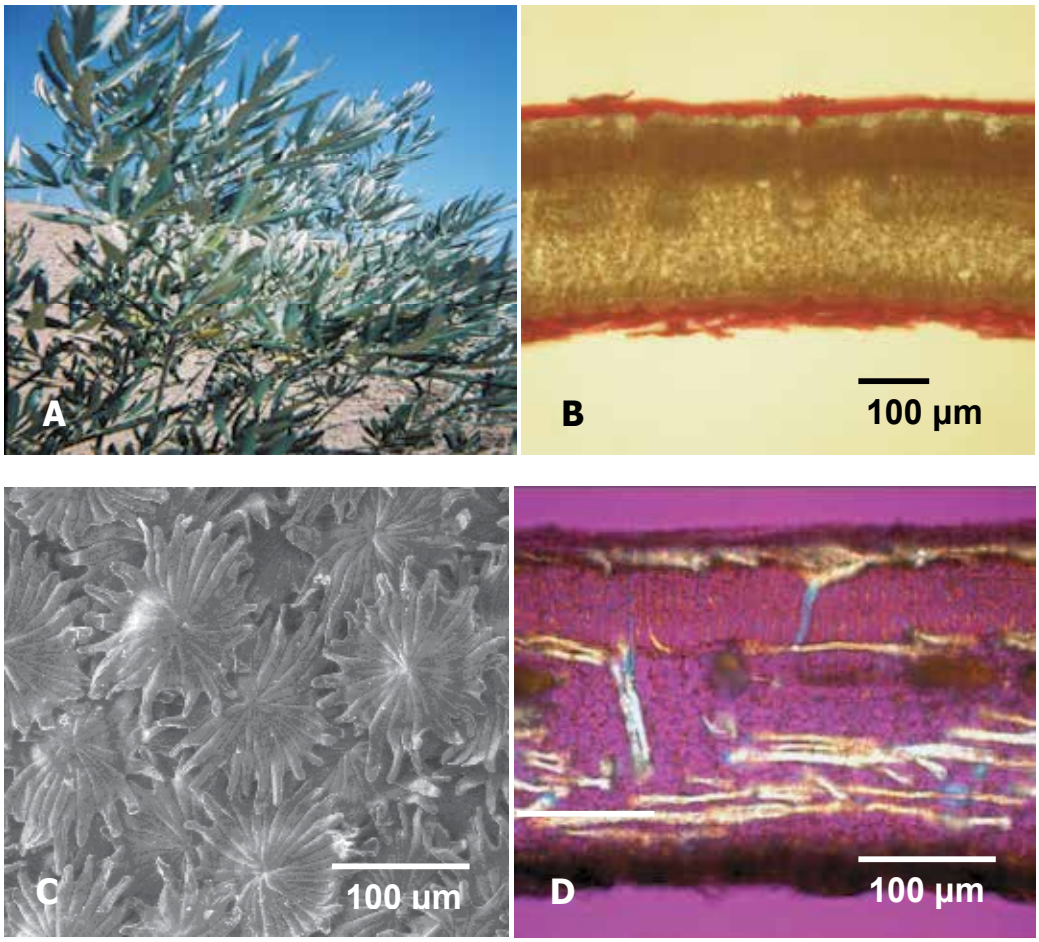

Fig. 1. Olive protections at leaf level against water loss and excessive irradiance. (A) Paraheliotropic movement under water stress; (B) Dense packing of the mesophyll layers and thick cuticle and epicuticular wax layers (optical micrograph); (C) Dense trichome layer of abaxial surface protecting the stomata (SEM micrograph); (D) Dense network of sclereids (optical micrograph, cross-polarized light).

The olive is a diffuse-porous tree having a dense wood with abundant fibers and little parenchyma (Fernández & Moreno, 1999). The large amount of fibers, which makes olive wood so hard, accounts for the low vessel lumina of the species in comparison with other diffuse-porous Mediterranean plants. Salleo et al. (1985) observed that the vessel lumina, when expressed as percentage of the total xylem cross-sectional area, was half that measured in other Mediterranean species such as *V. vinifera*. The low hydraulic conductivity

of olive xylem is a feature that seems to play an important role in the tree's water relations. Salleo and Lo Gullo (1993) observed losses of about 10% of hydraulic conductivity in 1-year-old twigs of young *O. oleaster* trees, when these became stressed, due to xylem cavitation. One consequence of this is that olive trees prevent excessive water loss on days of high water demand by closing their stomata soon after midmorning (Fernández et al., 1997).

During periods of water stress, olive tree typically experience reductions in transpiration, stomatal conductance and net photosynthesis (Giorio et al., 1999). Nevertheless, environmental and physiological factors do not affect H_2O and CO_2 exchange to the same extent, resulting in possible variations in water use efficiency in this species (Xiloyannis et al., 1988). Meanwhile, some differences in gas exchange responses to water stress between olive cultivars have been observed in previous experiments (Chartzoulakis et al., 1999a; Tognetti et al., 2002).

In moderate drought conditions, olive plants stop shoot growth but not photosynthetic activity and transpiration. This allows the continued production of assimilates as well as their accumulation in the various plant parts, in particular in the root system, creating a higher root/leaf ratio compared to well-watered plants (Xiloyannis et al., 1999).

Olive tolerates drought by maintaining turgor through osmotic adjustment and changes in cell wall elasticity (Connor, 2005). Active and passive osmotic adjustment plays an important role in maintaining cell turgor and leaf activities which depend on it (Xiloyannis et al., 1999). Mannitol and glucose play a major part in the osmotic adjustment of olive leaves (Cataldi et al., 2000). In addition, the osmotic adjustment observed in the root system allows maintenance of cell turgor, avoiding or delaying the separation of roots from soil particles (Xiloyannis et al., 1999). The accumulation of proline under drought stress in both leaves and roots of 2-year-old *O. europaea* (cv. Coratina) plants (Sofo et al., 2004b) also indicates a possible role of proline in drought tolerance.

Under field conditions, particularly in the Mediterranean regions, water stress is often accompanied by other environmental constraints, such as steep leaf-to-air water vapour gradients, and high irradiance and temperature (Osório et al., 2006). Measurements have revealed non-stomatal limitations to photosynthesis consistent with photoinhibition in olive leaves exposed to high irradiance (Angelopoulos et al., 1996). The synergic action of high irradiance level and water stress reduces the capacity of the photosynthetic systems to utilize incident radiation, leading to a higher degree of photodamage (Bacelar et al., 2007; Sofo et al., 2004a). The increase of malondialdehyde content and lipoxygenase activity, two markers of oxidative damage, observed by Sofo et al., (2004b) in both leaf and root tissues of olive plants during the progressive increment of drought stress, indicates that water deficit induces lipid peroxidation. This result suggests that higher activities of some antioxidant enzymes and non-enzymatic antioxidants are required for a better protection against oxidative stress related to water deficit.

2.2 Vine's response, tolerance and adaptation to abiotic stress

Most of the world Wine Regions, such as the Douro Region in Portugal, has a Mediterranean climate with a strong continental influence. In these regions the rainfall is mainly concentrated in the winter months and the springs and summers are characterized by exceedingly hot and dry. In these conditions grapevines are often subjected to periods of severe drought associated with strong light and high temperature (Chaves et al., 2002). Consequently, the vineyard experiences irreparable damage on physiology behaviour and

yield attributes. The implementation of cultural strategies, which aims a better adaptation to these conditions, is a major goal, especially in the current scenario of global climate change (IPCC 2007).

2.2.1 Abiotic stress response

Under summer stress and as the first limitation, the photosynthetic productivity is limited by the stomatal closure, either in response to a large decrease in leaf water potential or due to an increase in atmospheric vapour pressure deficit. Several studies undertaken in the Douro Region clearly have shown that grapevines growing under severe summer stress experience a significant decline in productivity, mostly owing to stomatal limitations to photosynthesis (Moutinho-Pereira et al., 2004).

Grapevine cultivars differ in the degree of control exerted by stomata under conditions of water limitation. While some varieties are genetically programmed to react to early signs of dryness in the air and/or soil, others may have greater difficulties in stomatal regulation (Moutinho-Pereira et al., 2007). For instance, under water stress conditions the water use efficiency and the correlation between net photosynthesis rate and stomatal conductance are significantly higher in 'Riesling' than that in 'Silvaner' (Düring, 1987). The ABA concentration, arriving from roots to leaves, is directly implicated in this behaviour (Correia et al., 1995). On the other hand, in grapevine the stomata response to ABA concentration is not uniform across the leaf surface. According to Düring (1992), this behaviour is related with the heterobaric anatomy (patchiness) of vine leaves, which makes the gas diffusion difficult in the intercellular spaces of the mesophyll and is responsible for non-uniform aperture of stomata over the leaf surface.

The photosynthetic apparatus is generally tolerant to water stress. However, if the imposition of dehydration of mesophyll cells is moderate but continued or severe but brief, a metabolic adjustment takes place through metabolic pathways, mainly related with RuBP regeneration and Rubisco activity (Medrano et al., 2002).

The structural integrity of chloroplasts and the photochemical reactions and electron transport chain do not seem to be much affected by low water potentials. Only the thickness of thylakoid lamellae seems to decrease (Chaves, 1991). In fact, Flexas et al. (1998) and Escalona et al. (1999) found that in its natural environment and under water stress the vine only developed a few signs of down-regulation of the photochemical activity. However, in Mediterranean climate, water stress is usually associated with many clear and hot days, which, in a synergistic action, leads to a significant down-regulation or photoinhibition of the photosynthetic apparatus (Osório et al., 1995). Under these conditions, the vineyard experiences irreparable damage. Frequently, some leaves display irreversible photoinhibition and chlorosis, followed by necrosis and leading to low grapevine water-use efficiency (Moutinho-Pereira et al., 2003). The air temperature increase will accelerate the grapevine phenology, leading to a reduction in the vegetative and reproductive period (Seguin & Cortazar, 2005).

2.2.2 Tolerance and adaptation

The ability of the vineyards to grow and produce satisfactorily in severe summer stress conditions depends on the development of morphological and physiological mechanisms, which allows them to retard the level of dehydration that is detrimental to cellular metabolism. In general, this is achieved through an improvement in water absorption by

roots and/or by reducing water loss. The formation of a deeper and dense root system by rootstock depends on the interaction of its genetic characteristics and is usually an effective strategy for grapevine to capture more water in periods of lower water availability (Palliotti et al., 2000). In this context, the selection of rootstock with these characteristics and a good soil preparation are one way to achieving these mitigation objectives.

One of the most widespread mechanisms to reduce grapevine water loss is achieved through lower vigor and/or partial senescence of leaves (Chaves, 1991). The increase in stomatal conductance mediated by the ABA concentration is another mechanism developed for the same purpose, especially in the periods of the day with deficits of higher vapor pressure (Iacono et al., 1998). The prevention of photoinhibition and overheating of the leaves in consequence of the lower leaf transpiration can also be undertaken by changing the leaf angle, e.g., from 53° to 80° (angle between the blade and petiole) (Smart, 1974).

The grapevine adaptations to dry and hot habitats seem to be strengthened by changes that occur at the level of vascular system, particularly the reduction in xylem section, which induce a significant decrease in hydraulic conductivity and thus minimize the susceptibility of these vessels to the phenomenon of cavitation (Lovisolo & Schubert, 1998; Schultz & Matthews, 1988).

The active accumulation of soluble sugars and other low molecular compounds is responsible for lower osmotic potential, allowing the cell turgor maintained as much as possible, with positive values. This process, known as osmotic adjustment, has been shown in vines gradually subjected to water stress, either in leaves (Düring, 1984) or in roots (Düring & Dry, 1995). Under prolonged drought, a decrease of 4 to 5 bars in osmotic potential, mainly more evident and rapid in young leaves than in adult leaves (Düring, 1984). The capacity for lowering the osmotic potential might be the dominant strategy for better restricting the leaf water losses in grapevines growing under water stress conditions (Patakas & Noitsakis, 1999).

3. Limitations of European chestnut growth at low latitudes

European chestnut (*Castanea sativa* Mill.) is characterized as a mesophilic species (Cortizo et al., 1996). Plants from this species are moderately thermophilic and well adapted to ecosystems with a yearly mean temperature ranging between 8 ^{o}C and 15 ^{o}C and monthly mean temperatures during 6 months over 10 ^{o}C. Unfortunatly, nowadays, chestnut tree growth shows some constrains which might be partially attributed to the climatic alterations.

In Europe, the chestnut is widespread. The Azores archipelago (25^{o} - 31^{o} W) is the most Occidental point for *C. sativa* and the Canary Islands is the most Southern point (27^{o} - 29^{o} N). Towards the north, chestnut fruit production reaches 52^{o} N latitude to the south of the United Kingdom, northern Germany, Poland and Ukraine.

It is found at sea level in some littoral areas above 39^{o} N latitude, as such the northern Iberian peninsula, north of Italy and Middle Eastern Greece due to sea influence. Below this latitude, still in the littoral areas, adequated climatic conditions for chestnut are found in higher altitudes, as it happens in Sierra Nevada (1500 m a.s.l., Granada, Southern Spain), Teide Mountain (2000 m a.s.l., Santa Cruz Tenerife, Canary Islands) or in Etna mountain (2000 m a.s.l., Sicily Island, Southern Italy). In the interior part of Europe, under continental climatic influence, chestnut only can grow above 500 m a.s.l. being the maximal altitude 1100 m a.s.l. in the highest mountains of Trás-os-Montes (Northeast of Portugal) or even to

1800 m in Caucasus Mountains, the former altitudes corresponding to the ancient orchards and the highest altitudes to the newest plantations (Gomes-Laranjo et al., 2005; Pereira-Lorenzo et al., 2010).

Below 600 m a.s.l. climate is hotter and dryer than the adequate conditions for chestnut, corresponding to a transition altitude, vineyards, olive tree and almond being now the main crops. Contrarely, above 1100 m a.s.l. climate is colder and wetter, and vegetative cycle is shorter than that needed for fruit production. So, typical climate is a continental temperate type, with mean annual values of sunlight and precipitation, 2400 to 2600 h and 600 to 1200 mm, with the total amount of temperature from lowest and highest altitude orchards ranging between 2800 ºD and 3400 ºD, respectivly (Gomes-Laranjo et al., 2007). Degree-days (°D) represent the amount of heat required, between the lower and upper thresholds (where T_0 = 6.0 ºC, see Fig. 2) for an organism to develop from one point to another in its life cycle (Cesaraccio et al., 2001; Zalom et al., 1983). For overall Portuguese varieties, half rate of photosynthesis (A_{50}) is found when temperature reachs 11ºC (T_{50m}) and 38ºC (T_{50M}), being the optimal value around 24ºC (Gomes-Laranjo et al., 2007). In relation to limitant radiation intensities, results suggest that it is a dimlight species, since 75% of maximal photosynthetic rate is found at 900 µmol.m^{-2}.s^{-1}, which corresponds almost at half full sunlight intensity, and A_{50} is at 400 µmol.m^{-2}.s^{-1}. So, European chestnut could be indicated to be included in restoration programes for the European forest, since adult trees save most of light in the top of their canopies and only low intensity will attain soil level. Identical conclusions have been drawn by Joesting et al., (2009) in relation to American chestnut (*C. dentata* (Marsh.) Borkh) with the aim to restore chestnut populations in eastern deciduous forest from Appalachian mountains.

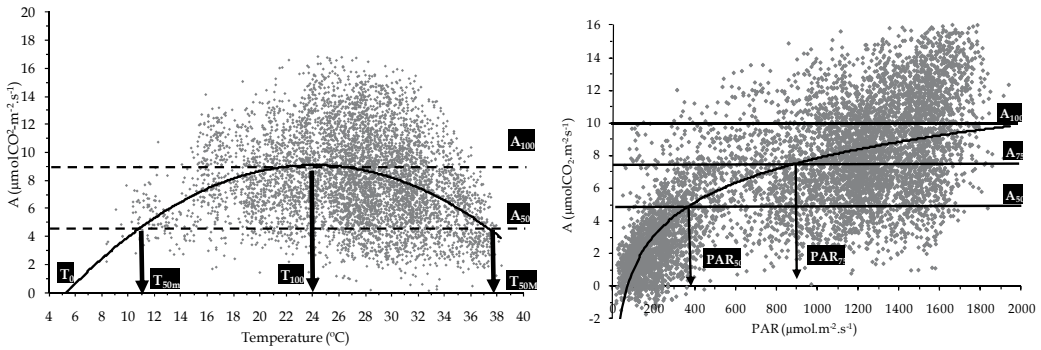

Fig. 2. Threshold temperature (left) and radiation (right) for photosynthesis rates in chestnut leaves. Study was done with 13 Portuguese varieties in Trás-os-Montes Region during 6 years. Concerning temperature study, T_0 represents the temperature value for vegetative zero growth, T_{50M} and T_{50m} the values that induce half rate (A_{50}) of the maximal photosynthesis (A_{100}). Values were obtained according to second polynomial curve, $y = -0.0253x^2 + 1.2349x - 6.2532$, $R^2 = 0.1094$. In relation to the radiation study, A_{100}, A_{75} and A_{50}, mean the maximal, 75% and half of the maximal value of photosynthesis rate, respectively, being PAR_{50} and PAR_{75} the respetive values of photosynthetic active radiation (PAR). These values were calculated from logarithmic equation, $y = 2.9628\ln(x) - 12.62$, $R^2 = 0.5335$ (n=8852).

In the interior regions of Europe, where chestnut grows under continental climatic influence, altitude is decisive to define adequated climatic conditions. As the Fig. 3 shows, photosynthesis increases with altitude, and inversly with temperature. So, maximal rates can be found above 800 m a.s.l. where temperature down to 25 – 22 ºC, the range of optimal temperature as has been refered above. In the lowest altitudes, photosynthesis rate decreases around 40%, indicating that chestnuts under these climatic conditions, nowadays start to suffer from abiotic stresses, mainly due to the heat stress. Regarding internal water balance, Martins et al., (2010) have demonstrated that adult trees can be saved from water stress, since they can continuosly absorb water from deep soil layers and so preserving predawn leaf water potential in the range of -0.6 to -0.9 MPa.

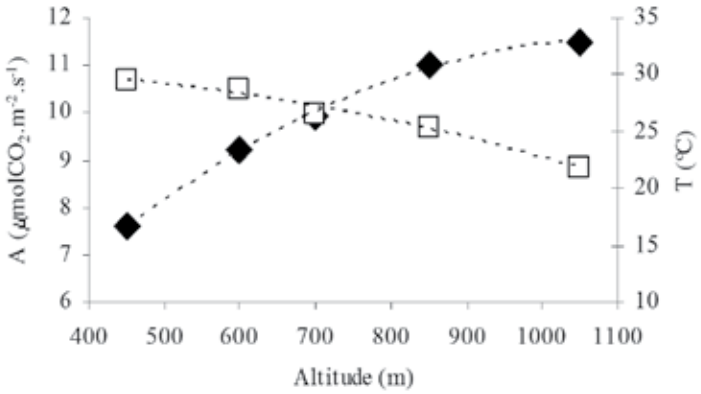

Fig. 3. Variation of mean daily photosynthesis rate (A, closed symbols) in leaves from chestnut (var. Judia) and air temperature (open symbols) as a function of altitude.

Additionally, the European chestnut ecotypes coming from wet sites are more locally adapted and less plastic than those from dry sites and hence more vulnerable to the climate changes (Villani et al., 2010). Five gene pools have been determined in Europe: three in Greece, one on the northwestern coast of the Iberian Peninsula and a large gene pool covering the rest of the Mediterranean basin (Martin et al., 2010; Mattioni et al., 2008).
The existence of some adaptative variation among populations from extreme conditions is proposed (Fernández-López et al., 2005): populations from Greece initiate growth earlier followed by those from South Italy and South Spain, while ecotypes from north Spain and Italy initiate later. A significant genetic variation between north and south Iberian ecotypes has also been confirmed (Fernández-López et al., 2005). The expected global climate changes are a great challenge for forest tree breeders. Eriksson et al., (2005) stablished a xerothermic index to characterize each one of those ecotype´s local origin and they found a negative correlation between it and plant growth at both 25ºC and 32ºC, but a positive correlation with carbon isotope discrimination, suggesting a large additive coefficient of variation for growth traits. This variation confers to the species good possibilities to respond genetically via natural or artificial selection to enviromental change. Dinis et al., (2011) working with plants from the portuguese Judia variety, have also concluded that the morphological and phenological differences among ecotypes are not only related to the small genetic differences, but are simply phenotypic adaptations to different climatic conditions.

Lowest altitudes and so, highest temperatures, seem to induce sun characteristics in leaves, demonstrated by low chlorophyll amount (Chl), since thermoinhibition might speed light saturation of the photosynthetic process (Dinis et al., 2011). On the other side, leaves present high Chla/b and low Chl/Car ratios are consistent with their acquired tolerance to warm and sunny conditions (Gomes-Laranjo et al., 2006; Pearcy, 1998). Chla is the main photosystem I pigment, which is located in exposed thyalakoid membranes, and carotenoids have the chlorophyll protection function against photoinhibition (DemmigAdams & Adams, 1996). Moreover, increase in Chla/Chlb suggests higher proportion of stacking thylakoid membranes, which in turn might induce higher photosynthesis rates, if any stress factor imposes (Anderson et al., 1988).

Altitude	Chltot	Chla/b	Chl/Car	Total fatty acid		PI	UI	Malonic aldehyde x10^{-4} (mM)	
(s.l.m)	mg.cm^{-2}			Saturated (%)	ınsaturated (%)			Control	ADP-Fe
1050	121.5 b	3.12 c	4.8 a	27.0	73.0	111.5	171.3	1.35	3.88
900	145.9 a	3.10 c	5.0 a	32.4	67.6	97.5	154.2	1.67	4.57
700	99.1 c	3.30 b	4.4 b	38.2	61.8	119.2	156.3	2.00	4.91
600	143.9 a	3.40 b	4.6 b	33.1	66.9	47.5	146.1	1.90	4.53
450	80.9 d	3.60 a	3.9 c	43.9	56.1	79.6	121.2	3.12	5.36

Table 1. Determination of photosynthetic pigment content (n=10), fatty acid composition (n=3) and malonic aldehyde (n=3) in chestnut chloroplast (var. Judia) (Gomes-Laranjo et al., 2005) isolated from leaves collected in the range of altitudes between 450 and 1050 m a.s.l

Altitude and consequently air temperature, also affects the thylakoid fatty acid composition (Table 1). In highest altitude locals, the unsaturation index is highest and inversely in the lowest ones that is the lowest. This adjustment is very important since hotness induces more fluidity in the membrane fatty acids and by this way, they must be more saturated in order to be more stable and consequently forming stable thylakoid membranes (Murata & Siegenthaler, 1998). In the Portuguese varieties, Judia, Longal and Aveleira, the most heat tolerant variety Aveleira has the lowest unsaturated fatty acid index (158.5) and viceversa Judia the least heat tolerant has the highest fatty acid index (175.1) (Gomes-Laranjo et al., 2006).
Additionally, deacrease in thiobarbituric reactive species (MDA) and ADP-Fe peroxidation in the leaves suggest the lower in peroxidation susceptibility the higher altitude (Table1).

4. Abiotic stress signalling

4.1 The role of abscisic acid (ABA)

Here we briefly introduce some of the recent research on the regulation of ABA levels in the aforementioned Mediterranean plants. ABA signal transduction will not be discussed in detail since the studies related to these plants are still scarce or almost inexistent, which undoubtedly open new frontiers for future investigations.
ABA, a phytohormone that plays a fundamental role in abiotic stress adaptation, is a small sequiterpenoid (C_{15}) that also plays important roles in plant growth and development, as well as in response and tolerance to dehydration. ABA is central in regulating the plant response to a variety of abiotic stressful conditions *e.g.*, drought, salt and osmotic stress (Marion-Poll & Leung, 2006). Environmental parameters are known to affect ABA and water status, which in turn affect physiological processes in plants (Kitsaki & Drossopoulos, 2005). ABA is related to both, long- and short-term responses of the plant to several environmental

stimuli. Environment parameters, such as dry soil conditions, temperature stress, relative atmospheric humidity, flooding, photoperiod, light intensity, salinity and wounding, as well as internal factors serving as developmental cues, affect ABA concentration in leaves and other plant organs. ABA regulates transpiration and water loss via stomatal closure (Rock et al., 2010). It is also noteworthy that nitric oxide (NO) functions as a second messenger in the ABA signaling pathway in guard cells (Acharya & Assmann, 2009).

Cramer et al., (2007) have demonstrated that a large number of transcripts involved in ABA metabolism or responsive to ABA are increased with water deficit or salinity over 16 days, suggesting that ABA plays a critical role in grape abiotic stress responses. Water deficit induced increases in ABA concentrations in the xylem sap and leaves of grapevine and changes in stomatal conductance are well correlated with ABA concentrations of the xylem sap (Pou et al., 2008; Soar et al., 2004). ABA also influences hydraulic conductance, aquaporin gene expression and embolism repair in grapevines (Cramer, 2010; Lovisolo et al., 2010). It is hypothesised that in the isohydric grapevine cultivar Grenache, the drought-induced root ABA biosynthesis increases apoplastic concentration because of a concomitance of events: an increase in suberisation of apoplastic barriers causes a reduction in water conductivity which is not compensated by aquaporin-mediated water transport (Lovisolo et al., 2010). Kaldenhoff et al., (2008) suggest an ABA-aquaporin interaction in the repair of grapevine embolism and in the aquaporin activation during water stress. The root and shoot ABA-mediated responses to water stress conditions, or, more generally, to abiotic stresses, are relevant to vine yield and productivity (Lovisolo et al., 2010). As described earlier by Keller, (2005), water stress influences ABA accumulation at the root, shoot and leaf level, and also affects berry quality. However, a connection between ABA and berry quality (sugar composition during fruit development) has not yet been clarified. Grapevine is among the first plant species in which a direct role of ABA in stomatal closure is demonstrated (Lovisolo et al., 2010). In effect, in different grapevine genotypes, during the gradual imposition of soil water stress (non-irrigation) or partial root drying, negative correlations are often observed between stomatal conductance, and either xylem or leaf tissue ABA contents (Lovisolo et al., 2010). These authors have pointed that ABA synthesis in grapewine shoots and leaves increases in response to soil water stress, which implies that some other root-based biochemical signal may trigger this response. Also, further work is required to clearly understand the role of hydraulics on stomatal regulation in grapevine (Lovisolo et al., 2010), in spite of that it is leaf ABA and not whole-plant hydraulic conductivity that determines stomatal conductance. Nevertheless, the primary role of a root-to-shoot hydraulic signal is generally followed by an increased ABA biosynthesis in the shoot that regulates stomata and leaf growth (Chaves et al., 2010).

Vvrd22, a dehydration-responsive gene has been recently isolated and cloned from grapevine of the Cabernet Sauvignon variety (Hanana et al., 2008). It is constitutively expressed at a low level in all analyzed tissues, not only responsible for drought stress but also responsible for salt stress. ABA induces Vvrd22 expression, even at a low level.

Gene expression of the ABA and ethylene pathways is particularly increased by stress compared with other hormone pathways and is negatively correlated with stem water potentials (Cramer, 2010). Using transcript and metabolite profiling, Deluc et al., (2009) have shown that water deficit has significant impacts on the metabolism of grape berries. Water deficit affects the metabolism of ABA in the grapewine cultivars Cabernet Sauvignon and Chardonnay in different ways: it increases ABA concentrations in Cabernet Sauvignon

berries, but doesn't in Chardonnay berries. Effectively, Cramer (2010) has recently emphasized that many of the grapevine responses to osmotic stress appear to be transcriptionally regulated, but proteomic studies indicate that there are post-translational controls as well. Also, metabolite profiling has revealed that accumulation of amino acids and polyamines (PAs) is dependent on ABA production, suggesting the integration of ABA signaling to accumulation of protective molecules (Toumi et al., 2010). The ABA signaling pathway integrates PAs and amine oxidases (AOs) in order to regulate the generation of hydrogen peroxide (H_2O_2), which signals further stress responses of the programmed cell death (PCD) syndrome. ABA enhances PA accumulation in grapes and, at the same time, induces the PA oxidation pathway, thus originating secondary protective effects *e.g.,* the stomata closure. Furthermore, PA's catabolism caused by enhanced expression of AOs, which is induced by ABA, generated H_2O_2 which correlated with the levels of peroxidases and phenolics during vascular differentiation (Paschalidis et al., 2009).

The olive tree tends to acclimatize to prolonged hot-dry periods by reducing the level of ABA at the end of summer, in spite of the low water potential (Kitsaki & Drossopoulos, 2005). During winter periods, leaf ABA content remains low, while water potential values are at their highest level. Concerning ABA content, young olive leaves to be more sensitive to most environmental parameters than old ones. Significant differences in water-stress-induced ABA accumulation have been observed between two *O. europea* cultivars, thus reflecting the degree of stress experienced (Guerfel et al., 2009). The drought tolerant cultivar 'Chemlali' accumulates lower levels of ABA in their leaves to regulate the stomatal control in response to water stress compared to the drought sensitive cultivar 'Chetoui', which accumulates ABA in large amounts. ABA in nutrient medium originates different olive carbohydrate spectra regarding abiotic stress type (salinity or low temperature) (Rejšková et al., 2007).

4.2 The role of nitric oxide (NO)

Reactive nitrogen species (RNS) are a family of reactive molecules derived from nitric oxide (•NO; hereafter called NO). The major RNS in the plant cell is NO (Besson-Bard et al., 2008). NO has the hability to cross cell membranes and can thereby transmit signals to other cells. A biologically important reaction of NO is *S*-nitrosylation, converting thiol groups (including cysteine residues in proteins) in *S*-nitrosothiols (RSNO). NO has an important function in numerous cell signalling processes, regulating cell growth, the hypersensitive response, the closure of stomata, plant response to stressors such as drought, high or low temperature, salinity, heavy metals and oxidative stress (Besson-Bard et al., 2008), and also has defense functions (Neill et al., 2008).

Concerning the Mediterranean species, the studies about NO are very scarce. In olive plants, salinity produces a 40% reduction in leaf fresh weight, induces oxidative stress and a dramatical increase in proteins that undergo tyrosine nitration (Valderrama et al., 2007). The specific NOS activity in olive leaves is dependent on L-arginine, NADPH and calcium (Valderrama et al., 2007). Salt stress induces an increase in the L-arginine-dependent production of NO, total RSNO and several proteins that undergo tyrosine nitration, thus functioning as good markers of nitrosative stress. Additionally, the vascular tissues could play an important function in the redistribution of NO-derived forms during nitrosative stress and in signalling-related processes. NO are produced in the olive reproductive organs in a stage- and tissue- specific manner (Zafra et al., 2010).

4.3 The role of MicroRNAs

4.3.1 MicroRNAs are ubiquitous gene regulators at post-transcriptional levels

MicroRNAs (miRNAs) are a newly identified class of 21-24 nucleotide (nt) (predominantly 21 nt) in length, endogenous non-protein-coding short RNAs (sRNAs) in animals, plants and viruses. They are derived from 70- to 500-nt long single stranded primary transcripts (pri-miRNAs), which are transcribed from miRNA genes (*MIR* genes) by RNA polymerase II, by the action of RNase III-like enzymes DICER-LIKE1 (DCL1) or DCL4 (Liu et al., 2009). The mature miRNA is loaded to the RNA induced silencing complex (RISC) to guide the complex to the target mRNAs (containing a stretch of perfect or near perfect complementary sequence). miRNAs have been considered one of the most important regulatory molecules, which regulate gene expression at the post-transcriptional levels via targeting mRNAs for direct cleavage of mRNAs, repressing mRNA translation, or small RNA-directed transcriptional silencing (Jones-Rhoades et al., 2006). Recently, the identified number of conserved and non-conserved species-specific plant miRNAs is rising at an accelerated speed by the newly developed deep sequencing technologies. The latest information on plant miRNAs can be obtained from the miRBase database maintained by Sanger Institute (http://microrna.sanger.ac.uk) (Kozomara & Griffiths-Jones, 2011).

MicroRNAs play crucial roles in essential biological processes, including developmental timing, stem cell differentiation, signaling transduction, human disease, and cancer (Couzin, 2008). In plant, miRNAs play a pivotal role in many aspects, such as organ development, phase change, signal transduction, and response to environmental stress (Shukla et al., 2008; Zhang et al., 2007). Many miRNAs are expressed in a cell- or tissue-specific manner during development of organisms and may contribute to the establishment and/or maintenance of cellular identity (Makeyev & Maniatis, 2008).

Laboratory molecular cloning and computational prediction of miRNA genes based on the conservation of sequence and secondary structure are two methods of plant miRNA study. Historically, most plant miRNA genes have been discovered by one or both of the two methods (Meyers et al., 2008).

MicroRNA study concerning the Mediterranean species is rare and almost all from vine. Recently, the grapevine genome of a highly homozygous genotype (Jaillon et al., 2007) and of a heterozygous variety (Velasco et al., 2007) has been published by two independent groups, respectively. These genome data provide a solid support for the study of sRNA-based regulatory networks in grapevine. By a computational-based BLAST search of sequences using *Arabidopsis* miRNAs' genes as references Jaillon et al. identified 164 miRNA genes with a medium size of 103.5 bp and total of 0.002 Mb in the homozygous grape genome (2007); Velasco et al. (2007) identified 143 miRNA genes representing 28 families in the heterozygous genome. They predicted 28 conserved and non-conserved miRNAs in grapevine. A total of 81 potential miRNAs have been computationally predicted; the length of miRNA precursors in grapevine varies from 68 to 207 nucleotides, with an average of 117 ± 42 (Y.D. Lu et al., 2008).

4.3.2 Progress in the study of grapevine miRNA on abiotic stress

Recently, attention has been paid on the role of miRNA in plant abiotic stress mediation, indicating that miRNAs participate in regulating various abiotic stress response, such as drought (Kantar et al., 2011; Xu et al., 2010), salt (Ding et al., 2009), cold (Zhou et al., 2008), heat (S.F. Lu et al., 2008), (see reviews by Phillips et al., (2007) and Shukla et al., (2008)). In

plants, miRNAs target regulatory proteins such as transcription factors, suggesting that miRNAs are master regulators (Phillips et al., 2007; Shukla et al., 2008). Stress-induced or upregulated miRNAs target negative regulators of stress responses or positive regulators of processes that are inhibited by stresses (e.g., cell division and expansion). Alternatively, stress downregulated miRNAs could repress the expression of positive regulators and/or stress upregulated genes. The existence of stress-related elements in miRNA promoter regions provides further evidence supporting its role in abiotic stress (Liu et al., 2008).
For the experimental analysis of miRNAs and other sRNAs, the first and most important step is the isolation of high-quality total RNA. High-quality RNA extraction from grapevine and other Mediterranean woody plants is problematic due to the presence of polysaccharides, polyphenolics and other compounds that bind or co-precipitate with the RNA. A rapid and effective cetyltrimethylammonium bromide (CTAB)-based method for RNA extraction from different tissues of grapevine and other woody plants including olive and chestnut has been reported (Gambino et al., 2008). Eighteen miRNA were computationally predicted to be responsible for abiotic stress in grape (Wei, 2009). Here we will summarize the identified and validated miRNAs of grapevine on abiotic stress.

4.3.2.1 miR398 and oxidative stress

miR398 down-regulates two closely related Cu/Zn-Superoxide Dismutase genes: cytosolic CSD1 and plastidic CSD2 that can detoxify superoxide radicals (Sunkar et al., 2007; Sunkar et al., 2006). It is expressed in a spatial- and temporal-specific manner under normal growth conditions finely tuning the expression of CSD1 and CSD2 transcripts and in turn regulating the levels of superoxide or other ROS required for signalling. miR398 expression is down-regulated transcriptionally by oxidative stress, and this downregulation is important for posttranscriptional CSD1 and CSD2 mRNA accumulation and oxidative stress tolerance. Computational prediction reveals that the miR398 family is represented by three members (MIR398a, MIR398b, and MIR398c) in grapevine (Y.D. Lu et al., 2008) and these members have been recently validated by deep sequencing analysis (Mica et al., 2010; Pantaleo et al., 2010). Among them, miR398b is at least 100 fold higher expressed in root than either leaf or early inflorescences. Furthermore, transgenic Arabidopsis plants overexpressing a miR398-resistant form of CSD2 accumulate more CSD2 mRNA than plants overexpressing a regular CSD2 and are consequently much more tolerant to high light, heavy metals and other oxidative stressors (Sunkar et al., 2006), which strongly demonstrates the role of miR398 in plant abiotic stress.

4.3.2.2 miRNAs and water deficit stress

A few miRNAs have been identified to regulate plant drought stress. Two drought-induced miRNAs, miR169g and miR-393, have been validated by microarray analysis in rice plants upon drought stress (Zhao et al., 2007). miR169g is induced more prominent in roots than in shoots. Two ABA-independent dehydration-responsive elements (DREs) exist in the upstream of the promoter region of the MIR169g gene, supporting its role in plant water deficit (Zhao et al., 2007). miRNA169a/c are found to be drought downregulated in *Arabidopsis thaliana* (Li et al., 2008). miR393 is a plant miRNA thought to regulate expression of mRNAs encoding the F-box auxin receptor, including transport inhibitor response1 (TIR1), which in turn targets AUX/IAA proteins for proteolysis by SCF E3 ubiquitin ligases in an auxin-dependent manner and is necessary for auxin-induced growth processes. Thus, miR393-mediated inhibition of TIR1 would down-regulate auxin signalling and seedling growth under abiotic stress conditions and further relate to drought stress. In addition,

miR1867, miR474, miR398, miR1450, miR1881, miR894, miR156, and miR1432 are upregulated in *Triticum dicoccoides* under drought stress (Kantar et al., 2011). It is expected that miRNAs that shut down processes involved in normal metabolism and growth are upregulated during drought stress, in order to conserve water and protect the cell. One good example would be miR156, which downregulates transcription factors involved in development and flowering. miR169g and miR169c have been computationally identified (Y.D. Lu et al., 2008), and 25 miR169 members (miR169a-y) have been validated in grapevine (Mica et al., 2010). miR393 has also been predicated to be involved in signalling pathways by regulating transport TIR1 (Y.D. Lu et al., 2008). Experimental work has shown that miR393 is expressed at a higher level in inflorescences than in tendrils in grapevine (Pantaleo et al., 2010). miR156 has been computationally identified (Velasco et al., 2007) and cloned (Pantaleo et al., 2010) in vine, respectively. In another independent work, it is predicted that ath-miR156 and vvi-miR157 share the same target SPL(Y.D. Lu et al., 2008).

4.3.2.3 miRNAs and nutrient deficiency stress

MicroRNA399 regulates phosphate stress responses. Upon Pi starvation, increased miR399 expression represses the expression of ubiquitin-conjugating E2 enzyme (UBC24) and consequently the repression of Pi uptake is alleviated in Arabidopsis (Aung et al., 2006). Where in grapevine miR399 is predicated to target AF2 (Y.D. Lu et al., 2008). miRNA399 is more highly expressed in roots (Mica et al., 2010) and at a very low level in leaves (Pantaleo et al., 2010), which may explain the absence of cleaved targets.

MiR395 is involved in sulphur accumulation and alocation by targeting both ATP sulfurylases and the sulfate transporter AST68 (SULTR2;1). During sulfate limitation, expression of miR395 is significantly up-regulated (Liang et al., 2010). In grapevine miR395 is predicted to target mRNAs coding for ATP sulphurylases (Y.D. Lu et al., 2008). The expression of miR395 family is higher at leaf than at tendrils, inflorescences and berry (Mica et al., 2010); and that is higher at tendrils than in inflorescences (Pantaleo et al., 2010).

In addition, several UV stress responding miRNAs and their target genes have been computationally predicted in grapevine (Wei et al., 2008).

5. Genetic modifications targeting improved plant abiotic stress

Several plant breeding approaches will likely be needed to improve the abiotic stress tolerance and maintain optimum yield levels of the Mediterranean crops in field conditions. The main method of crop improvement continues to be the conventional plant breeding through sexual hybridization, sometimes combined with classical cytogenetic techniques (Roy et al., 2011). Conventional breeding and marker assisted selection are being used to develop cultivars more tolerant to abiotic stress. However, these methods are time and resource consuming and germplasm dependent. On the other hand, improvement of stress tolerance by genetic engineering overcomes the bottlenecks of plant breeding methods.

Recently, efforts have been devoted to identifying potential target genes for use in genetic engineering for crop abiotic stress tolerance (Cushman & Bohnert, 2000). These include specific heat shock proteins, ion transporters, water transporters (aquaporins), as well as signalling components, such as, MAP kinases, Ca^{2+}-dependent protein kinases, transcription factors, like, DREB, CBF and Myb, and enzymes of plant hormone metabolism (Cushman & Bohnert, 2000; Mittler & Blumwald, 2010).

The use of mutation techniques in *Arabidopsis* to obtain knock out and up-regulated mutants, and the elucidation of stress defence mechanisms in yeast and humans, where these mechanism are highly conserved in eukaryotes, has also made a major contribution (Cassells & Doyle, 2003). Publication of the genome draft sequence of two grapevine genotypes (Jaillon et al., 2007; Velasco et al., 2007) offers new perspectives on genomic research in grapevine as well as in other trees species (Gambino et al., 2009).

Plant response to drought stress is quite complex, and is associated with a large number of physiological and biochemical changes. Some of those changes, such as osmotic stress adjustment, ABA accumulation, and root morphology, are known to be controlled by multiple genes (Khan et al., 2009; Lilley et al., 1996). One promising genetic path is the mapping of quantitative trait loci (QTL) that relate performance and yield to abiotic stress factors (Collins et al., 2008). Recently, QTLs for downy mildew resistance have been localized in the genetic linkage maps of two interspecific grape crosses (Moreira et al., 2011).

Besides Agrobacteria-mediated gene transformation, another advantageous transformation method is the particle bombardment, and indeed the only one available for many species (Altpeter et al., 2005). Although there is widespread belief that particle bombardment generates large, multi-copy loci prone to instability and silencing, refinements of the technology to produce clean transgene loci have demonstrated clearly that this is not the case, and that particle bombardment has many advantages for the production of commercial transgenic plants that perform well in the field and comply with all relevant regulatory processes (Altpeter et al., 2005). Protocol for olive somatic embryos genetic transformation by particle bombardment has been recently established (Perez-Barranco et al., 2009).

The induction of stress tolerance through engineering for over-expression of transcriptional factor genes is emerging as an attractive proposition (Yang et al., 2009). Transcription factors are regulatory proteins that modulate gene expression through sequence-specific DNA binding and/or protein– protein interactions. They are capable of acting as switches of the regulatory cascade by activating or repressing transcription of target genes. In plants, the C-repeat (CRT)-binding factor/dehydration-responsive element (DRE) binding protein 1 (CBF/DREB1) transcription factors control an important pathway for increased freezing and drought tolerance. Three CBF/DREB1-like genes, CBF 1-3, have been cloned from both freezing-tolerant wild grape (*V. riparia*) and freezing-sensitive cultivated grape (*V. vinifera*) (Xiao et al., 2008). The transgenic grapevine over-expressing DREB1b has been proved to have a significantly improved resistance to cold stress (Jin et al., 2009).

The grapevine WRKY transcription factor has 66% and 58% identity at the DNA and amino acid sequence levels, respectively, with Arabidopsis AtWRKY11 genes, and has been therefore designated VvWRKY11 (Liu et al., 2011). Transgenic Arabidopsis seedlings over expressing VvWRKY11 show higher tolerance to water stress induced by mannitol than wild-type plants. It is expected that a rapid progress in the development of stress tolerance genotypes in grapevines will be achieved in the near future, because there are large genetic resources for grapevine and there are a large number of high-throughput genomic tools available to conduct functional genomic analyses (Cramer, 2010).

cDNAs from *O. europaea* related to the aquaporin (AQP) gene family have been isolated and characterized (Secchi et al., 2007). The transcript levels of each AQP gene diminished strongly in plants submitted to drought. The down-regulation of AQP genes may result in reduced membrane water permeability and may limit loss of cellular water during periods of water stress (Secchi et al., 2007).

High temperature tolerance has been genetically engineered in plants mainly by over-expressing the heat shock protein (HSP) genes or indirectly by altering levels of heat shock transcription factor proteins (Singh & Grover, 2008). HSP70 has been cloned and characterized from olive tree (Drosopoulou et al., 2009). The presence and organization of many typical binding sites for the Heat Shock and GAGA factors in the sequence suggest that the promoter of this gene is highly heat-inducible and could be used for conditional expression in transformation systems (Drosopoulou et al., 2009).
Modern plant breeding involves novel technical approaches, and gene transfer is undoubtedly a powerful tool. However, genetic engineering does not always result in efficient transgene expression. Several cases have been reported, where transgene copy number does not correlate with the level of transgene expression (Gelvin, 2003).

6. Conclusions and future prospects

Olive tree and grapevine have evolved fine adaptation mechanism to drought, heat and high irradiation at morphological, anatomical, physiological and biochemical levels. Low altitude (consequently high air temperature) restricts the distribution of chestnut in Europe. An abiotic stress may initiate multiple signaling pathways in Mediterranean plants. Because ABA is involved in abiotic stress signaling, revealing how ABA is perceived certainly will help reveal how stress signals are sensed. The study of the newly identified signal molecules NO and miRNA on the Mediterranean crops is just emerging. The related data concerning chestnut is still absent. The elucidation of the interaction and crosstalk among NO, ROS and ABA, and their relation with miRNAs in regulating plant abiotic stress will give a novel panorama of the abiotic stress signaling networks. Introducing the most important genes involved in tolerance to the various abiotic stresses into sensitive Mediterranean species will allow the coordinated expression of these genes to improve abiotic stress tolerance.

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9

Molecular and Genetic Analysis of Abiotic Stress Resistance of Forage Crops

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1. Introduction

Abiotic stress, brought about by salinity, drought, extreme temperatures and oxidative stress are serious threats to agriculture and result in a huge reduction of production. Drought and salinity are becoming major threats throughout the world. Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and development (Wang et al., 2001). In order to survive from these harsh stresses, forage plants have developed precise and complicated tolerance mechanisms at the morphological, physiological and molecular levels.

Under serious threat, forage plants can change the shape of the leaves and roots to decrease the water loss. Some plants have evolved special structures, such as salt glands to excrete salt. At the physiological level, respiratory, photosynthesis metabolism and osmotic adjustments etc. all change in order to resist stress (Chaves, 1991; Sheng, 2010; Yang et al., 2007). In fact, all of these changes are related to gene expression. The complex plant response to abiotic stress involves many genes and molecular mechanisms. In the past several decades, multiple genes responding to drought, salt, low-temperature and oxidative stress have been identified. These genes are divided into two groups (Shinozaki et al., 2003). The first group functions to directly protect the plant against stress, involving key enzymes for osmolyte biosynthesis, LEA (late embryogenesis abundant) proteins, detoxification enzymes and enzymes involved in many metabolic processes. The other group consists of contained protein factors involved in the regulation of signal transduction, including various transcription factors, proteins kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules (Yamaguchi-Shinozaki & Shinozaki, 2006). Genes have been used extensively to improve the stress-tolerance in crop and forage crops. The investigation of stress-tolerance genes will increase our knowledge of tolerance mechanisms, which could in turn be used to promote improvements in forage crop plants tolerance.

2. The morphological response to abiotic stress

Plants often produce a visible response to certain types of environmental stress. Under drought stress, the leaf morphology changes in order to retain water and increase the water use efficiency in forage crops plants. In general, plants decrease the leaf area to limit water loss (Turner, 1979). Nobel investigated the relationship between leaf structure and water use

efficiency under water deficit, and found that if other conditions are invariant. The mesophyll cells become smaller per unit area under water deficit, conversely the larger the area of mesophyll cells, the higher is the water use efficiency (Noble, 1980). In an earlier study, tall fescue and turfgrasses were seen to rely primarily on a deep and extensive root system for drought tolerance because the longer root system had greater volume and surface area in contact with the soil, facilitating water and nutrient uptake under drought stress (Qian et al., 1997).

Facing salinity stresses, plants evolved special structures to survive. *Mesembryanthemum crystallinum* is a salt-secreting plant and possesses epidermal bladder cells in its aerial parts, which store Na^+ (Adams et al., 1998). While *Tamarix aphylla* uses a salt gland to excrete salt (Thomason et al., 1969).

Annual plants can escape drought by maturing before stress becomes severe. Some Fescue grass cultivars avoid drought stress through changes in leaf and root morphology (reducing the transpiration surface area and closing stomata) and probably through osmotic adjustment maintain sufficient turgor pressure in the growing zone for leaf elongation (Wang & Burghrara, 2008).

3. The physiological response to abiotic stress

Serious environmental threats stimulates forage and turf grassess to produce a physiological response. Photosynthesis and cell growth are the primary processes which are affected by stress (Chaves, 1991; Munns, et al., 2006). Under such stress, the rate of photosynthesis and assimilation of products of forage crops decreases remarkably, resulting in a slow down of growth. In a study of eight grass forage plants, chlorophyll content was found to decrease with increased water stress (Yang et al., 2007). Low-temperature has been found to decrease the chlorophyll content in *Poa pratensis* L.qinghai, *Roegneria thoroldiana* and *Elymus nutans*. The major reason for this is that the stress decreaseds the stability of the chloroplast and then destroyed them (Yan et al., 2007). Generally, the degree of decreasing chlorophyll content correlates with the degree of damage to the forage crop plants, so that the chlorophyll level can be used as a guide to stress tolerance in these plants.

Under a variety stresses, the respiration rate of forage plants becomes unstable. For example, the respiration rate dramatically decrease after suffering from freezing, heat, high-salinity or flooding. While after drought or chilling, the respiration rate increase initially, then sharply decreases (Sheng, 2010). The respiratory metabolism pathway also alters under stress. The Pentose Phosphate (PPP) pathway increases under drought and mechanical damage conditions in forage crop plants (Sheng, 2010).

3.1 Osmotic adjustment in response to abiotic stress

Different types of stress often produce interrelated effects and induce similar cellular damage. A general phenomenon is cell dehydration. Under the water deficit condition, plants sustain normal physiological processes through osmotic adjustment (OA). Osmotic adjustment is a major trait associated with maintenance of high cell turgor potential and water retention in response to dehydration stress (Hare et al., 1998; Ingram & Bartels, 1996). Osmotic adjustment can result in turgor maintenance, thereby sustaining cell elongation and leaf expansion as water deficits develop. Osmotic adjustment has been correlated with drought and salt tolerance in various forage and turfgrass species, including tall fescue (White et al., 1992), bermudagrass, buffalograss [Bouteloua dactyloides (Nutt.) Columbus] (Qian & Fry, 1997), *Cenchrus ciliaris* (Wilson & Ludlow, 1983), *Andropogon gayanus* var.

bisquamulatus (Geerts *et al*., 1998). Osmotic adjustment measurements can be used to select drought-tolerant cultivars (Morgan,1983). The extent of osmotic adjustment was higher in buffalograss and zoysiagrass (*Zoysia japonica* Steud) with a better drought tolerance than tall fescure (*Festuca arundinacea* Shreb.) (Qian & Fry, 1997).
Osmotically active solutes include amino acids (proline), sugars (e.g.,sucrose, fructans), polyols (e.g., mannitol), and organic ions(e.g.,potassium, sodium) (Chaves et al., 2003). Those solutes are associated not only with turgor maintenance, but also with the maintenance of membrane and protein structures and protection against oxidative damage (Crowe et al., 1992; Hoekstra et al., 2001). OA for creeping bentgrass and velvet bentgrass is associated with the accumulation of water soluble carbohydrates during the early period of drought and increases in proline content following prolonged a period; however, inorganic ions were not found to relate OA in these species (DaCosta & Huang, 2006). In alfalfa (*Medicago sativa* L.), salt stress induces a large increase in the amino acid and carbohydrate pools. Amongst the amino acids, proline shows the largest increase in roots, cytosol, and bbacteroides. Its accumulation is reflected in an osmoregulatory mechanism not only in roots but also in nodule tissue. The concentration of the carbohydrate pinitol is also increased significantly (Fougere et al., 1991). In many other forage and turfgrasses, glycinebetaine and proline makes a significant contribution to OA under abiotic stress (Girousse *et al*., 1996; Marcum, 1994).

3.2 Dormancy is another countermeasure to survive from stresses

Dormancy also is a mechanism by which forage crop plants become quiescent during prolonged environmental stress, especially drought. Grasses temporarily slow the growth of meristem to avoid drought damage and to allow survival (McWilliam, 1968). Poaceae forage plants, such as *Poa scabrella* (Laude, 1953), *Poa bullbosa* (Volaire *et al*., 2001), and some populations of forage grasses such as *Dactylis glomerata* 'Kasbah' (Norton *et al*., 2006), all exhibit summer dormancy.

4. The molecular and genetic response to stress

The plant responses to abiotic stress involves many genes and molecular mechanisms and stress-associated genes, proteins and metabolites from a complex regulatory network.
A large number of genes have been found to be associated with abiotic stress (Jin et al., 2010; Kang et al., 2010; Shinozaki and Yamaguchi-Shinozaki 2000; Thomashow 1999). Genes induced during stress conditions function not only in protecting cells from stress by producing important metabolic proteins, but also in regulating signal transduction in the stress response (Yamaguchi-Shinozaki & Shinozaki, 2006). These gene products are divided into two groups (Shinozaki et al., 2003; Yamaguchi-Shinozaki & Shinozaki, 2006). The first group functions in the direct protection of the plant against stress and includes key enzymes for osmolyte biosynthesis, LEA (late embryogenesis abundant) proteins, detoxification enzymes and enzymes involved in many metabolic processes. The second group contains protein factors involved in further regulation of signal transduction, including various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules (Yamaguchi-Shinozaki & Shinozaki , 2006).

4.1 Transcriptional factors

Transcription factor genes play important roles in stress survival by serving as master regulators of sets of downstream stress-responsive genes. Transcription factors regulate

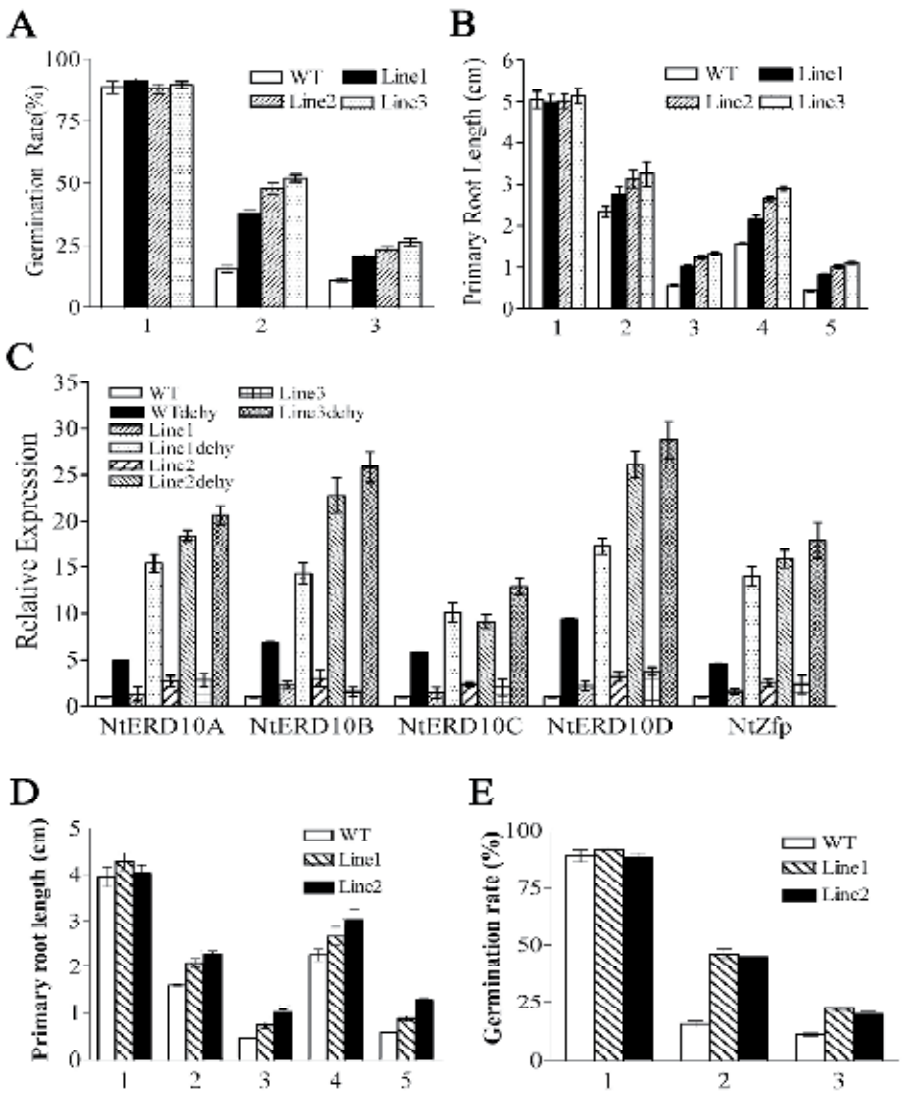
downstream gene expression via binding to specific elements (cis-elements) in target genes and consequently, enhance stress tolerance in plants (Chen & Zhu, 2004; Yamaguchi-Shinozaki & Shinozaki, 2006).

Transcriptional control of the expression of stress-responsive genes is a crucial part of the plant response to a range of abiotic stresses (Singh et al., 2002). Several hundred types of transcription factors have been isolated from higher plants. Important families of stress-responsive transcription factors include AP2/EFR, basic-domain leucine-zipper (bZIP), MYC/B, WRKY, Zinc finger, MADS, NAC (Liu et al., 1999; Yamaguchi-Shinozaki & Shinozaki ,2006).

In the Arabidopsis genome, 145 DREB/ERF related proteins are classified into five groups—the AP-2 subfamily, RAV subfamily, DREB subfamily, ERF subfamily, and others (Sakuma et al., 2002). Many AP2/EREBP transcription factor genes have been isolated from a variety forage crop plants (Chen et al., 2009; Niu et al., 2010; Wang et al., 2010; Wang et al., 2011; Xiong & Fei, 2006). In *Medicago falcate*, MfDREB1 and MfDREB1s encode an AP2/EREBP type transcription factor and are multi-copy genes. Also they are induced by low temperature stress, although hardly induced at all under salt and drought conditions (Niu et al., 2010). *LpCBF3*, encoding the transcription factor DREB1/CBF, is isolated form perennial ryegrass. *LpCBF3* is induced by cold stress, but not by abscisic acid (ABA), drought and salinity. Over-expression of *LpCBF3* in Arabidopsis was found to induce CBF3 target genes and enhance freezing tolerance by measuring electrolyte leakage (Xiong & Fei, 2006). *LpCBF3* can induce downstream gene expression in cold-tolerant perennial ryegrass accessions without cold treatment, but cannot be activated in cold-sensitive perennial ryegrass accessions. Also cold treatment can induce the downstream genes of CBF3 expression in these accessions. Over-expression of *LpCBF3* with a 35S promoter resulted in dwarf-like plants, later flowering and greater freezing tolerance (Zhao & Bughrara, 2008). *HsDREB1A* is isolated from xeric, wild barley in bahiagrass. *HsDREB1A* introduced into bahiagrass under the *HVA1s* promoter from barley, enhanced tolerance under severe salt stress and severe dehydration stress (James et al., 2008). The WXP1 gene from *Medicago truncatula* containing a AP2 domain was induced by cold, ABA and drought treatment mainly in shoot tissues. Over-expression of WXP1 in alfalfa not only induced a number of wax-related genes, but also significantly increased wax accumulation.Transgenic lines were found to enhance drought tolerance and quick recovery after re-watering (Zhang et al., 2005).

In our previous studies, we isolated and identified the AP2/EREBP genes from several forage crops, including *Caragana korshinskii*, *Galegae orientalis* (Chen et al., 2009; Wang et al., 2010; Wang et al., 2011) and *Ceratoides arborescens* (unpublished data).

CkDBF, a DREB like gene isolated from *C. korshinskii*, was confirmed as a transcription factor by one-hybrid experiments and located to the nucleus. CkDBF is induced by high salt, dehydration, low temperature and abscisic acid (ABA). Over-expression of *CkDBF* in transgenic tobacco induces the expression of downstream stress-responsive genes and increases tolerance under high salinity and osmotic stress (Wang et al., 2010) (figure 1). CkDREB, which contains a conserved AP2/ERF domain, is also isolated from *C. korshinskii*. It was located in the nucleus, and had a DRE element-binding activity and transcriptional activation ability. The expression of *CkDREB* is induced by a variety abiotic stress types including high salt, dehydration and low temperature. The over-expression of CkDREB in tobacco was found to enhance the tolerance for high salinity and mannitol stress by measuring the germination rate of seeds and primary root lengths (figure 1). The over-expression of *CkDREB* induces abiotic stress-response genes containing a DRE element in their promoters. These results show that CkDREB is involved in the regulation of stress-response signals (Wang et al., 2011).



a Germination rates of WT (control) and transgenic lines (CkDBF) grown on MS medium(1), or MS medium supplemented with 200mM NaCl (2) or 250mM mannitol (3) (n=100, each experiment was repeated three times). b Primary root growth of WT and transgenic lines tobacco seedlings (CkDBF) under normal condition (1), treated with150mM mannitol (2), 250mMmannitol (3), 100mM NaCl (4), or 200mMNaCl (5)(n=20). c Expression analysis of downstream genes NtERD10A, NtERD10B, NtERD10C, NtERD10D and NtZfp in transgenic tobacco (CkDBF) by using real-time PCR. Genes were amplified with specific primers. The ACTIN gene was used to normalize samples. Experiments were repeated three times. d Primary root growth of transgenic lines (CkDREB) and WT tobacco seedlings under normal condition(1), treated with 100 mM NaCl (2), 200 mM NaCl(3), 150 mM mannitol (4) or 250 mM mannitol (5) (n = 20). e Germination rate of WT(control) and transgenic lines seeded on MS media (1), or MS media supplemented with200 mM NaCl (2) or 250mM mannitol (3). Each experiment was repeated three times.

Fig. 1. Enhanced stress tolerance of transgenic tobacco carrying DREB transcription factor genes from *Caragana korshinskii*.

Previous studies with DREB transcriptional factor genes focused on DREB-1 and 2 types, which generally play important regulation roles. However, as an A-6 type DREB gene, *CkDBF* responds to a variety abiotic stress types , and the finding that over-expression could enhance the multiple stress-tolerance in transgenic plants indicates that the A-6 type factor also plays an essential role in transcriptional regulation, especially in forage crops.

In addition to DREB-type genes, other AP2/EREBP genes have also been investigated in forage crop plants. GoRAV, which was isolated from *Galegae orientalis*, belongs to the RAV family and has two AP2 and B3-like distinct DNA-binding domains. GoRAV is induced by cold, drought, high salinity and ABA (Chen et al., 2009).

Much researchs has been carried out with the aim of improving the stress-tolerance of forage crops through transgenic modification. Thus, introducing the Arabidopsis DREB1A/CBF3 gene into *Lolium perenne* can increase drpught and freezing stress tolerance, the increased stress tolerance being associated with increased activities of antioxidant enzymes (Li et al., 2011). In another experiment, GmDREB1 isolated from soybean, was introduced into alfalfa under the control of Arabidopsis Rd29A promoter and the transgenic lines induced by salt and showed high tolerance (Jin et al., 2010). Also the Arabidopsis *HARDY* gene, belonging to the stress-related AP2/ERF super family of transcription factors, was transformed into *Trifolium alexandrinum* L. By measuring fresh and dry weight, transpiration and sodium uptake in the transgenic lines and wild type, it was found that over-expression of *HARDY* improves drought and salt tolerance in transgenic plants (Abogadallah et al., 2011).

The zinc-finger motifs, which are classified based on the arrangement of Zinc-binding amino acids, are present in many transcription factors and play critical roles in interactions with other molecules (Chao et al., 2009; Sun et al., 2010). A number of zinc-finger transcription factors have been implicated in important biological processes and stress-tolerance regulation.

MsZFN, encoding a zinc-finger protein, was isolated from alfalfa. *MsZFN* is located in the nucleus, and is significantly induced by salt and reach a maximum level at 30 min (Chao et al. 2009). The Alfin1 gene from alfalfa which encodes a putative Zinc finger motif is specifically expressed in roots (Bastola et al., 1998; Winicov, 1993). The Alfin1 protein binds DNA in a sequence-specific manner in vitro, and can also bind to fragments of MsPRP2, which is considered to be root-specific and accumulates in alfalfa roots under a salt environment (Bastola et al., 1998). Over-expression of Alfin1 under the 35S promoter enhanced expression of the endogenous MsPRP2 gene in alfalfa and improved salinity tolerance (Winicov & Bastola, 1999). Alfin1 has been proposed as a root growth regulator, and transgene lines have increased in root growth under normal and saline conditions in alfalfa (Winicov, 2000). MtSAP1 (*Medicago truncatula* stress-associated protein1) encodes a zinc-finger domains, and It's expression is increased embryos during desiccation, and decreased significantly during the first hours of imbibing. MtSAP1 protein accumulated in the embryo axis under cold, hypoxia, ABA and desiccation related stress, but its expression is not notably changed under mild drought stress. RNAi studies showed that MtSAP1 storage proteins are very important for the success of germination (Gimeno-Gilles et al., 2011).

Other transcription factors also had been investigated. *AtHDG11* encodes a protein classified as a homeodomain-leucine zipper transcription factor number. Over-expression *AtHDG11* in tall fescue, with four 35S enhancers significantly enhance tolerance to drought and salt stress. The enhanced stress tolerance is associated with a more extensive root system, a lower level of malondialdehyde, a higher level of proline and superoxide dismutase (SOD) and catalase (CAT) (Cao et al., 2009).

In *Medicago truncatula*, the HD-Zip 1 transcription factor HB1 is expressed in primary and lateral root meristerms and is induced by salt stress and constitutive expression of HB1 in *M. truncatula* roots alters their architecture (Ariel et al., 2010).
These studies taken together demonstrate that transcription factors play an important role in the acquisition of stress tolerance in forage crops.

4.2 Osmoregulatory genes

In stress-tolerant plants, many genes are involved in the synthesis of osmoprotectants. Osmoregulation is believed to be the best strategy for abiotic stress tolerance, especially if an osmoregulatory gene can be triggered in response to drought, salinity or high temperature (Bhatnagar-Mathur et al., 2008). Osmotically active solutes include amino acids (e.g., proline, glycine betaine), sugars (e.g., sucrose, fructans), polyols (e.g.,mannitol), and organic ions(e.g., potassium, sodium) (Chaves et al.,2003). These active solutes accumulate in a large number of plant species under environmental stress conditions (Ashraf & Foolad, 2007; Chen & Murate, 2011; Mansour, 1998).
Proline plays a vital role in plants, especially in abiotic stress conditions (Ashraf & Foolad, 2007; Aubert et al., 1999; Schat et al., 1997). Many genes are involved in the synthesis and degradation of proline under a variety of stress conditions such as salt, drought and metal toxicity etc. A role for P5CS (Δ^1-pyrroline-5-carboxylate synthase) in the proline biosynthetic pathway under stress conditions, has been emphasized in the last two decades (Kishor et al., 1995; Zhu et al., 1998). In alfalfa, the expression levels of MtP5CS1 in the different plant organs closely correlated with proline levels but transcript abundance was not affected under osmotic stress condition; was seen to significantly accumulate although only in shoots under osmotic stress conditions (Armengaud et al., 2004). BADH (betaine aldehyde dehydrogenase) is a key enzyme in the biosynthesis of glycine betaine and a BADH gene, which originated from *Atriplex hortensis,* was transformed into alfalfa and was found to increase the salt tolerance of the transgenic plants (Liu et al., 2011).
Sugars also play an essential role in osmotic adjustment. Trehalose-6-phosphate synthase (TPS1) and trehalose-6-phosphate phosphatase (TPS2) from yeast, driven by the rd29A promoter, were transformed into alfalfa. The transgenic lines led to increased plant biomass and tolerance under drought, freezing, salt and heat stress conditions (Suárez & Iturriaga, 2009). In alfalfa leaves, sucrose phosphate synthase (SPS) and sucrose synthase (SS) were examined to explore sucrose metabolism under cold (5°C) and heat stress conditions and it was found that(33°C) genes expression was significantly changed in the cold but not the heat condition (Mo et al., 2011).
Putrescine aminopropyltransferase (PAPT) enzyme, highly specific for putrescine as the initial substrate, can yield multiple polyamine products which are associated with osmotic stress. PAPT activity from alfalfa was found to be mainly located in the meristematic shoot tip and floral bud tisseues. A scheme was proposed to comprehensively illustrate the role of PAPT in biosynthesis of several common and unusual polyamines in alfalfa (Bagga et al., 1997).

4.3 Detoxifying genes

In most of the aerobic organisms, elimination of ROS (reactive oxygen species) is needed under environment stress conditions. In order to control the level of ROS and protect the cells from oxidative injury, plants have developed a complex antioxidant defense system that includes various enzymes and non-enzymatic metabolites (Vranova et al., 2002). Key

enzymes for detoxifying ROS in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (Yang et al., 2006).

Superoxide dismutases (SODs) are important antioxidant enzymes that occur in virtually all oxygen-respiring organisms (Halliwell & Gutteridge, 1999; Scandalios, 1997). Until now, four types of SODs have been identified. Copper-zinc SOD is the most importance one, which is closely related to resistance to stress in plants (Feng et al., 2005; Song et al., 2006; Wang et al., 2005). PS-CuZn SOD from *Polygonum sibiricum* , which encodes a copper-zinc SOD, is a constitutively expressed gene and has different expression modes in different organs under salinity-alkalinity stress conditions (Qu et al., 2010). Our team isolated a copper-zinc SOD from *Galega orientails*. Expression of this gene, induced by drought and salt stress, indicated that the copper-zinc gene is involved in stress signaling (unpublished). Copper-zinc SOD can be divided into two forms, one is cytosolic and the other is associated with chloroplast isoenzymes (Sheri et al., 1996). Subcellular analysis showed that Cu-Zn SOD of *G.orientails* locates in chloroplast (unpublished).

Transgenic plants with over-expression of the SOD gene in alfalfa were found to be able to resist, and to possess markedly enhanced antioxidant capacities (Bryan et al., 1993; Bryan et al., 2000). The Mn-SOD gene from *Nicotiana plumbaginifolia* is introduced into alfalfa using two different plasmid vectors for targeting to mitochondria or chloroplast. The transgenic lines had enhanced SOD activity and increased re-growth after freezing stress (McKersie et al., 1993). Another study showed that the introduction of Mn-SOD improved survival, vigor and yield over three years in a natural field environment (McKersie et al., 1996). Further observations with many different transgenic plants in both laboratory and field evaluations show may that over-expression of Mn-SOD improve the winter survival and dry-matter yield, although some lines showed the converse. Although many of the transgenic plants had higher winter survival rates and herbage yield, there was no apparent difference in primary freezing tolerance of the cells in the taproot or crown of transgenic alfalfa (McKersie et al., 1999). In another study, a Fe-SOD gene from Arabidopsis, with a chloroplast transit peptide, was over-expressed in alfalfa under the cauliflower mosaic virus 35S promoter. The transgenic alfalfa show a higher superoxide-scavenging capacity and winter survival. The Fe-SOD activity is found to have a close relationship with winter survival, but not with the oxidative stress tolerance and shoot dry matter production in a 2 year trial. The higher winter survival may stem from reducing secondary injury symptoms and enhancing recovery after stress (McKersie, et al. 2000). In a further investigation of transgenic MnSOD in mitochondria of leaves and nodules, MnSOD in the Chloroplasts and FeSOD in the Chloroplasts; it was shown that transgenic lines had a 20% higher photosynthetic activity than the parental line under mild water stress conditions, however there were no major differences between the untransformed and the transformed alfalfa for most parameters examined under a water stress environment (Rubio et al., 2002). To explore two SOD transgenes influencing the SOD stress-tolerance mechanisms, the F1 progeny was generated through a sexual cross of a hemizygous Mit-MnSOD alfalfa and a hemizygous Chl-MnSOD alfalfa. The results showed that the F1 progeny with the two genes inserted had increased total SOD activity and significantly higher storage organ biomass compared with the non-transgene siblings, but had a lower biomass production compared to siblings having only one transgene (Samis et al., 2002).

Catalase is a unique hydrogen peroxide-scavenging enzyme. A catalase gene *Facat1* is isolated from *Festuca arundinacea* Schreb. Facat1 is up-regulated in cold and salt stress treated leaves, and reached an expression peak at 2 and 4 h, respectively. However, under ABA and drought treatment conditions, the expression was down-regulated (Yang et al., 2006).

Targeting the detoxification pathway is an appropriate approach for producing plants with multiple stress-tolerance traits (Bartels et al., 2001). It is expected that with increased understanding of this pathway, a breeding forage crop will be produced with multiple stress-tolerance.

4.4 Signal transduction

Plant cells sense stress through signaling pathways and transmit the signal to cellular machinery activating an adaptive response essential for plant survival. Molecular and biochemical studies suggest that abiotic stress signaling in plants involves receptor-coupled phosphorylation, phosphoinositol-induced Ca^{2+} changes, mitogen-activated protein kinase cascades and transcriptional activation of stress-responsive genes. In addition, protein post-translational modifications and adapter or scaffold-mediated protein-protein interactions are also important in abiotic stress signal transduction (Xiong & Zhu, 2001).

In alfalfa, $P44^{MMK4}$ kinase was specifically activated under drought and cold treatment conditions, but not induced by high salt concentrations or heat shock. Under ABA treatment, MMK4 transcription levels and $p44^{MMK4}$ kinase were not increased or activated (Jonak et al., 1996). In another study SIMK, an alfalfa mitogen-activated protein kinase (MAPK), was found to be highly regulated by salt stress, in terms of its levels and subcellular localization in roots (Baln ka et al., 2000). SIMK is a member of the family of MAPKs(mitogen-activated protein kinases) which are involved in transducing a variety of extracellular signals. It is transiently activated by NaCl, KCl, sorbitol, and in alfalfa reaches maximal activity between 8 and 16 min before a slow inactivation. Unlike other MAPKs in most mammalian and yeast cells, SIMK has a constituive nuclear localization and the activation is not correlated with nucleo-cytoplasmic translocation (Munnik et al., 1999).

The Msapk1 gene, harbouring a unique ankyrin repeat, can be detected in almost every organ of alfalfa. It is found to be induced in the roots of alfalfa upon osmotic stress (Chinchilla et al., 2003). Also MsCPK3, a calmodulin-like domain protein kinase (CPK), was identified in alfalfa. The expression of MsCPK3 is activated by 2,4-Dichlorophenoxyacetic acid (2,4-D), ABA and NaCl but not by kinetin, ABA or salt treatment. Measurement of the recombinant protein activity showed that MsCPK3 involved in auxin and stress related Ca^{2+} signalling pathways (Davletova et al., 2001).

The AnnMs2 gene, an annexin-like protein from alfalfa, is expressed in various tissues especially in buds, flowers and roots. It is activated in cells or tissues under osmotic stress or by ABA. The recombinant AnnMs2 protein is able to bind to phospholipids in the presence of Ca^{2+} and immunofluorescence studies showed that it is mainly localized in the nuclear (Kovács et al., 1998).

Another signaling element active oxygen species (AOS), can act as ubiquitous signal molecule in plants. It is a central component in the stress response and it's level determines the type of response (Vranova et al. , 2002).

The gene *Srl*k, from *M. trnucatula*, a leucine-rich repeat RLK (receptor-like protein kinases) is rapidly induced by salt stress in roots. The gene expression study and a *Srl*k promoter-β-glucuronidase (GUS) fusion location experiment suggested that Srlk is activated in the root epidermis. Through studies using RNAi and Srlk-TILLING mutants, Srlk would appear to be involved in the regulation of the adaptation of *M.truncatula* roots to salt stress (De Lorenzo et al., 2009).

Heme oxygenase is the rate-limiting enzyme in the breakdown of heme changing into carbon monoxide (CO), iron etc (Shekhawat & Verma, 2010) and it plays a vital role in stress

responses. The expression and protein levels of MsHO1 are higher in alfalfa stems and leaves than in germinating seeds and roots and are induced significantly by some pro-oxidant compounds including hemin and nitric oxide donor sodium nitroprusside (Fu et al., 2011).

4.5 Late embryogenesis abundant proteins

LEA proteins, which are suggested to act as desiccation protectants during seed desiccation and in water-stressed seedlings, can be induced by ABA and various types of water-related stress (Espelund et al., 1992). MtPM5, identified as an atypical hydrophobic LEA protein, during stress, is able to stabilize proteins, but is unable to protect cell membranes. MtPM25 is able to rapidly dissolve aggregates in a non-specific manner and sorption isotherms show that when it is unstructured, it absorbs up to threefold more water than MtEM6 (Boucher et al., 2010). In proteomic analysis of the germination of *M. truncatula* seeds associated to desiccation tolerance (DT), 11 polypeptides were identified as late embryogenesis abundant proteins. The abundance changes of MtEm6 and MtPM25 show the two proteins were related to DT (Boudet et al., 2006).

4.6 Transporter genes

Ion transporters selectively transport ions and maintain them at physiologically relevant concentrations. Sodium transporters in plant cells have been extensively studied. Sodium is compartmentalized into the vacuole, through the operation of the vacuolar Na^+/H^+ antiporter, down an electrochemical proton gradient generated by the vacuolar H^+-ATPase and H^+-Ppase (Blumwald et al., 2000). In both prokaryotic and eukaryotic cells, the Na^+/H^+ exchanger plays a key role in the regulation of cytosolic pH, cell volume and Na^+ homeostasis (Padan et al., 2001; Wiebe et al., 2001). Plant Na^+/H^+ antiporters have been isolated from Arabidopsis (Shi et al., 2000), rice (Fukuda et al., 1999) and forage plants (Li et al., 2009; Tang et al., 2010; Yang et al., 2005). MsNHX1, encoding a vacuolar Na^+/H^+ antiporter, was isolated from alfalfa. The expression of MsNHX1 was significantly up-regulated after treated by NaCl and ABA (Yang et al., 2005). MsNHX1 was located to the vacuolar membrane and can partly complement the NaCl-sensitive phenotypes of a yeast mutant. The expression of MsNHX1 in Arabidopsis enhances the resistance to salt stress (An et al., 2008). To investigate the mechanisms of *Medicago intertexta* and *Melilotus indicus* in salt stress, the expression of four genes coding for NHX-type Na^+/H^+ antiporters were measured. The result show that three genes are expressed in *M. intertexta* leaves and roots, and one gene in *M. indicus* roots. *NHX* gene expression may trigger *M. intertexta* to cope with tissue Na^+ accumulation, while in *M. indicus*, the low Na^+ content and the lack of correlation between growth in the presence of NaCl, Na^+ content and *NHX* gene expression indicates that different mechanisms are involved in coping with salt stress (Zahran et al., 2007). TrNHX1, isolated from *Trifolium repens* L., can complement the *Δnhx1* and *Δena1-4Δnhx1* yeast mutants by suppressing their observed phenotypes. A similar result is observed in the presence of LiCl and KCl. Under 150mM NaCl treatment, the expression level of TrNHX1 in roots, shoots and leaves is 1.7, 2.2 and 4.3 times, respectively, that of the controls. The expression levels and Na^+ content in organs also have a close relationship (Tang et al., 2010). SsNHX1, isolated from the halophyte *Salsola soda*, could significantly enhance salt- tolerance in transgenic alfalfa under the stress-inducible *rd29A* promoter even at 400 mM NaCl (Li et al., 2011). Also *GoNHX1* was induced by salt, drought and ABA treatment in *Galega orientalis* (Li et al., 2009).

When the AVP1 gene, a vacuolar H^+-pyrophosphatase gene from *A. thaliana*, was transformed into alfalfa, over-expression enhanced salt and drought tolerance. Compared to the wild-type, the transgenic plants accumulated more Na^+, K^+ and Ca^{2+} in leaves and roots, retained more solutes and water, maintained a higher root activity, had a protected photosynthetic machinery, and maintained a stable cell membrane under abiotic stress conditions (Bao, et al., 2009). Zhao's team reported that co-expressing *Suaeda salsa* SsNHX1 and AVP1 conferred greater salt tolerance to transgenic plants than did SsNHX1 alone (Zhao et al., 2006). These studies provide a promising way for improving salt and drought tolerance in forage crop plants.

4.7 Cold-acclimation specific gene

Many plants increase freezing tolerance upon exposure to low non-freezing temperatures, a phenomenon known as cold acclimation. Cold acclimation includes the expression of certain cold-induced genes that function to stabilize membranes against freeze-induced injury (Thomashow, 1999). In forage grasses, many genes (Mohapatra et al., 1989; Tamura & Yonemaru, 2010; Tominaga et al., 2001; Zhang et al., 2009) and proteins (Kosmala et al., 2009) are regulated during cold acclimation.

In alfalfa, some CAS (cold-acclimation-specific) genes are specifically expressed during cold-acclimation and their expression level measured by mRNA abundance is positively correlated with the freezing-tolerance of cultivars (Mohapatra et al., 1989). From alfalfa, MsaCIA, cas15 (cold acclimation-specific gene), cas17, and MsaCIC, induced by low temperature have been isolated (Castonguay et al., 1994; Laberge et al., 1993; Monroy et al., 1993; Wolfraim & Dhindsa 1993), although they were not induced by ABA or other stresses (Mohapatra et al., 1989). Encoding a putative nuclear protein, the cas15 transcript level is increased significantly with cold acclimation but is hardly detectable in the absence of cold acclimation. Thus, the accumulation of cas15 and the prolonged cold acclimation have a close relationship (Monroy et al., 1993). In red clover, the homolog of the alfalfa MsaCIA is induced by cold, but the homolog-like MsaCIB, MsaCIC genes were not induced by cold. The expression level of MsaCIA transcripts was 3 times higher in the cold-acclimated, regenerative, F49R (cold tolerant) genotype, compared to the cold-acclimated, non-regenerative, F49M (cold sensitive) genotype. It was also shown that enhanced expression of MsaCIA and the regenerative trait are either linked (Nelke et al., 1999). MsaCIC is similar to bimodular proteins that are developmentally regulated in other plant species (Castonguay et al., 1994). Calcium, a second messenger, can also play an important role in cold acclimation in alfalfa. The influx of extracellular $^{45}Ca^{2+}$ at 4 ^{o}C is 15 times higher than at 25 ^{o}C. The addition of a calcium ionophore or a calcium channel agonist caused an influx of extracellular $^{45}Ca^{2+}$ and induced the expression of *cas* genes (as reporters in low-temperature signal transduction) at 25^{o}C, while the addition of calcium channel blockers inhibited the influx of extracellular $^{45}Ca^{2+}$ as well as the expression of cas genes (Monroy & Dhindsa, 1995). Associated with winter survival, the cold acclimation-responsive gene, such as RootCAR1, may act as a molecular marker for identifying winter hardy plants in semi-dormant or non-dormant alfalfa germplasm in winter of the seed year (Cunningham et al., 2001).

Forage crop plants are the foundation of animal husbandry. In general, forage crops are located in harsh environments, especially in China. This implies that forage crop plants contain essential stress-tolerance gene resources. Over previous decades, the study of stress tolerance mechanisms mainly focused on physiology and morphology, the molecular and

genetic mechanism being less understood. The discovery and use of stress-tolerance-associated genes to confer forage plant stress tolerance, is clearly a promising approach. New studies aimed at revealing the signaling transduction, transcriptional regulation and gene responses in forage plants, will contribute to this end.

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10

Salt Stress in Vascular Plants and Its Interaction with Boron Toxicity

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1. Introduction

Among abiotic stresses, high salinity is the most severe environmental stress, impairing crop production on at least 20% of irrigated land worldwide. In addition, the increased salinity of arable land is expected to have devastating global effects, resulting in up to 50% land loss by the middle of the 21st century. Furthermore, there is a deterioration of about 2 million ha (1% of world agricultural lands) because of salinity each year (Mahajan & Tuteja, 2005).
Critically, the problem of salinization is increasing due to the accumulation of tons of salts into the soil as a consequence of bad agricultural practices (e.g., use of fertilizers on a massive scale), draining of aquifers, and a limited regional rainfall. Irrigated land is particularly at risk with approximately one-third being significantly affected by salinity. Despite its relatively small area, irrigated land is estimated to produce one-third of world's food (Munns, 2002), so salinization of this resource is particularly critical.
Water scarcity in arid and semi-arid regions has forced the increased use of recycled waste-water and desalination of salty groundwater resources for agriculture use. Current technologies to desalinate or purify recycled waste-water for agricultural use can effectively reduce the concentrations of most toxic elements with the significant exception of boron (B). Boron in recycled water is often concentrated substantially as a result of the recycling process and as such can significantly impact agricultural soils. Therefore irrigation with saline groundwater containing high B concentration occurs in parts of the world where there is a notable scarcity of water.
The relations between salinity and mineral nutrition of horticultural crops are extremely complex and a complete understanding of the intricate interactions involved would require the input from multidisciplinary team of scientists. Thus, although information about their independent effects is abundant, information about the combined effects of salinity and B is very limited.

2. Resistance to salinity - range of tolerance

Plants, due to their sessile nature, have developed several mechanisms to tolerate the various stresses to which that may be encountered during their life cycles. Most plants are

very sensitive to soil salinity and are known as *glycophytes,* whereas salt-tolerant plants are known as *halophytes*. In general, *glycophytes* cannot grow at 100 mM NaCl, whereas *halophytes* can grow at salinities over 250 mM NaCl.
These adaptations include the synthesis of compatible osmolytes, proper maintenance of ionic balance, the synthesis of detoxifying enzymes of reactive oxygen species (ROS), and other responses. Playing an important role in the proper induction of these responses are: at first, the Ca^{2+} signaling; then, the signaling mediated by abscisic acid (ABA) and, finally, the MAP (Mitogen-Activated Protein) kinases signaling cascade (Tuteja, 2007).

3. Problems caused by salinity

Salinity alters the smooth operation of the plant due to factors ranging from the cellular level to physiological level (Hasegawa et al., 2000; Mahajan & Tuteja, 2005; Munns, 2002), which are outlined as follows:

- Salinity affects the physiology and metabolism of the plant, as it causes both hyperosmotic and hyperionic stresses.
- Due to the high salt concentration, soil water potential (Ψ) becomes more negative, which hinders water uptake by the plant. These salt levels also affect the absorption of nutrients such as K^+, Ca^{2+}, or NO_3^- owing to competition of these ions with Na^+ and Cl^-.
- A physiological damage is observed in leaf transpiration and also a growth inhibition, which increases the toxic effects of the salt within the plant. These alterations might respond, according to recent findings in *Arabidopsis*, to a failure in cortical microtubule organization and helical growth of this plant. A salt buildup also occurs in the old leaves, and the death of them could be a key strategy for plant survival. It has also been observed a decline in photosynthetic activity.
- At cellular level, the presence of Na^+ alters the K^+/Na^+ cytosolic ratio, even though the Na^+ remained extracellular. The alteration in the levels of K^+, due to its importance for plant growth, causes a disruption of osmotic balance, the malfunctioning of the stomata and the inhibition of some enzyme activities.
- Also at cellular level, the entry of Na^+ alters the cell membrane potential, thereby allowing the entry of Cl^- ions. Thus, the Na^+ in concentrations around 100 mM is toxic to cell metabolism and may inhibit some essential enzymes, expansion and cell division, and membrane organization. Associated with this stress, many ROS are produced, with the oxidative damage that this entails (Grattan & Grieve, 1999).

There is therefore a link between the immediate cellular alterations to salt stress and physiological changes taking place in the plant, and which focalize the problems in ionic imbalance and an insurmountable decrease in water potential values for the plant.

4. The response to stress and cross-tolerance

When stress affects any plant a series of responses are triggered at both cellular and systemic level (Tuteja, 2007; Zhu, 2002). According to these authors, synthetically the stress response goes as follows:

- Different receptors (ion channels, receptors serine/threonine kinase or histidine kinase, or G-protein-coupled receptors) located in the plasma membrane perceived stress at that localization.

- These receptors trigger different signal transduction cascades, among which are found as secondary signals Ca^{2+}, inositol phosphate (IP), ABA and ROS.
- These signaling cascades are at the core of the induction of the expression of certain genes that are directly or indirectly involved in protection against this stress. According to the response time we distinguish between early and late response genes. The first, induced in minutes, are transcription factors that determine the expression of late genes, which themselves are involved in stress tolerance and detoxifying enzymes. They comprise ion channels, enzymes, metabolites synthesizing protective chaperone, among others.
- Some of the induced genes are involved in the synthesis of diffusible signals (e.g., ABA, ethylene, salicylic acid) acting in a second wave of signaling, which now affects to the whole organism and determines a global adaptation.

When we analyze the response of plants to stresses of different nature, there is a redundancy in the mechanisms deployed. Thus, plants show cross-tolerance, which means that a plant resistant to a particular condition can develop tolerance to other forms of stress. Although the mechanisms by which develops cross-tolerance occurs remain unknown, it is suspected that cross-tolerance between salinity, drought and cold stress are due to the common consequences (osmotic and oxidative stresses) (Mahajan & Tuteja, 2005; Tester & Davenport, 2003).

5. The case of salt stress

Salt stress is a complex problem, in which experiments show induction of 194 genes in *Arabidopsis*. It requires a perfect orchestration between genetic, epigenetic modifications, pre- and post-transcriptional regulation and also post-translational control. Below we expose the main signaling pathways and safeguard mechanisms induced by salinity (Tuteja, 2007).

5.1 Signaling of salt stress

Signaling is an area of greatest potential for plant research, either in relation to how to detect nutrients, abiotic factors or other organisms (symbionts, harmless or pathogenic). It also is of major interest to optimize the production in agricultural systems. In the case of salt stress we know that the Ca^{2+}-mediated signaling plays a crucial role, followed by ABA-mediated signaling, and with the participation of other routes, such as the MAP kinases (Mahajan & Tuteja, 2005).

5.1.1 Ca^{2+} signaling

Calcium levels in the cytoplasm are maintained below 1 μM by a delicate balance held by carriers that are present in the endoplasmic reticulum (ER), chloroplast and in the vacuole. This homeostasis is a prerequisite for the action of this cation as a second messenger.

In the presence of high salt concentration, at first place there is an increase in cytosolic Ca^{2+} level coming from the apoplast. Then, the entry of Ca^{2+} derived from cellular organelles takes place, which is determined by the action of inositol triphosphate (IP3) formed by the enzyme phospholipase C. This will generate several waves of Ca^{2+} to form a signaling pathway (so called "Ca signature"), which is decoded by several calcium-binding proteins. In *Arabidopsis*, three genes involved in this decoding activity have been described, known as

SOS1, *SOS2* and *SOS3* (Salt Sensitive Overlay), although only *SOS3* encodes a calcineurin B-like protein (CBL), that is, a calcium sensor. The pathway follows with the interaction of SOS3 with SOS2, a serine/threonine kinase that catalyzes the phosphorylation of SOS1 (an antiporter Na^+/H^+) and this determines its activation state. This antiporter is involved in maintaining ionic balance, as we will detail later. In addition of SOS1, other channels and transporters as HKT, NHX and CAX1 are regulated by the SOS2-SOS3 pathway. This pathway is the most important in salinity tolerance, as it seeks to restore the ion balance (Mahajan et al., 2008; Zhu, 2002).
Calcium levels also induce genes responsible for enzymes of ABA synthesis pathway, such as zeaxanthin oxidase, 9-*cis*-epoxycaotenoid dioxygenase, ABA-aldehyde oxidase and molybdenum cofactor sulfurase (Tuteja, 2007). The induction of these genes leads to increased levels of ABA, which acts as a second messenger, as explained later.
Maintenance and restoration of calcium homeostasis is determined by the channel CAX1, an H^+/Ca^{2+} antiporter present in the membrane of the vacuole. The main role of this network is the restoration of basal levels of Ca^{2+} in the cytoplasm, but is also important in order to maintain the osmotic balance during stress.

5.1.2 ABA signaling

The genes responsible for ABA synthesis are induced by salt stress. Once accumulated, this hormone mediates the induction of genes involved in both its own synthesis and its degradation. But when it comes from salt stress responses, genes such as *RD29A* or *RD22* (Responsive to Dehydration), *COR15A* or *COR47* (COld Responsive), the pea DNA helicase 45 (*PDH45*), or pyrroline-5 carboxylate synthetase (*P5CS*, involved in the synthesis of proline), among others, are regulated by ABA (Tuteja, 2007). However, there are activation pathways of those genes independently of ABA.
At the molecular level, there have been described several transcription factors regulated by ABA as well the *cis*-regulatory elements that they recognize. Genes involved in tolerance to salinity are under the control of these promoters and their transcription factors. There are several examples, such as the AREB transcription factor (a leucine zipper transcription factor) that recognizes the ABRE region; or as the DREB2A and DREB2B transcription factors that recognize the region known as DRE/CRT. Other regulatory elements are MYCRS and MYBRS regions recognized by RD22 and MYC/MYB factors that help in the activation of the response, and could be the bridge between different stresses, and an explanation for cross-tolerance (Mahajan & Tuteja, 2005).

5.1.3 Signalling by MAP kinases

The pathway of MAP kinases is well known as a signal transduction system, both intracellular and extracellular. Increases have been detected in the expression of different MAPK, MAPKK and MAPKKK in *Arabidopsis*, alfalfa, tobacco and others, suggesting that this signaling pathway is working on an appropriate response to salt stress (Zhang et al., 2006).

6. Response to salt stress

As already stated, the major damages of salinity resulting from the alterations of Na^+ occur at the ion balance and osmotic levels, which then lead to problems of toxicity and enzyme

inhibition, among others. The main mechanisms involved in resistance to high salt concentrations, namely, restoration of ionic balance, synthesis of compatible osmolytes (also called osmoprotectants, i.e., compatible solutes) such as proline or glycine betaine, expression of detoxifying enzymes of ROS, and helicases are shown in Figure 1.

6.1 Ionic balance

Excess of Na^+ ions produce an imbalance in the ionic equilibrium, which is maintained through the joint action of pumps, together with other ions, and mediated largely by Ca^{2+} signaling. Thus, during salt stress, the expression of numerous channels is modified by the calcium-dependent partner SOS2-SOS3, as already discussed.

There are channels of many different types of channels, which perform different functions for the maintenance of ionic and osmotic homeostasis. On one hand, we have those with greater selectivity for the ion K^+ than for Na^+, such as the KIRC channel (K^+ Inward-Rectifying Channel) that mediates the entry of K^+ after hyperpolarization of the membrane and accumulates this ion over ion Na^+; another example of this type of channel is the HKT channel (Histidine Kinase Transporter), a low affinity transporter for Na^+, which prevents the entry of Na^+ into the cytosol. On the other hand, the entry of Na^+ in plant cells is determined by the NSCC channel (NonSpecific Cation Channel). Another type of channel is provided by the KORC channel (K^+ Outward-Rectifying Channel), which is activated after depolarization of the membrane, mediates the efflux of K^+ and Na^+ entry, which thus is accumulated in the cytosol. To place this movement requires a number of carriers that generate the H^+ gradient needed for channel maintenance. In that sense, transporters as NHX (Na^+/H^+ exchanger) that allows the accumulation of Na^+ in vacuoles are necessary, alleviating the effects of stress; or as channel CAX1 (H^+/Ca^{2+} antiporter) responsible for the maintenance of Ca^{2+} homeostasis (Tuteja, 2007).

6.2 Proline and glycine betaine

Both are osmoprotectants synthesized by many plants in response to various stresses, including salt, and whose main function is to relieve the effects of this stress (Chen & Murata, 2008; Delauney & Verma, 1993). They are not the only ones; there are other osmolytes as polyols and alcohol sugars, whose functions are centered in the maintenance of the osmotic balance, the cell pressure and the protein folding.

Glycine betaine (GB) is synthesized naturally from the choline by the action of the choline monooxygenase and betaine aldehyde dehydrogenase enzymes. In plants where GB is not produced, the overexpression of GB synthesizing genes in transgenic plants resulted in the production of enough amount of GB. With the inclusion of these genes in various plants and other organisms, it has been shown greater tolerance to salinity. Likewise, direct foliar application of the compound also improves the plant response to a saline environment (Tuteja, 2007).

The synthesis of proline is a frequent response in salt stress. This osmolyte is accumulated in the cytosol and allows proper osmotic adjustment. Furthermore, this amino acid also stabilizes subcellular structures, buffers the redox potential and blocks free radicals. It is synthesized from glutamic acid by the action of enzymes pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). Again, the inclusion of these genes in various plants has improved the tolerance of these transgenic plants to high salt concentrations. The induction of P5CS gene seems be mediated by ABA, as its mRNA

accumulates quickly in response to the treatment with this plant hormone (Chinnusamy et al., 2005; Vinocur & Altman, 2005).

6.3 ROS detoxifying enzymes

Salt stress, like many other stresses, involves the development of ROS such as singlet oxygen (O_2^1), the superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$). These species are capable of producing lipid peroxidation, as well as DNA, RNA, and protein oxidative damages that compromise cell and plant viability. For detoxification of these ROS, plants have a battery of enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, and GSH reductase, all induced under salt stress. Furthermore, enzymes such as aldehyde dehydrogenase are also induced, allowing more tolerance to salt stress by eliminating aldehydes produced in reactions between ROS and lipids or proteins (Vinocur & Altman, 2005).

6.4 Helicases

Various stresses, including salinity, induce the expression of genes involved in gene expression machinery, such as several helicases with DEAD box. The answer is given in the presence of Na^+ ions and mediated by ABA. Furthermore, phosphorylation sites in these proteins have been described, which may be a contact point for Ca^{2+} signaling. These helicases have the function of the opening of duplex DNA or RNA, and in this second case we speak of an RNA chaperone function, to avoid unfavorable folds (Owttrim, 2006).

Although the actual mechanism by which helicases increase tolerance to salinity remains unknown, there are two prevailing hypotheses:

- They would stabilize the mRNA transcriptional or translational level. In response to various stresses, secondary structures in the 5' end of mRNA can be performed, and this which could be preventing the proper processing of the RNA.
- They would alter gene expression in association with protein complexes of DNA processing.

6.5 LEA proteins

LEA proteins were first discovered in seed embryogenesis and germination, because they are accumulated in the first phase, and constitute over the 4% of cytoplasmic proteins in some seeds. They constitute a diverse family, but their sequences are enriched in polar amino acid, charged or uncharged, as glicines, glutamic acid or lysines. This hydrophilic character can explain their diverse functions, which include water retention as the protein D-19 from cotton; stabilization of other proteins by hydrophilic and hydrophobic interactions as RAB proteins; formation of a solvation surface around proteins, similar to the sugar solvation surface induced by salinity too; and ion sequestering as HVA1 protein in barley. ABA is inducing LEA protein synthesis in embryogenesis, as has been revealed in some mutants in the ABA signaling, and the same procedure will be controlling the expression of these proteins under salinity (Chourey et al., 2003).

6.6 Other responses

As other stresses, salt stress induces more responses that mentioned above. A brief comment about chaperones and sugar metabolism follows.

Chaperones are involved in the folding of protein, under both normal and abnormal situations. Although it is depending of the kind of chaperones, the ability to isolate the nascent protein or unfolded protein from the high saline cytoplasm can help to achieve the folded state of essential proteins (Choi et al., 2004).

Sugar metabolism is the source of carbon backbones for the whole cell metabolism. Under salt stress a series of cell changes occurs, which include synthesis of osmoprotectants and induction of gene transcription and protein synthesis involved in stress tolerance. Interestingly, genes encoding gliceraldehyde-3-phosphate dehydrogenase, sucrose-phosphate synthase and sucrose synthase are upregulated in response to drought (Ingram & Bartells, 1996), and increased levels of these enzymes are also associated with other environmental stresses in plants. Therefore, given the cross-tolerance between drought and salt stress (Munns, 2002), a similar gene response is very likely that also occurs against salt stress, which would reflect increased energy and carbon backbone requirements.

Finally, careful genetic and biochemical analyses have demonstrated that maturation of *N*-glycans is necessary for plants stress tolerance, and that *N*-glycans are essential not only for protein folding but also for in vivo functions of plant glycoproteins (Kang et al., 2008).

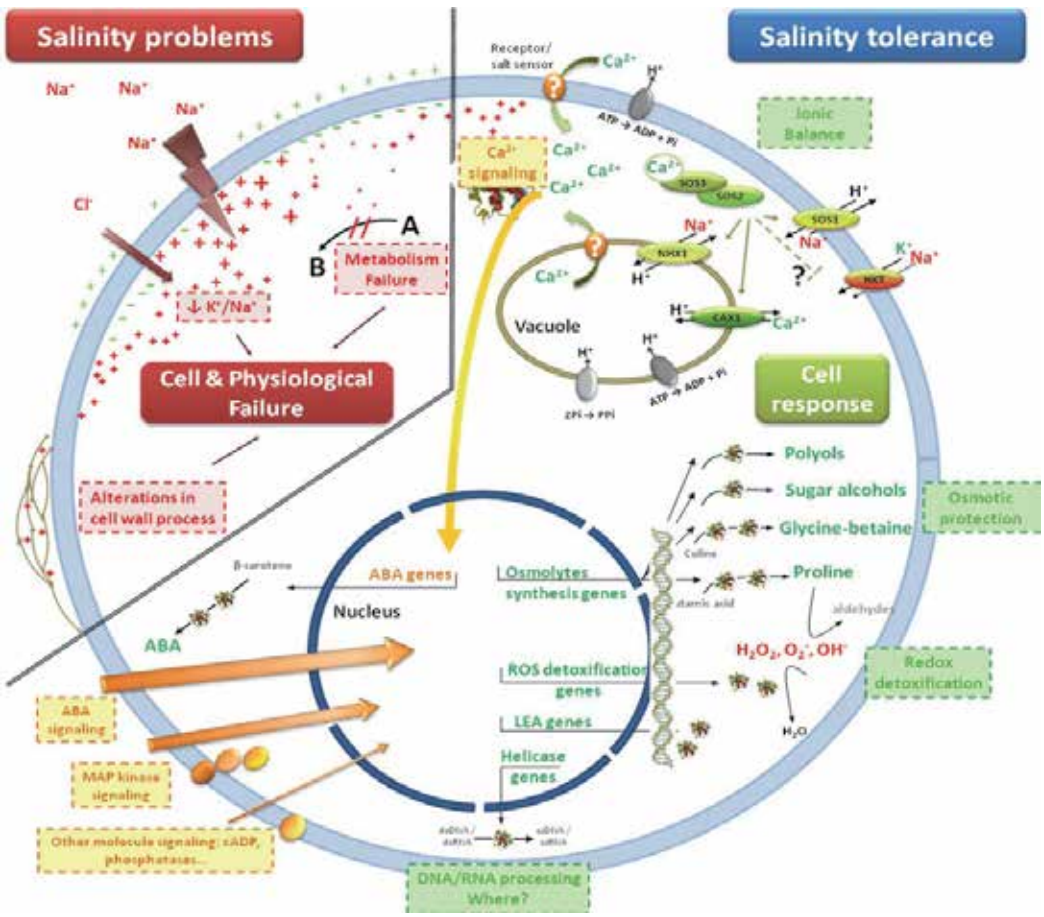

Fig. 1. Regulation of ion homeostasis by SOS pathway and other related pathways in relation to mechanisms of stress tolerance.

7. Boron toxicity

Boron (B) is likely the micronutrient whose concentration inside vascular plants must be kept within the narrowest range for achievement optimal growth and, for this purpose, excess B in soils is more difficult to manage than its deficiency, which can be prevented by fertilization (Herrera-Rodríguez et al., 2010). Although B is found in all cellular compartments (Dannel et al., 2002), its presence is particularly notable in the cell wall where most B is located forming complexes with pectic and galacturonic derivatives with a specific *cis*-diol configuration (Bonilla et al., 2010; Camacho-Cristóbal et al., 2008; O'Neill et al., 1996). Once B is inside the roots, it moves passively with the transpiration stream and it is accumulated in mature leaves, where it can be translocated depending on the presence or not of sugar alcohols capable of forming mobile B-polyol complexes through phloem (Brown et al., 1999). Overall increased B content in mature leaves indicates B immobility, whereas higher B content in young meristematic tissues suggests B mobility. In most plants, B mobility is restricted to the xylem, as they do not biosynthesize significant amounts of polyols (Brown et al., 1999; Brown & Shelp, 1997).

B toxicity is an agricultural problem that reduces crop yield worldwide. Toxicity takes place in vascular plants as B accumulates in shoots, generally following a pattern from leaf base to tip, that leads to chlorosis and necrosis (Marschner, 1995; Reid et al., 2004). As B toxicity alters metabolism and cell division, its symptoms are also manifested as a slowing-down and inhibition of growth, especially in roots, and reductions in yield, along with loss of fruit quality (Grieve et al., 2010; Nable et al., 1997).

Furthermore, high irradiance appears to increase the harmful effects of B toxicity, probably because elevated B contents may impair plant mechanisms to cope with photooxidation stress (Reid et al., 2004).

According to Reid et al. (2004), excess B would overbind compounds with hydroxyl groups in the *cis*-configuration that could explain how this mineral stress exerts its harmful effect. Thus, extra B may bind with pectic polysaccharides and thereby altering cell wall structure; also excess B could alter the structure of primary metabolic compounds through binding to the ribose moieties of ATP (adenosine triphosphate), NADH (nicotinamide adenine dinucleotide, reduced form), NADPH (nicotinamide adenine dinucleotide phosphate, reduced form); finally, high internal B concentrations may negatively affect plant development by binding to ribose of RNA.

Recent reports show changes in gene expressions as a consequence of high internal B concentration (Kasajima & Fujiwara, 2007; Öz et al., 2009; Pang et al., 2010). Although the mechanism by which excess B promotes these changes remains unknown despite many efforts are being made to elucidate it (Kasai et al., 2011), the involvement of a signaling cascade is likely.

8. Boron toxicity tolerance

Boron tolerance appears to be associated with the ability to limit B accumulation in both roots and shoots. Thus, *Bot1* expression, a gene providing tolerance to excess B, was localized in barley roots and youngest leaf blades (Sutton et al., 2007). Other genes encoding B efflux transporters and conferring tolerance to B toxicity in cultivars of wheat (cv. India) and barley (cv. Sahara), namely *TaBOR2* and *HvBOR2*, respectively, have also been reported (Reid, 2007). Therefore, boric acid/borate efflux transporters appear to be key determinants of plant B tolerance, and provide a molecular basis for the generation of highly B-tolerant crops (Takano et al., 2008).

9. Salinity and excess boron

As above mentioned, plant growth and yield are severely affected by salt stress in many regions of the world as a result of osmotic effects, ion toxicities, and mineral disorders (Hasegawa et al., 2000). The response of plants to salinity depends not only on the total ion concentration in the external medium, but also on the chemical nature of the involved ions (Curtin et al., 1993). Nevertheless, most experimental works for salinity studies in plants have been performed with NaCl as a salinizing chemical.

NaCl toxicity is apparent by ion toxicity and osmotic stress. Ion toxicity is due to specific damages by the high levels of Na^+ and Cl^-, reduction of K^+ uptake being a main consequence, among others. Moreover NaCl treatment can increase the electrolyte leakage in tomato roots, indicating that the integrity of their plasma membranes is altered with salt stress (Bastías et al., 2010).

Boron-rich soils with high contents of naturally occurring salinity, and irrigation with groundwater containing high concentrations of salts and B are two common ways by which plants can be subjected to a double stress: salinity and excess B (Nable et al., 1997). For instance, soils with salt and B accumulation occur in South Australia (Marcar et al., 1999) and Jordan River Valley in Israel and Jordan (Yermiyahu et al., 2008); irrigation water with high levels of salts and B has been well documented in San Joaquin Valley in California (Grieve al., 2010), and the Lluta Valley in Chile (Bastías et al., 2004b), among other places.

At first glance, one might think that between salt stress and B toxicity there would be an additive or synergistic relationship. In other words, either the outcome of the two stresses, when occur simultaneously, is equivalent to the sum of the effects of both stresses when applied separately (additive response), or the outcome of the both combined stresses is greater than the sum of them acting separately (synergistic response). However, interactions between salinity and B toxicity are rather complex and it has been reported that an antagonistic response can also exist when both stresses appear simultaneously (Bastías et al., 2004a; Yermiyahu et al., 2008). As summarized by Yermiyahu et al. (2008), there is no agreement regarding mutual relations between salt stress and B toxicity (Grieve et al., 2010).

Apparently, antagonism between salt stress and B toxicity can be a consequence of lower toxicity of NaCl in the presence of high B, lower toxicity of B in the presence of high NaCl, or both. Several reports support that diminution of Cl^- uptake owing to high B could reduce the salt toxicity, since the increased addition of B to the soil did not affect leaf Na^+ content in pepper plants (Yermiyahu et al., 2008), or even decreased it in wheat (Holloway & Alston, 1992). Therefore, B would positively affect to salt stress through decreased Cl^- accumulation in the leaves as a consequence of the reduced Cl^- uptake. Nevertheless, this amelioration is through an as-yet-unknown mechanism.

In turn, reduced leaf B contents with the increase of NaCl concentration in the irrigation water have been widely reported. As an explanation it has been proposed that reduced rates of transpiration would limit the leaf accumulation of B that is transported through the xylem (Yermiyahu et al., 2008).

Another alternative proposal is that the interaction between salinity and B toxicity could be related to aquaporin functionality (Bastías et al., 2004a; Martínez-Ballesta et al., 2008a, 2008b). Under excess external B, significant B transport takes place through the plasma membrane aquaporins (Dordas et al., 2000). The lower aquaporin functionality found in NaCl-treated plants could be related to the reduction of B contents in plants subjected to combined B and NaCl, in comparison with plants treated only with B, which could explain the beneficial effect of salinity against B toxicity (Bastías et al., 2004a). In turn, under salt

stress, the activity of specific membrane components can be influenced by B regulating the functions of certain aquaporin isoforms as possible components of the salinity tolerance mechanism (Martínez-Ballesta et al., 2008b).
In addition, it has been proposed that salt-tolerant plants may be more resistant to toxic B levels because their salt-exclusion mechanisms also contribute to reduce internal B concentrations (Alpaslan & Gunes, 2001).

10. Calcium and boron ameliorate salt tolerance

It is well known that salt stress leads to a Ca^{2+} and K^{+} deficiency and to other nutrient disorders (Cramer et al., 1987; Marschner, 1995). The external supply of Ca^{2+} to the soil ameliorates the damage caused by salinity (La Haye & Epstein, 1971), likely because the integrity of the membrane and its selective capacity is maintained by an adequate supply of Ca^{2+} (Cramer & Läuchli, 1996).
Interestingly, a balanced addition of B and Ca^{2+} together increased tolerance to salt stress in nodulated nitrogen-fixing pea plants, as extra Ca^{2+} can recover nodulation inhibited by salinity and extra B also contributes to nodule development and functionality (El-Hamdaoui et al., 2003a, 2003b). Furthermore, a proper B and Ca^{2+} supplement restores iron content in salt-stressed pea nodules (Bolaños et al., 2006), as well as the germination in salt-stressed pea seeds (Figure 2).
In addition, it has been proposed that interactions among NaCl, B and Ca^{2+} appear to be involved in the stability of the cell wall in plants (Cassab, 1998) and nodules (Bolaños et al., 2003).
It is widely known that both Ca^{2+} and B are needed for cell wall structure. Ca^{2+} stabilizes pectic polysaccharides in cell walls by ionic and coordinate bonding in the polygalacturonic

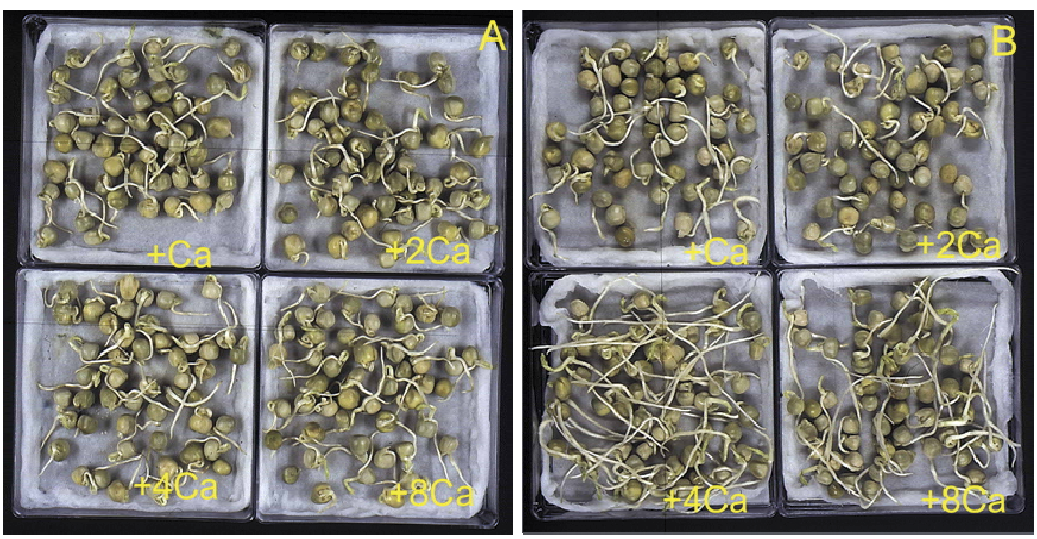

Fig. 2. Effects of supplement with combined boron (**A,** control: 9.3 µM; **B,** +6B: 55.8 µM) and calcium (+Ca: 0.68 mM; +2Ca: 1.36 mM; +4Ca: 2.72 mM; +8Ca: 5.44 mM) on germination of *Pisum sativum* cv. Argona seeds after 6 days under salt stress (75 mM NaCl). Boron was added as H_3BO_3 and Ca as $CaCl_2$. Seeds treated with combined +6B and +4Ca had a germination similar to that of those treated without NaCl (control seeds).

acid region (Kobayashi et al., 1999), and B is also essential to the structure and function of the cell forming borate ester cross-linked rhamnogalacturonan II dimer (O'Neill et al., 1996). Besides this structural feature, it has been highlighted that both nutrients share other characteristics, namely, preferential distribution to apoplast, scarce mobility, very low cytosolic concentration, and signaling functions (Bonilla et al., 2004; Camacho-Cristóbal et al., 2008; González-Fontes et al., 2008). Thus, it would not be surprising that Ca^{2+} and B within the cell could contribute co-ordinately to some physiological role, and that the combined addition of both nutrients can palliate the harmful effects caused by salinity.

11. Conclusion

Tolerance to salinity involves processes in many different parts of the plant and can occur at a wide range of organizational levels, from the molecular level to the whole plant. Consequently improvements in salinity tolerance result from close interactions among molecular biologists, geneticists, biotechnologists, and physiologists and benefit from feedback from plant breeders and agronomists. The mechanism of high salt tolerance is just beginning to be understood. However, much effort is still required to understand in detail each product of genes induced by salinity stress and their interacting partners to elucidate the complexity of the signal transduction pathways involved in high salinity stress.
On the other hand, the mechanism of the relationship between B and salinity is not yet elucidated. Nevertheless, the palliative effect of B under saline conditions may be due to an improvement of the functionality of aquaporins, to prevention of salt-induced nutrient imbalance, interactions between B and Ca with respect to cell wall stability, or to a lower Cl- uptake.

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11

An Efficient Method to Screen for Salt Tolerance Genes in Salt Cress

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1. Introduction

Arabidopsis thaliana is the most widely used model organism in plant molecular biology (Bressan et al., 2001) and it is an ideal model system for many reasons. Arabidopsis can be cultured in solid and liquid media and in soil. Greenhouses and growth chambers are suitable for Arabidopsis growth, meaning that different environmental conditions can be selected. Compared with crop plants, such as rice, wheat and tomato, Arabidopsis is more attractive due to its small size, high fecundity and short life cycle. Arabidopsis can be stably transformed using *Agrobacterium tumefaciens*-mediated transfer of T-DNA. Using the vacuum-infiltration procedure, transformants can be obtained at high efficiency. The small genome size of Arabidopsis meant that it was the first model plant to have its whole genome sequenced. Mutant lines, especially T-DNA insertion lines, of most Arabidopsis genes are obtained easily from several large seed stock libraries around the world. In conclusion, as a plant genetic model, Arabidopsis has played a significant role in characterizing the biological functions of plant genes, including salt stress-related genes.

Nevertheless, Arabidopsis is a typical glycophyte in that does not display tolerance to intense salt stress. Thus, to solve this problem, a halophytic model system needs be developed. Any new model plant must provide experimental expediency similar to that of Arabidopsis. Salt cress (*Thellungiella salsuginea*), which is closely related to Arabidopsis, has emerged as a candidate (Amtmann, 2009). Like Arabidopsis, salt cress meets certain criteria that are important for any model plant, such as small size, short life cycle, self-pollination, high seed number, small genome and efficient transformation. Salt cress can withstand dramatic salinity shocks up to 500 mM NaCl and it can grow in high salt environments that are lethal to Arabidopsis (Bressan et al., 2001). Salt cress does not produce salt glands or other complex morphological alterations either before or after salt adaptation. Expressed-sequence tag (EST) analyses of several hundred salt cress clones have shown that there is approximately 90 to 95% identity between salt cress and Arabidopsis cDNA sequences

(Zhang et al., 2008). As a result, more than 10 years ago, salt cress started to be used for studies examining the mechanisms underlying salt tolerance.

2. Importance

Forward and reverse genetic studies of salt cress were encumbered by a lack of genomic information and poor mutant line storage. So, a new method is required that combines the advantages of Arabidopsis and salt cress, while avoiding any important disadvantages. Here, an overexpression system is presented, which has been used previously in Arabidopsis (LeClere and Bartel, 2001). A similar system has been development by another group independently to mine stress tolerance genes from salt cress (Du et al., 2008). In our lab, a cDNA library of salt cress was generated from salt-treated seedlings and that was driven by double cauliflower mosaic virus (CaMV) 35S promoters. Using *A. tumefaciens*-mediated transformation, the salt cress cDNA library was randomly overexpressed in Arabidopsis. T1 transgenic plants were grown in soil, treated with NaCl and the survival rates of transgenic lines were monitored. The salt cress cDNAs expressed in these lines were identified by PCR amplification and sequencing. To confirm the initial screening results, both salt cress genes and their homologs in Arabidopsis were re-overexpressed in Arabidopsis Salt tolerance ability of the off-spring of these re-transformed lines and mutants of the homologous genes in Arabidopsis was examined. After this screening, candidate genes were chosen for further investigation of their biological functions.

3. Results

3.1 Generation of salt cress cDNA library

Binary Ti vectors are the plasmid vectors used for the *Agrobacterium*-mediated plant transformation because they are able to replicate in both the *E. coli* and *Agrobacterium* species. High level efficiency of *in vivo* recombination is needed for the construction of cDNA libraries, but the large size of the common binary Ti vectors limit the *in vivo* recombination efficiency. Moreover, the size of binary Ti vectors needs to be limited to allow for large pieces of DNA to be transferred into plants. Plasmid manipulations and cDNA library construction are also easier if the vectors replicate in *E. coli* to high copy number. A binary Ti vector with double cauliflower mosaic virus (CaMV) 35S promoter and NOS terminal, named pGreen, fulfilled these demands and was used in the present study (Hellens RP, 2000). To keep the size of pGreen to a minimum, *RepA* and *Mob*, which were necessary for plasmid replication in *Agrobacterium*, were removed. The cloning site of pGreen was based on the well known plasmid, pBluescript, meaning that it was relatively simple to rearrange selective marker and reporter genes.

For wide coverage of salt cress transcripts in relation to salt stress, whole plants were collected at different time points after NaCl treatment. Total mRNA was purified from a mixture of plant tissues and at different time points the cDNA library was constructed in pGreen. Approximately 1 x10^6 colonies were collected from LB agar for the construction of the primary library. The titer of the cDNA library in the host bacteria, *E. coli* XL1-blue MRF ' (Stratagene), was ca. 1 x10^7 colonies/μL. To analyze the insert size and recombination rate in the primary library, 100 colonies were randomly selected and the plasmids were digested by *Eco*RI/*Xho*I. Insert sizes ranged from 500 bp to 2 kb (mean size of ca. 1 kb), while efficiency of recombination was ca. 89%.

3.2 Generation of a library of Arabidopsis transgenic lines overexpressing salt cress genes

Salt cress cDNAs were constructed into pGreen and driven by the double CaMV 35S promoters. A selectable marker gene (*NPTII*) was chosen to identify positively transformed plants. As *RepA* and *Mob* were removed from pGreen, it was unable to replicate in the *Agrobacterium*. To remedy this problem, a co-resident, pSoup, was used to provide the replication function *in trans* for pGreen.

The plasmids isolated from the cDNA library were mixed with pSoup and introduced into *Agrobacterium* strain EHA105. Wild type Arabidopsis plants were transformed and T0 seeds were sown. With efficient kan resistance selection, a total of 2,000 individual transgenic lines were obtained and T1 seeds of these lines were harvested.

3.3 Screening for salt tolerant lines from the transgenic Arabidopsis library

High salt stress has multiple adverse effects on plant growth and development, such as inhibition of seed germination, retardation of plant growth and acceleration of senescence. To evaluate whether a plant is tolerant to salt, the following parameters can be monitored: seed germination, root elongation, fresh weight increase and survival rate.

Seed germination rate has been used for isolating abiotic stress tolerant or abscisic acid insensitive genes in many previous reports. However, seed quality and storage time can influence germination rate, in spite of their genetic background. In addition, fast germination is not necessary or sufficient for salt tolerance, as some lines that germinate faster than the wild type plants under high salinity conditions ultimately were found to be more sensitive to salt stress. Thus, germination rate is not sufficient for identifying salt tolerant plants. Another method for screening for salt tolerance plants is to germinate seeds on media containing salt and then monitor growth or survival rates. This method is better than the seed germination method. However it needs a consistent treatment and high quantities of seeds. Root elongation is a parameter used widely for the identification of salt stress tolerant plants. Seeds are germinated on agar plates without salt. Then, 4-days-old seedlings are transplanted onto new agar plates containing the desired concentrations of salt. Finally, root length is measured at several time points to calculate the growth rate. However, while this method is efficient for identifying salt sensitive mutants, it is not able to identify salt tolerant mutants. If using the rate of fresh weight increase as the salt tolerance parameter, the plants should be grown in liquid medium and be kept alive after being weighed. This method is time consuming and unsuitable for high-throughput screening.

Therefore, a new strategy had to be applied to avoid these disadvantages. To reduce the influence of seed quality on plant growth, plants were grown in soil before salt treatment. Among all the parameters used to evaluate salt tolerance ability, the most important one was selected in this present study, specifically survival rate. If most lines contain a single insertion, 75% of transgenic plants would contain one copy of an expressed salt cress gene. When this gene is related to salt tolerance, 75% of plants should have the phenotype. To save screening time. T1 plants were analyzed because salt cress cDNA would be expressed in approximately 75% of T1 plants.

A high-throughput screen system was set up to isolate transgenic lines with the ability to tolerate high salinity. Using wild type plants as the control, seeds of transgenic lines were germinated on MS plates and then transplanted into soil. Approximately three weeks after

germination, plants were treated with 200 mM NaCl. It was a robust screening, as most wild type plants died. The lines with a survival rate of greater than 70% were identified to be putative salt tolerance lines because about 25% of the T1 plants were wild type. We routinely screened more than 1,000 lines in soil and yielded a total of 20 candidate lines.
For confirming the salt tolerant ability of the candidate lines, the T2 seeds of each line were subjected to a secondary screen. Plasmolysis often occurs when plants are treated with high salinity without pre-conditioning. These plants tend to die of plasmolysis, rather than ionic or osmotic toxicity. This situation was avoided by adding salt in steps of 50 mM to allow the plants to adjust to the increasing salinity. Ten high salt stress tolerance lines of the 20 candidate lines were confirmed at this stage.

3.4 Isolation of the inserted salt cress cDNA expressed in the salt tolerant lines and identification of homologs in Arabidopsis

After screening, the next step was to identify the salt cress genes in these salt tolerant lines. Insert sequences were amplified by PCR and sequenced. Homologous genes in Arabidopsis were identified using BLAST.

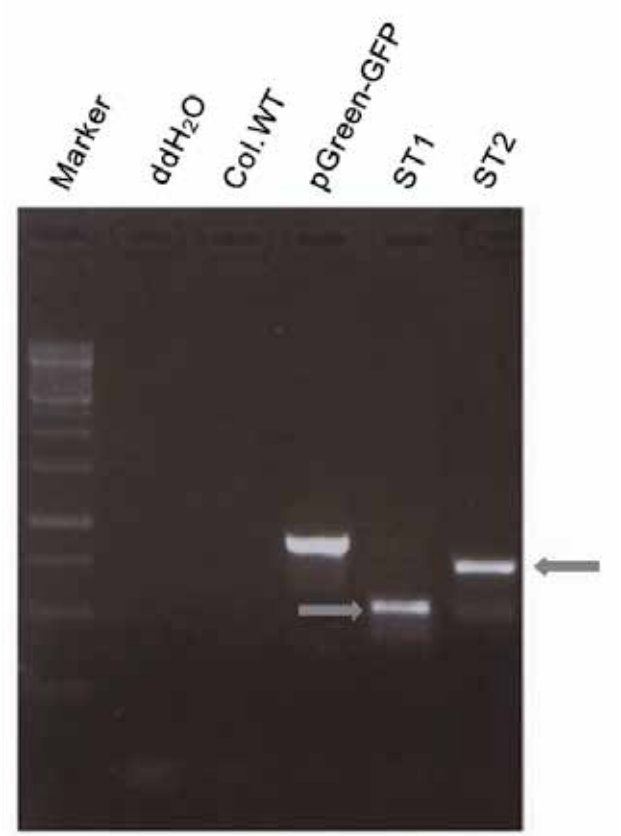

Fig. 1. Isolation of insertion sequences by PCR. ST1 and ST2 are two salt tolerant lines. The plant overexpressing pGreen-GFP was used as a positive control. Arrows indicate the amplified sequences from transgenic lines to be sequenced.

3.5 Validation of the salt-tolerance functions of the isolated candidate genes

The salt tolerant phenotype of the plants must be confirmed by other methods for the following reasons. First, environment factors, such as illumination and irrigation, are not identical for every plant, and can lead to variations among plants, even when the salt treatment is not added. Second, there is often more than one insert identified in a single transgenic line. By using the screening result alone, it is not clear which gene is responsible for the salt tolerance. Third, co-suppression can often occur. Thus, it is possible that the homologous gene in the transgenic plants is expressed at lower levels than in wild type plants, and the salt tolerant phenotype is due to reductions in the expression of these genes.

Two different strategies were adopted to solve these problems. The first approach was re-overexpression. The candidate salt cress genes and their homologs in Arabidopsis were overexpressed in the Arabidopsis wild type plants. Then the salt tolerability of these transgenic lines was checked as described above. If transgenic plants gained salt tolerant ability, the genes were considered to be related to salt stress tolerance. The second approach was to check the phenotype of homologous gene mutant lines of Arabidopsis, and RNAi lines of salt cress. If these genes were indispensable for salt tolerance, elimination or reduction of their expression level should result in increased salt sensitivity of the plants.

4. Conclusion

In this present study, a simple high-throughput method to mine salt tolerance genes from salt cress has been developed by ectopically expressing salt cress cDNA in Arabidopsis. This method does not require any genomic or cDNA sequence information for salt cress. It is convenient for gene cloning by a single PCR instead of mapping. Moreover, based on the information on homologous genes in Arabidopsis, the functional analyses of candidate salt cress genes is much simpler. Finally, gain-of-function mutants can uncover gene functions that would never be revealed by conventional loss-of-function approaches. This approach could be applied to mine genes related to interesting phenotypes in other stress or developmental conditions.

5. Materials and methods

5.1 Plant material

A. thaliana Columbia and salt cress (*T. salsuginea*) were used in this study. Seeds of Arabidopsis and salt cress were surface-sterilized with 10% bleach and then washed three times with sterile water. Sterile seeds were plated on MS medium plus 1.5% sucrose. Arabidopsis seeds were stratified in darkness for 2 to 4 d at 4 ^{o}C, while salt cress seeds were stratified for three weeks under the same conditions. Then, the seeds were transferred to a tissue culture room at 22 ^{o}C operating a 16-h light/8-h dark photoperiod. After two weeks, seedlings were potted in soil and placed in a growth chamber at 22 ^{o}C and 50% humidity operating a 16-h light/8-h dark photoperiod.

5.2 Generation of the salt cress cDNA library

For a wide coverage of transcripts related to salt stress, when salt cress plants in soil had generated four to six true leaves, they were treated with 400 mM NaCl. Leaf and root

tissues of plants were collected at 0, 15 min, 30 min, 1 h, 3 h, 6 h and 12 h. Rosettes were cut off from the plants directly and the roots were washed with water before collection. The collected tissues were mixed together, frozen in liquid nitrogen and ground to a powder. The powder was resuspended in extraction buffer (50 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 2% sodium dodecyl sulfate [SDS], 10 mM LiCl; pH 6.0) and mixed with an equal volume phenol/chloroform (1:1, pH 6.0) at 65 ^{o}C. This mixture was vortexed and centrifuged at 4 ^{o}C for 15 min at 10,000 *g*. The supernatant was phenolized twice and precipitated with an equal volume of 4 M LiCl. After centrifugation, the RNA pellet was resuspended in Tris-EDTA (TE) buffer. The supernatant was phenolized again and then precipitated by adding 0.1 volumes of 3 M NaAc (pH 5.3) and three volumes of pure ethanol. The RNA pellet was washed and resuspended in TE buffer.

Total mRNA was purified, and double strand cDNA was synthesized from 5 µg of mRNA using a cDNA synthesis kit (Stratagene, CA, USA). The double-stranded cDNA, containing *Eco*RI and *Xho*I ends, was ligated into the *Eco*RI/*Xho*I-digested pGreen vector (Hellens RP, 2000). After ligation, the library was dialyzed with distilled water and electroporated into *Escherichia coli* XL1-Blue MRF′ (Stratagene) .The transformants were plated on Luria Bertani (LB) agar containing 50 µg/mL kanamycin sulfate (kan).

5.3 Transformation of the cDNA library into Arabidopsis

Plasmids of the salt cress cDNA library were collected from *E. coli* XL1-Blue MRF′ and electroporated into *Agrobacterium* strain EHA105 with the co-resident plasmid pSoup. The transformed agrobacteria were selected on LB agar containing 50 µg/mL kan and 100 µg/L rifampicin. All agrobacterial colonies were washed off the agar plates, resuspended and then diluted in agrobacterial infiltration medium to an OD600 of 0.7 for Arabidopsis transformation. When plants were at their peak of flowering, the Arabidopsis flowers were dipped using a vacuum infiltration method as described previously (Bechtold and Pelletier, 1998). For maintaining a high level of humidity, plants were covered for 24 h after dipping. Then, the plants were transferred to a greenhouse and allowed to grow to maturity. T0 seeds were harvested in bulk and germinated on half-strength MS medium containing 50 µg/mL kan. The positive transgenic seedlings were transplanted into soil for the production of T1 seeds.

5.4 Screening for salt tolerance lines from the transgenic Arabidopsis library

T1 seeds were surface-sterilized, germinated and cultured as detailed in the 'Plant material' Section above. For each line, 18 plants were chosen at random and transplanted into two pots. Wild type plants were used as the control. By approximately three weeks after germination, each plant had generated four to six true leaves. Then, the plants were treated with 200 mM NaCl. Normally, *A. thaliana* Columbia wild type plants are unable to withstand such a high concentration of NaCl. T1 lines with survival rates greater than 70% were chosen as salt tolerant candidates.

5.5 Confirmation of salt tolerance in the candidate lines by re-screening

To confirm the salt tolerant phenotype, T2 seeds were used. T2 seeds were surface-sterilized, germinated and cultured as detailed in the 'Plant material' Section above. Wild

type plants were used as the control. About three weeks after germination, plants had generated four to six true leaves. Then, the plants were treated with 50 mM NaCl. Salt concentration was increased by 50 mM every four days until a final concentration of 200 mM. Plants were allowed to keep on growing under these conditions. The final survival rate of salt tolerant lines would be expected to be much greater than that of wild type plants.

5.6 Isolation of the inserted salt cress cDNA and identification of homologous genes in Arabidopsis

Since salt cress cDNAs were introduced to Arabidopsis by T-DNA insertion, the flanking sequences of cDNAs were conserved in the genome of transgenic plants. Using appropriate primers (pGreen-sense: 5′-GGAACTACTCACACATTATTATGGAG-3′; and pGreen-antisense: 5′-CATTTGGAGAGGACACGCTG-3′), cDNA inserts were amplified by PCR. PCR products were cloned into T-vectors and sequenced. If more than one band emerged from the PCR as a result of multiple insertions, each band should be sequenced. According to previous studies, most salt cress genes had homologous genes in Arabidopsis. Arabidopsis homologs were identified using BLAST.

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Impact of Drought Stress on Peanut (*Arachis hypogaea* L.) Productivity and Food Safety

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1. Introduction

Peanut (*Arachis hypogaea* L.) is one of the world's most important legumes. It is grown primarily for its high quality edible oil and protein. Peanut is grown on 35.5 million ha across 82 countries in the world. More than half of the production area, which accounts for 70% of the peanut growing area fall under arid and semi-arid regions, where peanuts are frequently subjected to drought stresses for different duration and intensities (Reddy et al., 2003). An annual estimated loss in peanut production equivalent to over US$520 million is caused by drought. Further, drought is also known to predispose peanut to aflatoxin contamination (Blankenship et al., 1984; Cole et al., 1989) making them unfit for human consumption. Yield losses due to drought are highly variable in nature depending on timing, intensity, and duration, coupled with other location-specific environmental stress factors such as high irradiance and temperature. In the United States peanuts contribute to more than $4 billion to the country's economy each year. In USA majority of the peanut are grown under rain-fed conditions and only limited acreage is irrigated. Frequent failure of rains late in the season has resulted in decreased yield, poor quality peanuts and aflatoxin contamination. Furthermore, increased worldwide demand for water due to rapid population growth and irrigation practices have resulted in declines in aquifers limiting availability of water for irrigation. To meet future food-supply demands, crop production will have to increase, but it must do so under the constraints of less water and, most likely, less farm land. Agricultural Research Service (ARS) scientists with the Plant Stress and

Germplasm Development Research Unit, Lubbock, Texas, the National Peanut Research Laboratory (NPRL) in Dawson, Georgia, and ICRISAT, India are working with cooperators to help peanut farmers maintain and improve their production in a changing environment. Drought-stressed plants lose moisture from pods which leads to the reduction in the seeds physiological activity, thereby increasing the susceptibility to fungal invasion. Besides affecting food quality, drought stress is also known to alter nutritional quality of peanut seed proteins. Since peanut lack desirable genetic variation in drought and aflatoxin tolerance several conventional as well as molecular breeding techniques were adopted to improve drought and aflatoxin tolerance (Mehan et al., 1986; Dorner et al., 1989; Holbrook et al., 2000). Recently several advanced molecular tools have been developed to screen drought tolerance in peanut genotypes. Effect of drought stress on peanut is being studied at the molecular and cellular level, which has generated enormous amount of genomic and proteomic data that displays the mechanism by which peanut plants respond to drought stress. Engineering peanuts to withstand drought stress has been achieved *via* different strategies, while few of them have succeeded in developing improved peanut genotypes that withstand drought stress others are in the process of developing advanced genotypes. This chapter will highlight selected as well as most significant achievements made to understand and overcome drought stress in peanuts.

2. Effect of drought on plant performance

2.1 Drought stresses reduce plant productivity

Drought stress has been the major environmental factor contributing to the reduced agricultural productivity and food safety worldwide. Drought stress perceived by the plant from its surrounding environment varies spatially and temporally at several different scales. Drought affects membrane lipids and photosynthetic responses (Lauriano et al., 2000) and yield in peanuts (Suther & Patel, 1992). Water deficit affects thylakoid electron transport, phosphorylation, carboxylation and photosynthesis. Changes in the lipid content and composition are common in water-stressed plants and this increases membrane permeability. This causes damage and membrane disruption as well as reduction in photosynthesis. Maintaining membrane integrity under drought conditions will determine the plants resistance towards stress. Plants have several mechanisms for adaptation to water and heat stress including stomatal conductance, paraheliotropism, and osmotic adjustments. Arid and semi-arid environments typically have hot days and cool nights. Since there is a lack of water vapor in the air, the temperature at night drops making the night cooler but the day hotter. This can be stressful to the plant.

2.2 Plant responses to drought

Drought stress has adverse influence on water relations (Babu & Rao, 1983), photosynthesis (Bhagsari et al., 1976), mineral nutrition, metabolism, growth and yield of groundnut (Suther & Patel, 1992). In addition, drought conditions influence the growth of weeds, agronomic management and, nature and intensity of insects, pests and diseases (Wightman et al., 1989). Parameters like relative water content (RWC), leaf water potential, stomatal resistance, rate of transpiration, leaf temperature and canopy temperature influences water relations in peanut during drought. Stressed plants have lower RWC than non-stressed plants. For example, relative water content of non-stressed plants range from 85 to 90%, while in drought stressed plants, it may be as low as 30% (Babu & Rao, 1983). Peanut leaves

show large diurnal variation with high values in the morning when solar radiation and vapor pressure deficits are low, followed by low values around midday and gradual increase after midday (Erickson & Ketring, 1985). Osmotic potential follows the same pattern but ranges less widely than leaf water potential. Transpiration rate generally correlates to the incident solar radiation under sufficient water availability. However, drought stressed plants transpire less than unstressed plants. Subramaniam & Maheswari, (1990) reported that leaf water potential, transpiration rate and photosynthetic rate decreased progressively with increasing duration of water stress indicating that plants under mild stress were postponing tissue dehydration. Stomatal conductance decreased almost steadily during the stress period indicating that stomatal conductance was more sensitive than transpiration during the initial stress period. Stirling et al., (1989) found that under water deficit conditions the leaves exhibited marked diurnal variation in leaf turgor, while pegs showed less variation and maintained much higher turgor levels largely because of their lower solute potentials. Marked osmotic adjustment occurred in growing leaves but not in mature ones, allowing them to maintain higher turgor during periods of severe stress. This adjustment was rapidly lost when stress was released (Ali Ahmad & Basha, 1998). Bhagsari et al., (1976) reported that water potential of leaves and immature fruits were similar under drought stress conditions. It is a general observation that under severe moisture stress conditions, young pods lose their turgor and shrivel. Azam Ali (1984) reported that stomatal resistance of older leaves was greater than that of younger leaves. Leaves also become thicker under moderate drought stress (Reddy & Rao, 1968). The developing leaves of groundnut have an unusual thick layer of cells devoid of chloroplasts with lower epidermis below the sponge parenchyma. Cells of this layer are considered to be water storage cells (Reddy & Rao, 1968). During moisture stress, the opposing leaflets of tri-foliate leaf come together and orient themselves parallel to incident solar radiation, in an effort to reduce solar radiation load on the leaf. Leaf expansion is more sensitive to soil water deficit than stomatal closure (Black et al., 1985). Drought reduces leaf area by slowing leaf expansion and reducing the supply of carbohydrates. Reddy and Rao (1968) reported that severe drought stress decreased the levels of chlorophyll *a*, *b* and total chlorophyll. The decrease in chlorophyll was attributed to the inhibition of chlorophyll synthesis as well as to accelerated turnover of chlorophyll already present.

Periodic water stress leads to anatomical changes such as a decrease in size of cells and intercellular spaces, thicker cell walls and greater development of epidermal tissue. Nitrogen fixation by leguminous plants is reduced by moisture stress due to a reduction in leg haemoglobin in nodules, specific nodule activity and number of arid regions. In addition, dry weight of nodules is significantly reduced in moisture stressed plants. Moisture stress also delays nodule formation in leguminous crops (Reddi & Reddy, 1995). There is considerable evidence that N, P and K uptake of peanut is reduced by drought stress (Kulkarni et al., 1988).

Leakage of solutes as a consequence of membrane damage is a common response of groundnut tissue to drought stress. Metabolic process is affected by water deficits. Severe water deficits cause decreases in enzymatic activity. Complex carbohydrates and proteins are broken down by enzymes into simpler sugars and amino acids, respectively (Pandey et al., 1984). Accumulation of soluble compounds in cells increases osmotic potential and reduces water loss from cells. Proline, an amino acid, accumulates whenever there is moisture stress. Accumulation of proline is greater in the later stages of drought stress and

therefore its concentration is considered a good indicator of moisture stress (Reddi & Reddy, 1995).

2.3 Effect of drought during flowering and pod formation

2.3.1 Flowering

The start of flowering is not delayed by drought stress (Boote & Ketring, 1990). The rate of flower production is reduced by drought stress during flowering but the total number of flowers per plant is not affected due to an increase in the duration of flowering (Gowda & Hegde, 1986; Janamatti et al., 1986; Meisner & Karnok, 1992). A significant burst in flowering on alleviation of stress is a unique feature in the pattern of flowering under moisture stress, particularly when drought is imposed just prior to re-productive development (Janamatti et al., 1986). When stress is imposed during 30–45 days after sowing the first flush of flowers produced up to 45 days do not form pegs during that time, however, flowers produced after re-watering compensated for this loss (Gowda & Hegde, 1986).

2.3.2 Pod formation

Peanut plants may experience water stress during pegging and pod development and then may have adequate amount of water (Jogloy et al., 1996). This would result in a drastic reduction of crop yield, and the magnitude of reduction would depend on peanut cultivars. Not only the yield of peanut but also the quality of products decreases under drought stress (Rucker et al., 1995). Peg elongation, which is turgor dependent, is delayed due to drought stress (Boote & Ketring, 1990). Pegs fail to penetrate effectively into air-dry soil, especially in crusted soils. Often, within 4 days of withholding water, the soil surface becomes too dry for peg penetration. Skelton & Shear (1971) reported that adequate root zone moisture could keep pegs alive until pegging zone moisture content is sufficient to allow penetration and initiation of pod development. Once pegs are in the soil, adequate moisture and darkness are needed for pod development. Adequate pod zone moisture is critical for development of pegs into pods and adequate soil water in the root zone cannot compensate for lack of pod zone water for the first 30 days of peg development. Dry pegging zone soil delayed pod and seed development. Soil water deficits in the pegging and root zone decreased pod and seed growth rates by approximately 30% and decreased weight per seed from 563 to 428 mg. Peg initiation growth during drought stress demonstrated ability to suspend development during the period of soil water deficit and to re-initiate pod development after the drought stress was relieved (Sexton et al., 1988). It has frequently been reported that under water stress, pegging and seed set responses of various peanut cultivars varied substantially, this leads to a large reduction in pod yield, and the reduction percentage also varies among peanut cultivars (Haris et al., 1988, Nageswara Rao et al., 1989).

2.4 Relationship of drought tolerance and aflatoxin contamination

Drought stress has a strong effect on biocompetitive (phytoalexins, antifungal proteins) or protective compounds (phenols), which influence the growth of *Aspergillus* fungus and aflatoxin synthesis, as well as the proper maturation of peanut seeds. Aflatoxin contamination threat increases with increasing seed maturity. As the seed moisture content decreases during drought, the capacity of seed to produce phytoalexins decreases resulting in *Aspergillus* invasion and aflatoxin production. Some of the enzymes that are induced in response to fungal attack such as chitinases, osmotins, peroxidases, and proteases are also

adversely affected during drought stress through cell membrane-mediated mechanisms. Drought stress and drought stress mediated-fungal infection compromise peanut defense and exacerbate aflatoxin formation in the seeds (Guo et al., 2005). Thus, breeding for drought tolerance has been accepted as one of the strategies for developing aflatoxin-tolerant peanut cultivars, which would not only minimize water usage but also help expand peanut production in marginal and sub-marginal soils. Success in this effort has been slow due to lack of genetic resources and lack of information on the relationship or interaction between the pathways affected due to drought and or pathogen invasion. However, to date, few peanut cultivars with natural pre-harvest resistance to aflatoxin production have been identified through field screening.

3. Breeding for crop improvement

3.1 Breeding towards drought tolerance

Efforts to improve peanuts that focus on yield as the only environmental method for screening of tolerance are seen to have a high variability in yield as well as differences in exactly reproducing stress conditions. A more-integrated approach for peanut breeding is needed to offer success in developing stress-tolerant varieties. Understanding physiological and molecular genetics may lead to the understanding of stress response and aid in development of new varieties with stress tolerance. So, a high-yielding cultivar that continues to produce well under drought conditions is a priority to enable stability of production. That is why much research for the last decade has attempted to improve performance by selecting plants with good pod yield under adverse conditions. As well as spending time testing plants in large-scale trials under different conditions, a study of plant physiology has revealed the features of the plant that correlate best with drought tolerance.

Research in the previous decade had developed low-cost, rapid and easily measured indicators for three significant physiological features of drought-tolerance *viz.* amount of water transpired (T), water-use efficiency (W) and harvest index (HI), thus allowing their potential quantification in large numbers of breeding populations. The application of this physiological model in peanut-breeding programs has not been possible because of practical difficulties associated with measurement of the traits under field conditions. The USDA germplasm collection numbers over 9000 accessions of *A. hypogaea* (Holbrook, 2001) and about 800 accessions of *Arachis* species. Large *Arachis* species collections are also maintained at Texas A&M University and N. C. State University. The US breeding program is focused more on yield, grade, seed size and developing disease tolerant germplasm, and less on drought tolerance. Identifying drought tolerant genotypes with emphasis to reduce preharvest aflatoxin contamination is being conducted at the USDA-ARS, Tifton, GA., The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India and The National Center of Genetic Resources (CENARGEN), Brazil. The largest collection of domesticated peanut germplasm is located at ICRISAT, where there are 14,310 accessions from 92 countries while CENARGEN has 413 accessions of *Arachis* species (Upadhyaya et al., 2001a). A new drought tolerant groundnut variety, ICGV 91114, is becoming very popular in Anantapur district in Andhra Pradesh, India, where it is now replacing a 7-decade old variety TMV 2. ICGV 91114 has also been released in Orissa, India and is doing very well in Karnataka, India.

In another study at Main Oilseeds Research Station, Junagadh Agricultural University, Junagadh, around 130 genotypes/crosses from different breeding trials (these were identified as potential drought tolerant with the help of visual observations such as retention of greenness at harvest, thickness of foliage, dwarfness combined with greenness, etc.) were screened for higher yield than local check varieties under simulated drought conditions in the summer season of 1999, 2000 and 1997. In the second phase of investigation, yield performance of these selected crosses/entries was assessed in comparison with three varieties GG-2, GG-5 (local checks) and J-11 (national check) at three naturally drought prone locations viz., at Targhadia (Main Dry Farming Research Station), Manavadar, Nanakandhasar and Jamkhambhalia, Gujarat, India in terms of pod yield. The basic advantage in selecting yield as the selection criteria is that it integrates all the additive effects of many underlying mechanisms of drought tolerance. Seven crosses and two genotypes with three controls (check varieties) were grown in a randomized complete block design with four replications for three consecutive Kharif seasons – 1999, 2000 and 2001. The results clearly indicated that the selected crosses/genotypes are at par with the local cultivated varieties of groundnut with respect to pod yields. In fact, they could even be termed superior because under extreme conditions of water deficit during 1999 and 2000 they recorded significantly higher pod yield than the local checks. Hence, the crosses GG-2 x NCAC 17135, GG-2 x PI 259747, J 11 x PI 259747, S 206 x FESR-8, Kisan x FESR-S-PI-B1-B, and the genotypes JB 223 and 224 could be termed as drought tolerant genotypes. Hence, it is suggested that these lines/genotypes could be grown under regions of limited rainfall. These lines may be also used as parents in breeding programs for developing drought tolerant groundnut cultivars.

3.2 Limitations of traditional breeding

Crop improvement in terms of production, desirable traits and resistance to drought stress is a pre-requisite in modern day agriculture. Conventional breeding for developing drought-tolerant crop varieties is time-consuming and labor intensive due to the quantitative nature of drought tolerance and difficulties in selection for drought tolerance (Ribaut et al., 1997). Combining high levels of resistance into higher yielding cultivars with acceptable market traits continues to be difficult (Holbrook & Stalker, 2003). Breeding programs, aimed at incorporating resistance genes from wild *Arachis* relatives have proved largely unsuccessful due to genetic incompatibility. Due to limitations of conventional peanut breeding either because of limited gene pool or the restricted range of organisms between which genes can be transferred, new *omics* techniques in addition to conventional methods are needed to develop peanut cultivars with resistance to drought and pre-harvest aflatoxin contamination.

4. Applications of molecular breeding tools for crop improvement

4.1 Genomic approach

Peanut is a polyploid with a large genome size, complete sequencing will be too expensive and labor intensive to perform with current resources. Research with molecular aspects of the peanut genome began in the 1980s when protein and isozyme variation in *A. hypogaea* was determined to be of little use for characterizing variation within the cultivated peanut. Although large numbers of polymorphisms were detected among other species in the genus (Lu & Pickersgill, 1993; Stalker et al., 1994), the number of markers was too small to be routinely used in breeding programs.

4.1.1 Molecular markers

Improvement of drought tolerance is an important area of research for groundnut breeding programs. Recent advances in the area of crop genomics offer tools to assist in breeding (Varshney et al., 2005, 2006). The identification of genomic regions associated with drought tolerance would enable breeders to develop improved cultivars with increased drought tolerance using marker-assisted selection (Ribaut et al., 1996). To make selection on large populations of progeny for breeding work, the accessions must be grown out and tested for traits. This is time consuming and subject to environmental variability. The scarcity of DNA polymorphism in cultivated peanut posses a considerable obstacle in genetic mapping of peanut. The Texas Peanut Breeding and Genetics Program is working on a long-term program to integrate modern physiological and molecular methods with plant breeding, to develop peanut varieties that can be grown efficiently under reduced water inputs and high heat stress. There are RFLP (Restricted Fragment Length Polymorphism) maps of wild type x cultivar crosses but the polymorphisms are too low for a cultivated x cultivated species cross; therefore, new markers are needed (Burow et al., 2001). Restricted Fragment Length Polymorphism markers also have disadvantages of using radioisotope, and results take longer to obtain than the use of PCR-based methods. Burow et al., (2001) study focused on finding traits useful in selecting genotypes for drought and heat tolerance. Heat stress was determined by fluorescence from cultivars grown in a high thermal stress greenhouse environment. Selections were made for drought and heat tolerance and crosses were made for further progeny evaluation. Further, they suggested that the research would entail sequencing cDNA in mapped RFLP clones to start the development of molecular markers in peanut.

A considerable number of SSR sequences have been identified from peanut genome by several research groups (Hopkins et al., 1999; He et al., 2003; Ferguson et al., 2004; Moretzsohn et al., 2005; Proite et al., 2007; Cuc et al., 2008). SSR markers developed from these repeat sequences offer promising genetic and genomic tools in peanut research. Genetic diversity of peanut germplasm has been studied in Valencia (Krishna et al., 2004), mini-core collection (Barkley et al., 2007), and in Chinese (Tang et al., 2007) and Japanese peanut germplasm collections (Naito et al., 2008) using SSR markers. Genetic linkage maps with SSR markers have been constructed for diploid AA genome (Moretzsohn et al., 2005), BB genome (Moretzsohn et al., 2009), tetraploid AABB genome derived from a cross of cultivated with amphidiploids (Fonceka et al., 2009), and tetraploid AABB genome in the cultivated peanut (Hong et al., 2008, Varshney et al., 2009; Hong et al., 2010). Although an exceedingly large number of SSRs have been identified, the polymorphic SSR markers may not be sufficient for the construction of a saturated linkage map in the cultivated peanut, provide enough meaningful markers for marker-assisted selection in peanut breeding programs, or sufficient coverage of important domains of the peanut genome for functional genomics research.

To identify the genomic regions suitable for marker-assisted breeding strategies, it is important to establish accurate phenotyping methods, develop highly saturated molecular marker-based genetic linkage maps, and then identify QTLs (quantitative trait loci) associated with traits of interest. Several studies were conducted in the past that reported identification of QTLs for drought tolerance or related traits. A RIL mapping population comprising of 318 F8/F9/F10 lines derived from a cross of TAG 24 x ICGV 86031 was phenotyped for transpiration (T, g plant-1), transpiration efficiency (TE, g biomass kg-1 water transpired), SLA (cm2 g-1), SCMR, leaf area (LA, cm2 plant-1), shoot plus pod dry

weight (DW, g plant-1), and total dry matter (TDM, g plant-1, which includes root dry weight) and carbon discrimination ratio (d13C) during post-rainy season in 2004 and 2005 by Ravi et al., (2011). A genetic map containing 191 SSR loci based on a single mapping population (TAG 24 9 ICGV 86031), segregating for drought and surrogate traits was developed. This study suggests deployment of modern approaches like marker-assisted recurrent selection or genomic selection instead of marker-assisted backcrossing approach for breeding drought tolerance in peanut.

4.1.2 Gene expression during drought stress in peanuts

Abiotic stress is a growing concern for peanut cultivation. Many production areas are in semiarid environments or have unreliable rainfall, and global climate changes and growing demand for fresh water pose major challenges. Physiological adaptation and selection for drought tolerance have been studied by many researchers (Reddy et al., 2003). Study of peanut genomics has been limited by biological constraints, and many basic tools of genomics have yet to be developed (Gepts et al., 2005). The peanut genome is large, making insertional mutagenesis and whole-genome sequencing expensive using current technology, and requiring large genomic libraries for physical mapping and positional cloning. To date, 136,901 peanut sequences, including 87,688 ESTs from cultivated peanuts and 39,866 nucleotide sequences have been deposited in the NCBI EST database. Out of which 52 nucleotide sequences and 25,914 EST sequences are available in response to drought treatments.

One of the major molecular responses that plants exhibit to drought stress is altered expression of genes, related to different pathways associated with stress perception, signal transduction, regulators and synthesis of a number of compounds (Ramanjulu & Bartels, 2002; Sreenivasulu et al., 2007). Several hundred genes that respond to drought stress at the transcriptional level have been identified in model crop *Arabidopsis* by microarray technology and other means (Seki et al., 2002; Shinozaki and Yamaguchi- Shinozaki., 2007). The adaptive mechanisms under stress are a net effect of altered cell metabolism resulting from regulated expression of stress responsive genes. The resurrection plants have better capabilities to cope with severe drought conditions; hence, several studies have been conducted to discover what key genes are involved in enabling these plants to survive desiccation.

Differential display reverse transcriptase PCR was used to identify genes induced and suppressed in peanut seed during drought. A total of 1235 differential display products were observed in irrigated samples, compared to 950 differential display products in stressed leaf samples (Jain et al., 2001). In another experiment, seven transcripts were found induced following stress of which two transcripts were suppressed in drought stressed immature pods of tolerant variety K1375 (Devaiah et al., 2007) (Fig. 1). These products demonstrated qualitative and quantitative differences in the gene expression during drought stress in peanuts.

Subtractive hybridization was used to identify about 700 genes from cDNA library prepared from peanut plants that were subjected to gradual process of drought stress adaptation (Govind et al., 2009). Further, expression of the drought inducible genes related to various signaling components and gene sets involved in protecting cellular function has been described based on dot blot experiments. Many families of transcription factors including AP2/EREBP (AhWSI 279), bHLH (AhWSI 111, AhWSI 40), bZIP (AhWSI 20), CCAAT box (AhWSI 117), Homeobox (AhWSI6 11), Jumonji (AhWSI 72, AhWSI 116), NAC (AhWSI 153,

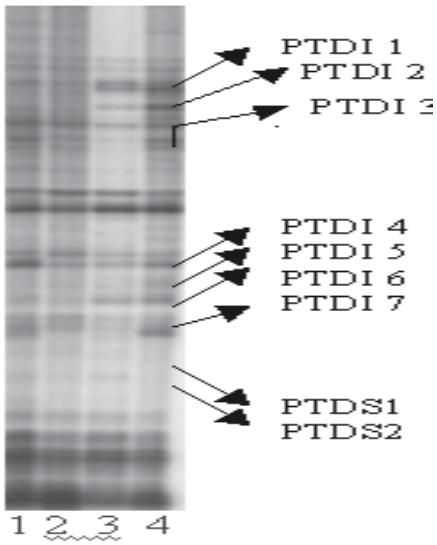

Fig. 1. DDRT PCR cDNA amplification from drought susceptible (JL-24) and drought tolerant peanut genotype (K1375). Arrows pointed upwards show peanut transcripts drought induced (PTDI) and arrows pointing downwards show peanut transcripts drought suppressed (PTDS).

AhWSI 308) and several zinc finger protein transcripts are preferentially induced under drought treatments in peanut plants. Also among the upstream signaling components they observed induction of transcripts of calmodulins (AhWSI 227, AhWSI 228), G protein (AhWSI 551), MAPKK (AhWSI 28) and several receptor kinases during drought treatments. In addition, specific upregulation of hormone responsive genes such as auxin-repressed proteins (AhWSI 306, AhWSI 468, AhWSI 467), brassinosteroid responsive BRH1 (AhWSI 36), cytokinin-repressed protein CR9 (AhWSI 465), GA like proteins (AhWSI 291, AhWSI 464) was observed during drought treatments. Insights gained from this study would provide the foundation for further studies to understand the question of how peanut plants are able to adapt to naturally occurring harsh drought conditions. Guo et al., (2006) identified a novel PLD gene in peanut, encoding a putative phospholipase D (a main enzyme responsible for the drought-induced degradation of membrane phospholipids in plants). PLD expression was induced faster by drought stress in the drought-sensitive lines than in the drought-tolerant lines, suggesting that peanut PLD may be involved in drought sensitivity responses, which could be useful as a tool in germplasm screening for drought tolerance. Gene expression in leaves of peanut plants submitted to progressive drought stress was studied by Drame et al., (2007). This study revealed that a good correlation exists with the agronomical and physiological responses during drought in peanuts. This study demonstrated that phospolipase Dα and LEA transcripts accumulation could contribute to reduced water loss and protection of cellular components.

4.1.3 Microarray based screening for monitoring gene expression during drought

Microarray technology employing cDNAs or oligonucleotides is a powerful tool for analyzing gene expression profiles of plants exposed to abiotic stresses such as drought,

high salinity, or cold, or to ABA treatment (Seki et al., 2001, 2002a, 2002b; Kreps et al., 2002). There are two predominant varieties of microarray technology available; the cDNA microarray (Seki et al., 2001, 2002a, 2002b) and the oligonucleotide microarray. cDNA Microarray was used to screen peanut genotypes by Luo et al., (2005). In this study, resistance genes in response to *Aspergillus parasiticus* infection under drought stress were identified using microarray and real-time PCR. A peanut genotype (A13) which is believed to be tolerant to drought and pre-harvest aflatoxin contamination was used to study gene expression. A total of 52 up-regulated genes were detected in response to drought apart from genes that were expressed due to biotic stress. Reactive oxygen scavengers glutathione S-transferase GST, superoxide dismutase (Cu–Zn), lactoylglutathione lyase, ascorbate peroxidase, lipoxygenase 1, Lipoxygenase 1, lactoylglutathione lyase, superoxide dismutase (Cu–Zn), stress proteins like drought-induced protein RPR-10, cytochrome P450, NOI protein, cold-regulated LTCOR12, low temperature and salt responsive protein, LTI6B, auxin-induced protein, ultraviolet-B-repressible protein, embryonic abundant protein, salt tolerance-like protein, proline-rich protein APG isolog, 10 kDa protein precursor, salt tolerance-like protein, NOI protein, embryonic abundant protein, ultraviolet-B-repressible protein, auxin-induced protein, osmotin-like protein, cell-autonomous heat shock cognate protein 7 and heat shock protein 81-2 were observed to be induced during drought.
High-density oligonucleotide microarray was developed for peanut using 49,205 publicly available ESTs and the utility of this array were tested for expression profiling in a variety of peanut tissues (Payton et al., 2009) to identify putatively tissue-specific genes and demonstrate the utility of this array for expression profiling in a variety of peanut tissues, transcript levels in pod, peg, leaf, stem, and root tissues. A set of 108 putatively pod-specific/abundant genes, as well as transcripts whose expression was low or undetected in pod compared to peg, leaf, stem, or root was detected. The transcripts significantly over-represented in pod including genes responsible for seed storage proteins and desiccation (e.g., late-embryogenesis abundant proteins, aquaporins, legumin B), oil production, and cellular defense were also observed. This Microarray chip represents sequences available from various drought stress treatments and hence, can be used as tool to monitor gene expression profile in genotype screening for drought tolerance.

4.1.4 Micro RNA could modify regulator gene expression during drought in peanuts

Micro RNAs are a new class of small, endogenous RNAs that play a regulatory role in the cell by negatively affecting gene expression at the post-transcriptional level. MicroRNAs have been shown to control numerous genes involved in various biological and metabolic processes. Recently MicroRNAs (miRNAs) were isolated in peanuts by Zhao et al., (2010). In this study, they used next generation high through-put Solexa sequencing technology to clone and identify both conserved and species-specific miRNAs in peanut. Next generation high through-put Solexa sequencing showed that peanuts have a complex small RNA population and the length of small RNAs varied, 24-nt being the predominant length for a majority of the small RNAs. Combining the deep sequencing and bioinformatics, they discovered 14 novel miRNA families as well as 75 conserved miRNAs in peanuts. All 14 novel peanut miRNAs were considered to be species-specific because no homologs have been found in other plant species except ahy-miRn1, which has a homolog in soybean. qRT-PCR analysis demonstrated that both conserved and peanut-specific miRNAs were expressed in peanuts. This study led to the discovery of 14 novel and 22 conserved miRNA families from peanut. These results show that regulatory miRNAs exist in agronomically-

important peanuts and may play an important role in peanut growth, development, and response to environmental stress.

4.2 Proteomic approach

4.2.1 Protein expression during drought stress

Proteomics studies have been carried out in leaf and immature peanut pods in response to drought stress. Identification and development of drought-tolerant genotype/s is the potential means to reduce aflatoxin contamination. Difference in biochemical response of peanut genotypes with varying degree of drought tolerance was monitored by withholding irrigation for various intervals. Changes in seed protein composition in response to drought stress were measured using two-dimensional electrophoresis followed by Mass spectroscopy. Mass spectroscopy analysis revealed down-regulation of methionine rich proteins (MRPs) and arachin proteins in drought-susceptible (DS) genotypes, while these proteins continue to express in drought-tolerant (DT) genotypes. Up-regulation of mRNA transcripts in DT genotypes indicated their association with stress tolerance. Continued expression of these proteins seems to enhance drought tolerance, reduce aflatoxin level and enhance nutritional value of peanut. These studies have revealed that drought stress suppresses expression of several seed storage proteins such as arachin, methionine-rich proteins, conarachin, etc (Basha et al., 2007).

Changes in the seed protein content and composition during 14 days of desiccation was determined by Mazhar and Basha (2002) using a combination of electrophoretic and immunochemical techniques. Following desiccation, the protein content of 'white' (most immature) and 'orange' (Intermediate maturity stage: Drexler and Williams, 1979) seed increased, while that of the 'brown' (more mature) seed were not affected. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed no major qualitative differences in protein composition during desiccation. However, immunoblotting with anti-dehydrin antisera revealed presence of several new proteins in the desiccated samples compared with the controls. One of the dehydrin-like proteins, was found to be related to water-stress, while the other proteins appeared to be the storage proteins accumulated as the seed matured *in vitro*. Capillary electrophoresis (CE) showed major changes in protein quantity and quality of 'white' seed (Immature) during the 0–14 days of desiccation. In contrast, in the 'orange' and 'brown' seeds (more mature) changes in protein composition were less significant. Their results indicated that several dehydrin-like proteins expressed in peanuts during desiccation but not all of them are related to drought stress.

In 2007, Basha and his co-workers carried out a study to determine changes in seed polypeptide composition among drought-tolerant (Vemana and K-1375) and drought-susceptible peanut genotypes (M-13 and JL-220) following water stress (WS) for 7, 14 and 28 d. They found that water stress had variable effect on peanut seed polypeptide composition (Fig. 2A) among the DT and DS genotypes. WS affected polypeptides with apparent molecular weight (Mr) around 70, 35, 25, 20, 18 and 14 kDa, and isoelectric points between 4.0 and 6.0 pH. The maximum response to WS occurred between 0 to 7 d, and additional periods (14 and 28 d) of stress caused only limited changes in seed polypeptide composition. These responses included over-expression, suppression, and appearance of new proteins in water-stressed seed compared to irrigated control. These data revealed that seed polypeptide composition of drought-tolerant peanut genotypes (Vemana and K-1375) was least affected while that of drought-susceptible genotypes (M-13 and JL-220) significantly altered due to WS (Fig. 2).

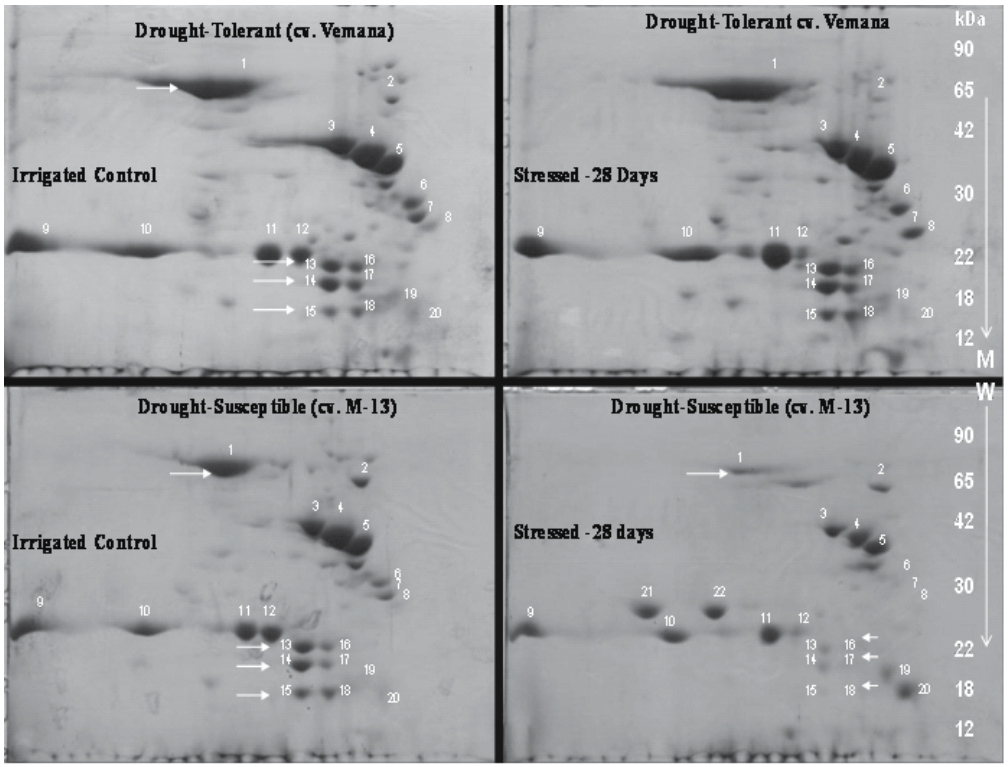

Fig. 2. Differential response of seed proteins of Drought Tolerant and Drought Susceptible Peanut Genotypes to Water Stress

Recently, Kottapalli and co-workers (2009) analyzed peanut genotypes from the US mini-core collection for changes in leaf proteins during reproductive growth under water-deficit stress. One and two-dimensional gel electrophoresis (1- and 2-DGE) was performed on soluble protein extracts of selected drought-tolerant and drought-susceptible genotypes. A total of 102 protein bands/spots were analyzed by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS) and by quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) analysis. Forty-nine nonredundant proteins were identified, implicating a variety of stress response mechanisms in peanut. Lipoxygenase and 1L-myo-inositol-1-phosphate synthase, which aid in inter and intracellular stress signaling were found to be more abundant in tolerant genotypes under water-deficit stress. Acetyl-CoA carboxylase, a key enzyme of lipid biosynthesis increased in relative abundance along with a corresponding increase in epicuticular wax content in the tolerant genotype, suggesting an additional mechanism for water conservation and stress tolerance. They also found a marked decrease in the abundance of several photosynthetic proteins in the tolerant genotype, along with a concomitant decrease in net photosynthesis in response to water-deficit stress. In contrast, Katam et al. (2007) found up-regulation of leaf proteins following drought stress in DT genotypes and down-regulation in DS genotypes. Differential regulation of leaf proteins involved in a variety of cellular functions (e.g. cell wall strengthening, signal transduction, energy metabolism, cellular detoxification and gene regulation) indicates that these molecules could affect the molecular mechanism of water-deficit stress tolerance in peanut.

5. Transgenic peanut tolerant to drought

The mechanisms of drought response have been investigated extensively in *Arabidopsis* (Bray et al., 1997; Shinozaki et al., 2003). However, the response of peanut to drought stress has not been extensively studied using genetic engineering. Classical breeding for drought tolerance in peanut is difficult because of variability in time, intensity, and duration of stress. In certain breeding programs, plants with genetic variability to drought have been identified and used to introduce this trait in genotypes with desirable agronomic characteristics. Thus in peanut classical breeding has and continues to have some success but the process is slow and limited by the availability of suitable genes for breeding. Beyond this there has been limited progress in breeding for drought tolerance because of limited characterization of associated traits and the fact that potential component traits of drought tolerance such as Transpiration, Transpiration Efficiency, or Harvest Index (Passioura, 1977) do not have simply additive effects (Bhatnagar-Mathur et al., 2007) in peanut. Molecular markers have been used to aid in the breeding process, but the low level of polymorphism in cultivated peanut has interfered with this approach. Although peanut germplasm with reduced drought tolerance have been identified and screened in breeding populations (Holbrook et al., 2000), peanut growers currently cannot rely fully on the available drought tolerant cultivars, as they are location specific. Therefore, the use of genetic engineering technology to over-express drought tolerant genes in peanut is an attractive prospective way to improve tolerance.

5.1 Developing drought tolerant peanut through genetic engineering

Development of drought tolerant peanut by genetic engineering requires the identification of key genetic determinants underlying stress tolerance in peanut plants, and introducing these genes into peanut crops. The effect of drought can trigger a wide array of physiological responses in plants, and this can affect a large number of genes. For example, Sahi et al., (2006) through their gene expression experiments have identified several hundred genes which are either induced or repressed during drought. *Arabidopsis* has played an important role in the elucidation of the basic processes underlying stress tolerance, and the knowledge achieved has been transferred to several food crops (Zhang et al., 2004). Most of the genes that are known to be involved in stress tolerance were initially isolated from *Arabidopsis*. Several stress induced genes that have been introduced in other plants by genetic engineering have resulted in increased tolerance of transgenic plants to drought. Therefore the same techniques that have been used in other crops can be used in peanut.

5.2 ABA-independent gene regulation to drought stress

There are two transcription factors *DREB1* and *DREB2*, which have been identified to be important in the ABA-independent drought tolerant pathways that induce the expression of drought tolerant genes. When the native form of *DREB1* and the constitutively active form of *DREB2* are over-expressed, tolerance of transgenic *Arabidopsis* plants to drought is increased. Even though these genes were initially identified in *Arabidopsis* plants, their existence and function in stress tolerance have been reported in many other important plants, such as tomato, barley, rice, canola, maize, rye, wheat, maize and soybean. This is an indication that these genes are conserved, and they perform a universal stress defense mechanism in plants. This is why the *DREB* genes can be used as suitable targets for peanut improvement for drought tolerance through genetic engineering.

5.3 Peanut transformation systems

Peanut transformation has been accomplished by several different methods. Ozias-Akins et al., (1993) reported the first successful transformation of peanut with accompanying plant regeneration by utilizing the microbombardment technique. Micro-bombardment has since been completed in peanut with a number of genes conferring disease resistance (Ozias-Akins & Gill, 2001; Magbanua et al., 2000; Yang et al., 1998; Higgins et al., 2004; Athmaram et al., 2006). However, its efficiency levels remain low and the process takes several months from when the initial transformation event is induced until plant maturity (Egnin et al., 1998). A highly-efficient and faster technique is needed to transform peanut, and *Agrobacterium*-mediated transformation appears to offer the possibility to achieve this goal. Cheng et al., (1996) used this method on a Valencia-type peanut, but other investigators have been unable to expand the methodology to other genotypes thus restricting its usefulness. To date, biolistic methodologies are more reliable in peanut than other transformation methodologies and single constructs can be inserted into the peanut genome. Individual genes that confer agronomic traits have been integrated into the peanut genome such as bialophos resistance (*bar*) for herbicide tolerance (Brar et al., 1994), *Bacillus thuringiensis* (*Bt*) toxin *cryIA(c)* for insect resistance (Singsit et al., 1997), viral nucleocapsid or coat protein genes for virus resistance (Higgings et al., 2004), chitinase, glucanase, and oxalate oxidase to control fungal diseases (Chenault et al., 2005; Livngstone et al., 2005; Rohini and Rao, 2001). But, in studying drought tolerance in transgenic peanut plants, Bhatnagar-Mathur et al., (2007), introduced a transcription factor *DREB1A* from *Arabidopsis thaliana*, in a drought-sensitive peanut cultivar JL-24 through *Agrobacterium tumefaciens*-mediated gene transfer (Fig.3). The stress inducible expression of *DREB1A* in these transgenic plants did not result in growth retardation or visible phenotypic alterations. They were successful in developing transgenic events of peanut with the *DREB1A* transcription factor that is specifically expressed under a stress responsive promoter such as *A. thaliana rd29A*. Thus, their study opens ways to other scientist to dwell more on producing transgenic peanut with drought tolerance.

6. Future research

Classical plant breeding programs, which are relatively inexpensive, are not well adapted for utilizing advanced technologies associated with genomics. Hence, a large percentage of scientists who perform genomic research are mainly interested in the molecular function of specific genes or processes and are usually less interested in marker development for phenotypic selection applications. On the other hand, plant breeders need markers to facilitate selection and are generally not interested in developing large data sets for sequencing specific genes. Although the gap between the producer of genomic information (molecular biologist) and the user (plant breeders) is very wide, there is enormous potential for interactions among disciplines for plant improvement. Indeed, increasing research efforts in engineering for production of drought-tolerant peanut crops should be employed. There are certain genes that are expressed at elevated levels when a plant encounters stress, and it is important to understand that tolerance to drought is a complex process, and it is unlikely to be under the control of a single gene. Therefore, it is wise to combine conventional screening efforts, marker assisted selection and genetic engineering to switch on a transcription factor regulating the expression of several genes related to drought tolerance.

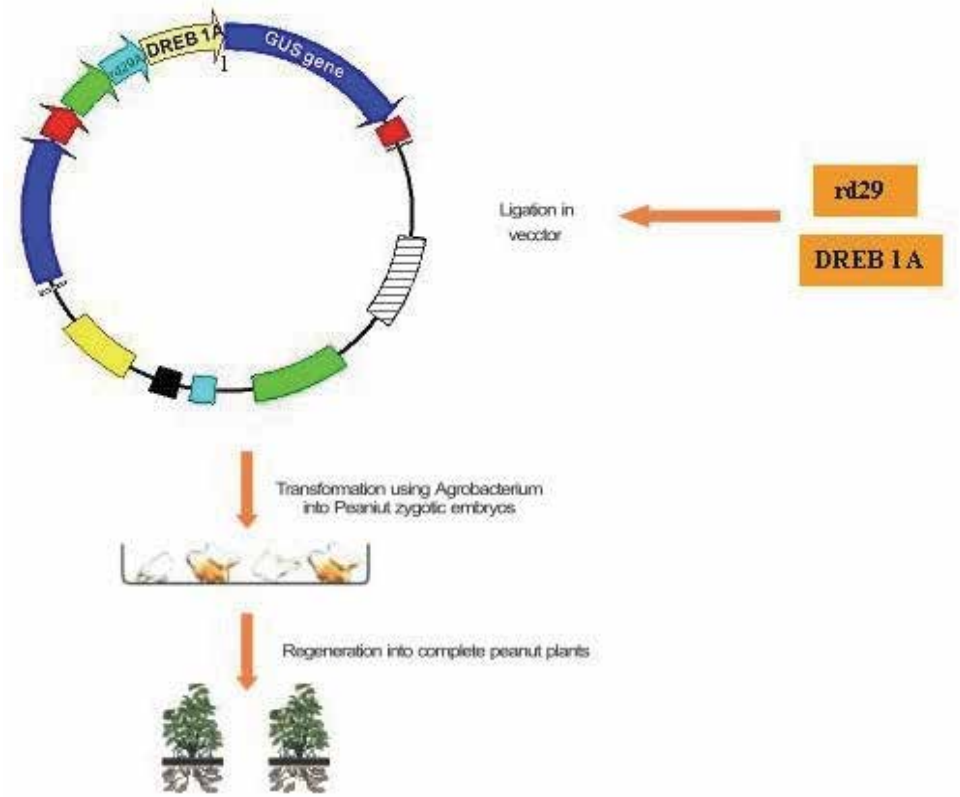

Fig. 3. Schematic representation of Cloning and Agrobacterium mediated genetic transformation in peanut

7. Conclusion

Although significant progress is being made to elucidate the genetic mechanisms underlying drought tolerance in peanut, considerable challenges still remain. In field conditions, peanut plants are subjected to variable levels of multiple stresses, and hence, the response of peanut to a combination of stresses deserves much more attention. In other words, the response of plants to multiple stresses cannot be inferred from the response to individual stress. Therefore, it is very important to test newly developed varieties to multiple stresses, and to perform extensive field studies under diverse environments to assess their tolerance.

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Changing environmental condition and global population demands understanding the plant responses to hostile environment. Significant progress has been made over the past few decades through amalgamation of molecular breeding with non-conventional breeding. Understanding the cellular and molecular mechanisms to stress tolerance has received considerable scientific scrutiny because of the uniqueness of such processes to plant biology, and also its importance in the campaign "Freedom From Hunger". The main intention of this publication is to provide a state-of-the-art and up-to-date knowledge of recent developments in understanding of plant responses to major abiotic stresses, limitations and the current status of crop improvement. A better insight will help in taking a multidisciplinary approach to address the issues affecting plant development and performance under adverse conditions. I trust this book will act as a platform to excel in the field of stress biology.

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