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Symbiosis

Edited by Everlon Cid Rigobelo

SYMBIOSIS

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Meet the editor

Dr. Everlon Cid Rigobelo graduated from Agronomy School, Universidade Estadual Paulista, Campus of Jaboticabal, Brazil, in 2000. He received his MSc degree in Animal Science Microbiology from the same university in 2002. He obtained his PhD degree in Microbiology and works as a researcher in the same university. Rigobelo has experience in genetics and epidemiology and is active in the following subjects: microbial biotechnology, molecular genetics and bacterial genomics. He works with plant growth-promoting rhizobacteria, and he previously worked with probiotic strains to reduce the spread of *Escherichia coli* in animal production.

Dr. Rigobelo worked with probiotics bacteria in the past, but currently, he is working with plant growth-promoting rhizobacteria such as *Azospirillum brasilense*, *Herbaspirillum seropedicae*, *Bacillus subtilis* and *Bacillus pumilus* in several crops such as sugarcane, maize, soybeans and cotton. He has worked with 8 students as an adviser and has advised 10 researchers until now, all of them trying to understand the marvelous world of plants and microorganisms.

Contents

Preface

Symbiosis is an interaction between plants and microorganisms that have been responsible for the plant colonization worldwide since they appeared on earth. Symbiosis is a specific, complex, evolved and sophisticated process, which brings advantages for both organisms involved. One of its advantages is the improvement of nutrient uptake, thus promoting plant development. This interaction is useful to farmers facing strong challenges regarding crop production such as soil fertility loss, climate changes, high cost of production and yield loss. Moreover, food production requires the use of the alternatives to be environmentally friendly. In this context, the symbiosis between plants and microorganisms can be technologically, environmentally and economically appropriate.

This book provides information about the symbiotic relationship between a fern and *Azolla*, plant control over thread development during legume-rhizobia symbiosis, bacterial leaf nodule symbiosis in flowering plants and the potential of rhizobium strains in improving nitrogen fixation and legume yields. We hope this information will be useful to all people working on a hot topic.

I would like to thank my wife Fernanda, my daughter Maria Eduarda and my son João Henrique who make my life happier.

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Chapter 1

Introductory Chapter: Symbiosis - A Successful Association between Plants and Microorganisms

Everlon Cid Rigobelo

Additional information is available at the end of the chapter

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1. Introduction

1.1. Plant growth promoting

In the last century, the use of chemical fertilizer has increased annually in the agriculture crops, and also the demand for food production has been increased.

Since the green revolution, the plants have been modified to become each more productive; therefore, the need for chemical fertilization has been increased.

However, the efficiency of plants to use or to uptake these nutrients from chemical fertilizers is too small, and the losses of these nutrients are too high. The agriculture system is based on the input and the output. Moreover, the problem becomes higher when the amount of fertilizer is higher than necessary to offset your losses. This condition causes many environmental and human health problems. Moreover, when the chemical fertilizers have been used, the ability of the plant roots to look for the nutrients and the abilities of many microorganisms to provide them for plants are ignored.

Since the emergence of plants, the association between plants and microorganisms has become strong. Moreover, both plants and microorganisms have gained with this association.

The plant and its microbiota interactions initiated since the emergences of the plant on Earth. These associations were required because the soil fertility when the plants emerged was very low and the microorganisms presented efficient abilities to uptake these low nutrients from the soil. These associations were responsible for the success of the plants to colonize on Earth.

The associations between plants and microorganisms continue till date. The symbiosis is most elevated and complex association between plants and microorganisms. It represents an evolutionary

success, in which many mechanisms at both plants and microorganisms are required to be developed such as molecular interactions, cell surface receptors, metabolic interactions, and maybe the important characteristic the symbiosis had been is the gene expression control by microorganisms.

Interestingly, in the first moment, maybe the symbiotic microorganisms were saprophytic. This characteristic requires that these microorganisms should obtain nutrients for their survival from the dead organic matter. These kinds of microorganisms are not able to obtain nutrient from the live tissue. It is certain because they are not able to suppress the plant protection mechanisms. When in the dead organic matter, the difficulty would be to just solubilize the organic matter.

Beneficial or parasitic interactions between plants and microorganisms are similar. In both situations, the microorganisms need to overcome the defense mechanisms of plants, and the difference among them is the beneficial interactions the microorganisms learned their capacity to get nutrients and metabolic from the plant without damaging.

The dynamic nature of the soil is a direct manifestation of soil microbes such as bio-mineralization and synergistic co-evolution with plants. The microorganisms succeeded in colonizing several plant's organs such as root and leaf.

Both the leaf and root microbiota contain bacteria that provide indirect pathogen protection and serve as additional host functions through the acquisition of nutrients from the soil for plant growth. In this context, the plant microbiota emerges as a fundamental trait that includes mutualism through diverse biochemical mechanisms, as reported by several studies on plant growth promoting and plant health. Although there is a wide variety of plant growth promoting rhizobacteria, members of plant's microbiota, their roles, and usages for sustainable agriculture remain controversial and restricted.

Some microorganisms have many beneficial characteristics that can be used to promote plant.

The largest part of the nitrogen is on the air, and few organisms are able to obtain and use it. Therefore, some microorganisms have the ability to get atmospheric nitrogen and transform it into an organic molecule. This phenomenon is termed nitrogen biological fixation. By this way, the plant can uptake and use this nutrient for its growth. Other microorganisms have the ability to produce organic acids or enzymes such as phosphatases and phytases. These microorganisms can solubilize the phosphorus adsorbed into the soil, mineralizing it and providing it for plants.

Other groups of microorganisms are able to synthesize phytohormones. It is an interesting characteristic for both microorganisms and plants. The microorganisms produce phytohormones because it cancels the plant protection mechanisms against the microorganisms and additionally promotes the root and shoot growth. When the root grows, the microorganisms become more efficient to uptake the nutrients released from the root. Consequently, the root growth promotes plant development, the plant becoming more resistant to hydric and nutritional stresses.

Another important characteristic of some microorganisms is the interference of *quorum sensing* between bacteria. The individuals of microbial community communicate with each other

through some molecules termed elicitors. Interestingly, when the concentration of these elicitors is low, the communication between bacteria does not happen. When the concentration of these elicitors is high, some genes belonging to the bacteria of the microbial community are expressed, and the new microbial characteristic arises. When the beneficial microbes are inoculated into the plant, some bacteria produce some molecules that destroy these elicitors, impairing the communication between pathogenic bacteria and avoiding any damage to plants.

Finally, some beneficial bacteria produce several antimicrobial molecules against many pathogenic microorganisms. This characteristic promotes a reduction of damage caused by pathogenic bacteria against plants.

In this context, this book entitled symbiosis had to report some different aspects related to this complex biological phenomenon between plants and microorganisms as aim, demonstrating the importance of it for plants and how it could be used as a strategy to improve the yield in crop production.

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Chapter 2

The Unique Symbiotic System between a Fern and a Cyanobacterium, *Azolla-Anabaena azollae*: Their Potential as Biofertilizer, Feed, and Remediation

Ana L. Pereira

Additional information is available at the end of the chapter

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Abstract

The free-floating aquatic fern *Azolla* is small and heterosporic, with a worldwide distribution in quiet waters (rivers, dams, creeks, etc.) and is considered an invasive species. This is the only known fern with a permanent symbiotic association with the heterocystic nitrogen-fixing cyanobacterium *Anabaena azollae* where the cyanobiont is transmitted through the *Azolla* generations without a *de novo* infection. The cyanobiont and other bacteria genera inhabit an ovoid cavity in each dorsal lobe of the leaf. The cyanobiont has a high rate of nitrogen fixation and thus this symbiosis was analyzed regarding its biofertilization (incorporated in soil or as manure). In addition, due to the amino acids and protein contents, this fern can also be used as food, and due to the high ability to uptake heavy metals and other pollutants, it can be used as phytoremediator. Since this fern is grown in tropical and subtropical zones where most of the countries have problems regarding the living conditions (health, sanitary, and food, among others) of people, this fern can be a very useful and cheap tool to cope with the severe problems that they face.

Keywords: *Azolla*, *Anabaena azollae*, symbiosis, fern, cyanobiont, bacteria, biofertilizer, food, phytoremediation

1. Introduction

The prokaryotic organisms (cyanobacteria and bacteria) appeared on Earth about 3 billion years ago, and during their evolution, both formed associations with other organisms. Such associations are a "combination of different organisms" living in a body that is developing throughout the life of another body [1], which should be named as symbiosis.

The cyanobacteria can form symbioses with several organisms such as sponges, amoebae, diatoms, fungi, and plants (bryophytes, ferns, gymnosperms, and angiosperms). The most common cyanobacteria (called cyanobionts) that form those symbioses belong to the genus *Anabaena* and *Nostoc*, which are able to fix the atmospheric nitrogen in specialized cells (called heterocysts) through the nitrogenase complex enzyme and thus convert it into the ammonium ion (NH_4^+). On the symbioses, both partners get benefits of the association. The prokaryote has a continuous supply of nutrients and protection against herbivores and desiccation, while the host has a provision of all or almost all of the nitrogen needed for its development, allowing the colonization of nitrogen-poor environments (aquatic or terrestrial) [2–4].

Although all the symbioses are very interesting and there is still no complete knowledge of all of them, this chapter focuses on the unique symbiosis between the aquatic fern *Azolla*, the cyanobacteria *Anabaena azollae,* and some bacteria with a description of the symbiotic morphology and the potential uses of this fern in agriculture, feeding, and remediation of contaminated wastewaters. Also, the challenges of their use will be addressed.

2. The partners *Azolla*, *Anabaena azollae*, and bacteria and their relationship

This fern is widespread in the world, from tropical to subtropical and temperate regions flourishing in calm waters (rivers, dams, creeks, and others), which shows their adaptability to several environmental conditions, from very hot regions to moderate temperature or even freezing temperatures [5–7]. This adaptability makes this fern to be considered as an invasive species in many countries especially in Europe such as Germany, Portugal, Spain, Great Britain, and others. When the environmental conditions (light, temperature, photoperiod, and nutrients) are optimal, *Azolla* overgrows forming a dense thick mat causing many problems such as the clogging of water pumps, the drowning of livestock, or the reduction of water flow in the irrigation channels [8]. However, the control of *Azolla* spread is difficult since this species can be bought online as a plant for a fish aquarium.

2.1. Genus *Azolla*

Priest Louis Fevillé made the first description of *Azolla* in botanical literature in 1725 from a Peruvian record, giving it the name of *Muscus squamosus aquaticus elegantissimus*, but the genus *Azolla* was described by Lamarck in 1783 [9]. The name *Azolla* derives from two Greek words, αξω (dry) and οδδνω (kill), which reflect the inability of this fern to survive in dry environments meaning that it has to always be in contact with water.

For many years, the genus *Azolla* was placed in the family Azollaceae, but recent research shows that this genus belongs to the family Salviniaceae [10]. The genus has seven species: (1) *A. pinnata* and *A. nilotica* belonging to the section *Rhizosperma* and (2) *A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. microphylla*, and *A. rubra* belonging to the section *Azolla*. The taxonomy of *Azolla* is mostly made by morphological characters (vegetative and reproductive), but in the last years, the gene sequencing contributed to the clarification of the relationships between the species although some controversies still remain about the number of species [11–14].

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The oldest *Azolla* fossil is dated from the superior cretaceous period (Mesozoic Era), but the majority of the fossil records are dated from the Tertiary and Quaternary periods (Cenozoic Era) [15].

2.2. Morphology of the symbiosis

The multibranched sporophyte of *Azolla* has round (**Figure 1A**) or pinnate (**Figure 1B**) shape, and the main rhizome has alternate lateral ramifications fully covered with alternate, imbricate, and deeply bilobed leaves that form a dorsal (**Figure 1A**) and a ventral lobe (**Figure 1C**). The adventitious roots emerge from the ventral side of the rhizome at ramification points (**Figure 1C**) and hang down into the water. The dorsal lobe is aerial and chlorophyllous and has papillae and stomata (**Figure 1D**), and the ventral lobe is partially submerged and hyaline (**Figure 1C**). When environmental conditions are favorable, a red color develops in the leaves and rhizome due to the synthesis and accumulation of anthocyanins [5, 16–18].

This symbiotic association is permanent, meaning that it occurs at all stages of the life cycle of the pteridophyte, whether the propagation is made through vegetative fragmentation or by sexual reproduction. On the vegetative life cycle, there is a synchronous development of both symbionts (*Azolla* and *A. azollae*) from the apical meristem until the leaves become fully developed (**Figure 2A**). During this development, some cyanobiont filaments become entangled on primary branched hairs at the apical meristem (**Figure 2B**) and are partitioned into the forming cavities. On fully developed foliar cavities, the filaments of the cyanobiont occupy a very narrow space at the periphery of the leaf cavity (**Figure 2C**) and encircle the secondary branched hairs (**Figure 2D**) and simple hairs (**Figure 2E**) [5, 6, 18–20].

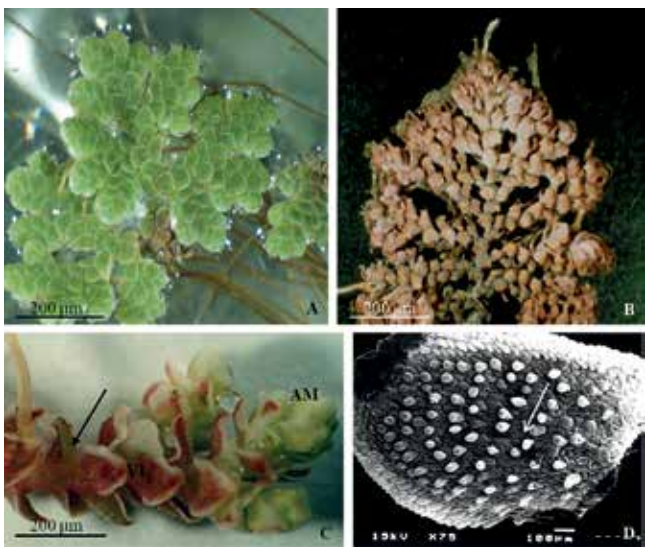

Figure 1. Morphology of the sporophytes of *Azolla*. (A) Round shape of *Azolla filiculoides* with chlorophyllous dorsal lobes. (B) Pinnate shape of *Azolla pinnata*. (C) Ventral view of the rhizome with the hyaline and ventral lobes (VL), curved apical meristem (AM), and adventitious root (arrow). (D) Upper surface of the dorsal lobe covered with papillae (*) and stomata (arrow).

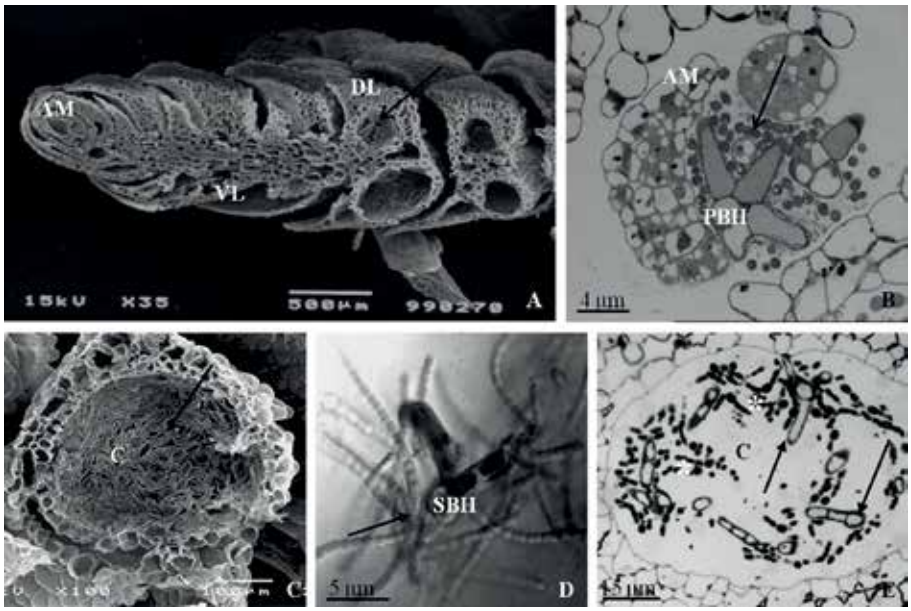

Figure 2. Morphology of the symbiosis between *Azolla* and the cyanobiont. (A) Cross section of the sporophyte showing the curved apical meristem (AM) until the fully developed dorsal lobe (DL) with an ovoid cavity (arrow) and the ventral lobes (VL). (B) Detail of an apical meristem (AM) with the cyanobiont (arrow) entangled in the primary branched hair (PBH). (C) Filaments of *A. azollae* (arrow) on the periphery of the leaf cavity (C). (D) Secondary branched hair (SBH) of the mature cavity surrounded by filaments of the cyanobiont (arrow). (E) Simple hairs (SH) of the mature cavity (C) surrounding with filaments of the *A. azollae* (*).

In the sexual reproduction, the sporocarps are differentiated on the ventral lobe when the environmental conditions are adverse to the vegetative propagation or survival of the fern. Since this fern is heterosporic, it forms a female sporocarp called macrosporocarp (**Figure 3A**) and a male sporocarp called microsporocarp (**Figure 3B**). The macrosporocarp has an indusium or cavity where it harbors a small inoculum of the cyanobiont (**Figure 3C**). When the environmental conditions become favorable, the fertilized macrosporocarp germinates and the inoculum of the cyanobiont is partitioned into the new leaves [21, 22].

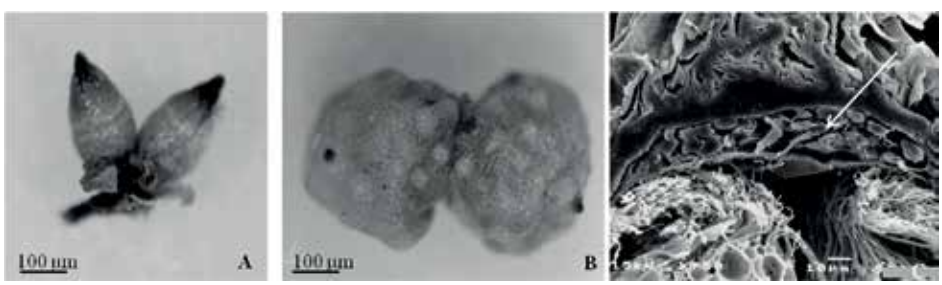

Figure 3. Sporocarps of *Azolla*. (A) Macrosporocarps. (B) Microsporocarps. (C) Indusium with cells of the cyanobiont *A. azollae* (arrow).

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The cyanobiont of this symbiosis is Gram-negative, colonial, filamentous, and nitrogen-fixing having vegetative cells, heterocysts, and akinetes. Since the development of the cyanobiont and foliar cavities are synchronous, the filaments on the apical meristem only have vegetative cells (**Figure 4A**) without nitrogenase activity, while in the foliar cavities, the cyanobiont filaments have vegetative cells, heterocysts, and akinetes (**Figure 4B**) and can fix nitrogen [16, 23]. The fixed nitrogen is excreted into the leaf cavity as ammonium ions, which are assimilated by the hairs that exist in the foliar cavities and incorporated as amino acids in the fern [6, 17, 24, 25]. Although the cyanobiont is known as *A. azollae*, studies on the taxonomy of cyanobacteria indicated that it can be classified as *Anabaena*, *Nostoc*, or *Trichormus* [26, 27].

The bacteria found in the apical meristem and foliar cavities of the *Azolla* sporophyte (**Figure 5**) were identified by several researchers as *Aeromonas*, *Agrobacterium*, *Alcaligenes faecalis*, *Arthrobacter*,

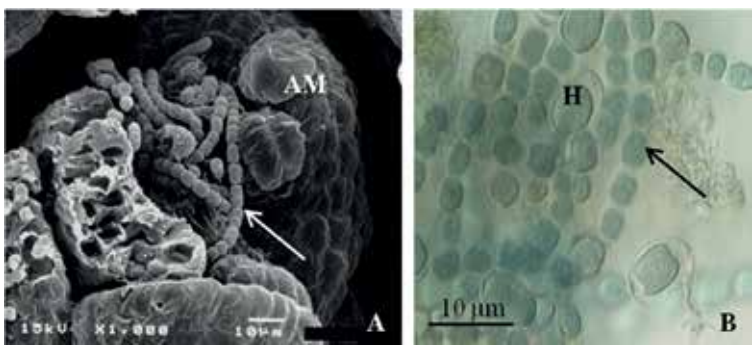

Figure 4. Filaments of the cyanobiont *A. azollae* in the sporophyte of *A. filiculoides*. (A) Filaments of vegetative cells (arrow) on the apical meristem (AM). (B) Filaments with vegetative cells (arrow) and heterocysts (H) in the mature leaf cavities.

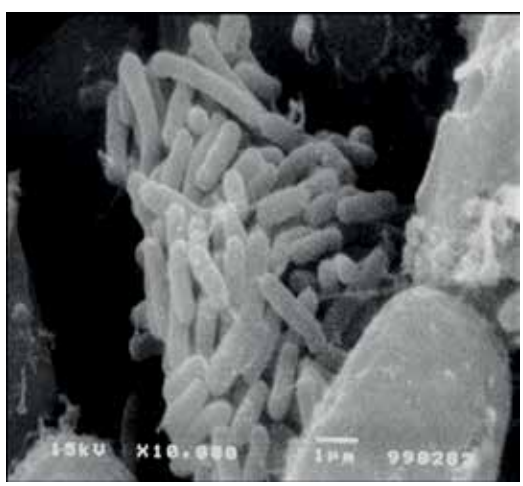

Figure 5. Bacteria inhabiting mature foliar cavity of the *Azolla* sporophyte.

Bradyrhizobium, *Caulobacter fusiformis*, *Flavobacterium ferrugineum*, *Pseudomonas*, and *Xanthomonas* [28–35]. At first, the bacteria that inhabit the foliar cavities were not considered as nitrogen-fixing, but the presence of nitrogenase was detected in some bacteria [36], but their role and importance in the symbiosis are not clear.

3. Applications

The world distribution, the resilience to several environmental conditions of light and temperature, and the formation of thick mats when the environmental conditions are optimal make the *Azolla* symbiosis a good candidate to be used in agriculture as biofertilizer, to the phytoremediation of contaminated water, and as nutritional supplement, among others.

3.1. Biofertilizer

In a world of increasing demand for biofertilizers especially nitrogen for a high productivity in agriculture, the use of industrial fertilizers increases not only the production costs but also the environmental impacts of the runoff with a high content of nitrogen to the water bodies and their eutrophication. Therefore, a system that reduces the costs and allows a more green agriculture without loss of production is desirable and welcome. Although the fuel crisis in 1970 of the twentieth century stimulates the finding of new alternatives and sources of fertilizers to be used in agriculture, nowadays only a few countries such as India and China still do research for the use of *Azolla* as biofertilizer.

The high rate of nitrogen fixation by the cyanobiont *A. azollae* and thus the content of nitrogen of the fern [5–7, 37] make this symbiosis suitable for use as biofertilizer, thus limiting the use of synthetic nitrogen.

Azolla is used for a long time in Asian countries as crop biofertilizer especially in paddy fields and also more recently in Africa [5–7, 37]. But, the use of *Azolla* can be limiting since large amounts of this fern are necessary. In controlled conditions of nutrients, light, and temperature, *Azolla* can grow and provide sufficient amount of biomass [38, 39]. Another way to have a large biomass is growing *Azolla* in treated domestic effluents, but the content of nitrogen, phosphorous, and heavy metals must be below the admitted limits. That way, the fern does not bioaccumulate those compounds above the limits and hence *Azolla* can be used to fertilize soils [40]. For large scale production of *Azolla* biomass, ponds, ditches, channels, small creeks, and tanks, among others can be used with costs of less than one dollar per week [7].

Azolla biomass can be used in rice fields as partial or total replacement of synthetic fertilizers because this fern can accumulate up to 2–3 Kg N/ha/day [41]. But, the sole use of *Azolla* biomass to fertilize soils is not enough to cover the high demands in nitrogen uptake by crops and thus it is always necessary to use chemical fertilizers [7, 42–46]. However, since it is necessary to use less amount of synthetic fertilizers, the production costs become lower.

When *Azolla* is applied in the soil of paddy fields, there is an increase in income of grain yield, caryopsis, straw, and dry matter [7, 42, 47]. However, the method of incorporation of the fern

in rice fields seems to be important. So if *Azolla* is included in the soil as co-culture (dual crop), before transplanting rice, the biomass of rice grows faster in this stage, but it needs synthetic fertilizer for the grain production when the rice needs more nutrients [48, 49]. The amount of nitrogen in caryopsis increases after the application of *Azolla* in the intercropping system (between cultures) [50]. With the incorporation of 40 Kg N/ha as dried *A. filiculoides* into the soil, the rice grain yield was similar to the application of an equal quantity of sulfate of ammonia. However, it should be around 228 Kg N/ha of *Azolla* to equal the income obtained from 160 Kg N/ha of ammonium sulfate [37, 41]. Also, the geometry of rice fields is important [51]. So, the time and quantity of *Azolla* incorporation into the paddy fields depend on whether the farmer wants high nitrogen at the beginning of the culture or high amount of nitrogen when the rice blossoms and at the formation of the grain, thus having a higher grain yield.

Although research was mainly made in rice, this fern can also be used as biofertilizer on other plants such as water bamboo (*Zizania aquatica*), taro (*Colocasia esculenta*), and wheat (*Triticum aestivum*) [6, 7, 49, 52]. But, there are studies regarding other crops.

The incorporation of *Azolla* in the soil also improves the physical properties of soil such as the organic and nitrogen content, the availability of phosphorus, soil texture, pH, and porosity, among other soil properties [7, 46].

Yet, in a modern agriculture where a high crop yield is necessary, a great amount of *Azolla* that almost replaces the chemical fertilizer needed for the crop production is required. The traditional way to produce biomass, while valid, is not suitable. So, the domestication of this fern through the induction of sporulation, the collection of spores, storage, and their germination to have more biomass is needed. This is a not well-known process and difficult to obtain in controlled conditions, but the recent sequencing of the RNA of the sporocarps and sporophyte of *A. filiculoides* is a major breakthrough [53].

3.2. Phytoremediation

The continuous degradation of water bodies due to the continuous discharge of contaminants (heavy metals, nitrogen, dyes, cyanotoxins, and others) drove researchers to new remediation methods using plants.

The presence of heavy metals in the environment (soil or water) poses health treats since they cause intoxication and several diseases. The seven species of *Azolla* can remove by uptake and accumulate from 20 to 95% of a vast array of heavy metals such as arsenic [54], mercury [55, 56], zinc [57–62], lead [57, 61–64], chromium [56, 65, 66], copper [67–69], gold [70, 71], strontium [72], uranium [67], cadmium [61, 62, 67, 69], and nickel [61, 62].

The use of *Azolla* to the phytoremediation of soils and water is due to the high ability to uptake and chelate and due to biosorption of the compounds. In fact, the high biosorption capability of *Azolla* was used to make a biofilter with packed dried *Azolla* in order to provide an efficient tool to decontaminate industrial and domestic effluents [73, 74]. However, the efficient uptake of heavy metals by dried *Azolla* depends on time, the flux of the effluent, pH, and temperature, and *Azolla* species is turning difficult to make an optimized and efficient system to be applied in large-scale decontamination of wastewaters. Another application is the recovery of heavy

metals, which can be made by electroplating or by using solvents and reusing them. This method could be used in small or large scale to diminish the depletion of the natural resources [58, 59, 62, 65, 68]. However, this technology was only tested in the laboratory.

The research on *Azolla* for the treatment of domestic wastewater has also been hypothesized. For instance, *A. filiculoides* can grow in a sewage pond of a wastewater treatment plant to remove nitrogen and phosphorous [75–77] and thus make the water suitable to be discharged into rivers, dams, and others or to be used as irrigation water. But again, as in the case of heavy metals, the application is hampered by the lack of large-scale research.

In fish aquaculture, it is usual to add sulfadimethoxine to prevent diseases in animals but the not absorbed fraction of this antibiotic is excreted through the fish feces and thus contaminates the water. The decontamination of water can be performed using *A. filiculoides* since it absorbs (until 88.5%) and degrades this antibiotic [78] and thus cleans the water, which can be used to irrigate farmland or can be dumped into the normal sewage, and the wastewater treatment plants will no longer need to remove this antibiotic from the water.

Another problem is the discharge of dyes from the textile industry, which contaminates water bodies such as rivers. In laboratory studies, the dyes Acid Red 88, Acid Green 3, Acid Orange 7 [79], and Basic Orange [80] can be removed from water up to 80% by dried *A. filiculoides*.

In most recent years, the water blooms of harmful cyanobacteria that synthesize cyanotoxins (microcystins, cylindrospermopsins, and others) have been increasing due mainly to water eutrophication. Some authors hypothesize that the *A. filiculoides* could uptake and remove those cyanotoxins from the water. However, the fern did not uptake nor accumulate microcystins [81] and cylindrospermopsin [82], making this fern not suitable to the phytoremediation of those environmental contaminants.

3.3. Food

Azolla has been studied for its possible use as feed not only for livestock but also for humans since it is rich in fibers, proteins, fatty acids, amino acids, polyphenols, vitamins, minerals, and others [83–90]. It can also be combined with other foods to create balanced diets. Although *Azolla* is rich in amino acids, methionine and cysteine are present in small amounts [83–86] meaning that the diet must include another source of these two essential amino acids.

Another aspect to consider is the digestibility and smell of *Azolla*, which can be a feeding deterrent. This fern has good digestibility [83, 85, 91, 92], but considering the smell, fresh *Azolla* has a green, mold odor due to some volatile compounds such as alcohols, ketones, and aldehydes that can contribute to its unpleasant smell [93].

Replacing 20% of the commercial feed by *Azolla* induced a weight gain in chickens and also gain in costs [94, 95]. When feeding laying hens with *A. pinnata* mixed with other food sources, there was an improvement in the production of eggs and the color of yolk [96].

Ducks fed with 20% of *A. microphylla* showed no reduction of growth but a lower cost production and higher profits [97].

Regarding fishes, the investigations have been focused on *Oreochromis*, *Tilapia*, and *Cichlasoma*. The fish *Cichlasoma* consumes preferably *A. microphylla* and *A. pinnata*, while *Oreochromis* consumes *A. filiculoides* [98, 99]. In the case of *Tilapia*, about 20% of the commercial feed can be replaced by dried *A. filiculoides* with fish having a weight gain similar to their feeding only with commercial fish feed [88]. However, *Oreochromis* can be fed with up to 40% of *Azolla* and still gained weight with lower production costs and higher profits [100]. Moreover, black tiger shrimps (*Penaeus monodon*) fed with meals with 40% of *Azolla* increase their weight, meaning that this can be an alternative to soybean meals [101].

Although *Azolla* can be useful for human consumption, there are very few reports about it. This fern can be integrated into soups and meatballs [6, 7] and pancakes [102], which proved to be acceptable in terms of taste and smell. A more extensive research with *Azolla* for human ingestion was made for space travels, which reveals that it would be beneficial to include steam sterilized leaves and roots in the space diet of astronauts to fulfill the human nutritional requirements [103, 104].

4. Future perspectives

The research on *Azolla* is vast and has been made for several decades, but only some countries such as India, China, and other Asian countries try to apply the outcomes of this research to benefit populations and especially agriculture practices. One of the problems is the use of *Azolla* collected from the environment for biofertilization and as a food supplement since if it can grow in polluted water bodies and since *Azolla* can accumulate contaminants such as heavy metals this would be a health problem due to human exposure to those contaminants. So, the strategy to follow depends on the ultimate use of *Azolla*.

If the fern is only used in the phytoremediation of water, the local populations can send this fern to an industry to recover the heavy metals and receive a monetary compensation or small villages can build a small recovery factory and sell the heavy metals to advanced factories. This would help in the economy of these small populations and also the environment. However, the technology to recover the heavy metals from this fern was only tested in the laboratory.

However, if the purpose is to include the fern as biofertilizer and/or as food for livestock or humans, there are two approaches:

(1) Since a continuous supply of *Azolla* devoid of contaminants is necessary, it will be better to grow *Azolla* in a small water pond. The water of the pond should be analyzed for the presence of harmful contaminants;

(2) If it is decided to use the *Azolla* that grow in water bodies, the water and fern must be analyzed in terms of contaminants since if it surpasses the legal amounts (provided by each country, WHO, FAO, or other) it cannot be used. For that reason and given that in many countries the populations are far away from cities or do not have scientific resources, it would be useful to discover a cheaper, simple, and rapid method to detect such compounds.

Another approach is the implementation of integrated farms with the cultivation of *Azolla*, fish in aquaculture, and agriculture. The water from fish tanks can be cleaned from any contaminants with *Azolla*, and in turn, this water can be used to grow *Azolla* or to irrigate crops, and *Azolla* can be used to feed fish or other animals and also humans. However, this means a good availability of water, which in many tropical countries especially in Africa is not possible due to severe dryness.

In conclusion, although many researches regarding the potential use of *Azolla* as biofertilizer, food, and phytoremediation and applications are made by farmers in India, China, or Vietnam, there are still many gaps and the research still does not really meet the needs of the populations of the countries that might benefit from this natural tool. For the effective application of this fern in the field, probably it will be beneficial to have a partnership with FAO, which has many people in the field of many countries and drives the research to fulfill specific demands from populations.

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Chapter 3

Plant Genetic Control over Infection Thread Development during Legume-Rhizobium Symbiosis

Anna Viktorovna Tsyganova and
Viktor Evgen'evich Tsyganov

Additional information is available at the end of the chapter

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Abstract

Legumes possess by unique possibility to interact with soil proteobacteria, known as rhizobia, forming on the roots the special organs called symbiotic nodules, where nitrogen fixation takes place. To form the nodule, rhizobia should penetrate inside root tissue, where they colonize a nodule primordium, formed from the reactivated root cells. One of the ways of root infection by rhizobia occurs via formation of a transcellular tubular structure, termed infection thread (IT), which grows through the cytoplasm by apical deposition of primary cell wall material. Numerous mutants impaired in the infection thread development were obtained in different legumes. Genetic analysis has revealed that mutants belong to different complementation groups; this means the existence of precise genetic control over infection thread development. Moreover, it was suggested that infection and nodule organogenesis are regulated with independent but coordinated genetic programs. Using the model legumes, a set of plant genes, controlling infection thread development was identified. These genes encode transcriptional factors, LysM receptor kinases, E3 ubiquitin ligases, SCAR/WAVE actin regulatory complex, nitrate transporter, remorins, flotillins, proteins involved in membrane biogenesis and traffic, and some other components. In this review, we briefly summarized our current knowledge about genetic control over developmental processes associated with infection thread.

Keywords: symbiosis, nitrogen-fixing nodule, infection, cytoskeleton, symbiotic mutants

1. Introduction

As a result of the interaction of leguminous plants and soil bacteria, collectively called rhizobia, a specialized new plant organ, the symbiotic nodule, is formed. The interaction is based on the exchange of signal molecules between the plant and bacteria, resulting

in the coordinated expression of the symbiotic genes of both partners. On the plant side, flavonoids serve as the primary signal, and lipo-chitooligosaccharides, called Nod factors, on the part of rhizobia [1]. The perception of Nod factors by the plant is mediated with the specific receptors. They are represented by transmembrane serine/threonine receptor-like kinases with extracellular domains containing three LysM motifs [2, 3]. In *Lotus japonicus*, the receptors are encoded by the genes *Nod factor receptor kinase 1* and *5* (*LjNfr1* and *LjNfr5*). In *Medicago truncatula* and *Pisum sativum* orthologous pairs of the genes *Nod factor perception* (*MtNfp*) and *PsSym10* [4] and *LysM domain-containing receptor-like kinase* (*MtLYK3*) [5] and *PsSym37* [6] were revealed. The receptors form heteromeric complexes, and it is assumed that not all the components of such complexes have been identified. In addition to the previously described receptors, localized on plasma membrane, a receptor-like kinase with leucine-rich repeat (LRR) encoded by the genes *DOES NOT MAKE INFECTION2* (*MtDMI2*) in *M. truncatula* [7], *SYMBIOSIS RECEPTOR-LIKE KINASE* (*LjSYMRK*) in *L. japonicus* [8], and *PsSym19* in pea was identified [8, 9].

It was shown that MtDMI2 interacts with 3-hydroxy-3-glutaryl coenzyme A reductase 1, an enzyme of mevalonate biosynthesis [10]. Mevalonate is a secondary messenger that transmits a signal from the components of the signal pathway localized on the plasma membrane to the nucleus, resulting in the generation of nuclear and perinuclear calcium oscillations [11].

Calcium oscillations in the nucleus are generated by the functioning of several channels localized on the nuclear membrane. The complex of MtDMI1 and MtCNGC15 regulates the sustained calcium oscillation [12], and calcium ATPase MtMCA8 returns calcium back to the lumen of the nuclear envelope [13]. Calcium oscillations in the nucleus activate calcium and calmodulin-dependent protein kinase LjCCaMK (MtDMI3 in *M. truncatula*), which phosphorylates the transcription factor LjCYCLOPS (MtIPD3 in *M. truncatula*) to stimulate the expression of symbiotic genes [14, 15]. It is likely that the complex MtDMI3 and MtIPD3 can be linked to the complex of GRAS domain-containing transcription factors MtNSP1 and MtNSP2 via the MtDELLA transcription factor, which is also necessary for the expression of symbiotic genes [16].

The first morphological changes observed in the action on legume plants of Nod factors are deformations and curling of the root hairs. They are accompanied by an active reorganization of the actin and tubulin cytoskeleton [17–22]. The further stage of infection is the formation of an infection thread (IT), a special tubular structure that ensures the penetration of rhizobia into the root [23, 24] (**Figure 1a–d**). The process of the IT development will be discussed in detail in the subsequent sections of the review. In parallel with the induction of the infection process in the root, cortical cell divisions are activated, resulting in the formation of nodule primordium [19] (**Figure 1b**). When the IT reaches the primordium cells, specialized outgrowths of the IT, devoid of cell wall and surrounded by only a plasma membrane, called infection droplets are formed [23] (**Figure 1e**). From these outgrowths, bacteria are released into the plant cell cytoplasm (**Figure 1f**). Bacteria are separated from the cytoplasm by a peribacteroid (symbiosome) membrane. As a result of differentiation of rhizobia, a specialized nitrogen fixation form, called bacteroid, is formed. A bacteroid surrounded by a peribacteroid membrane is known as a symbiosome [25].

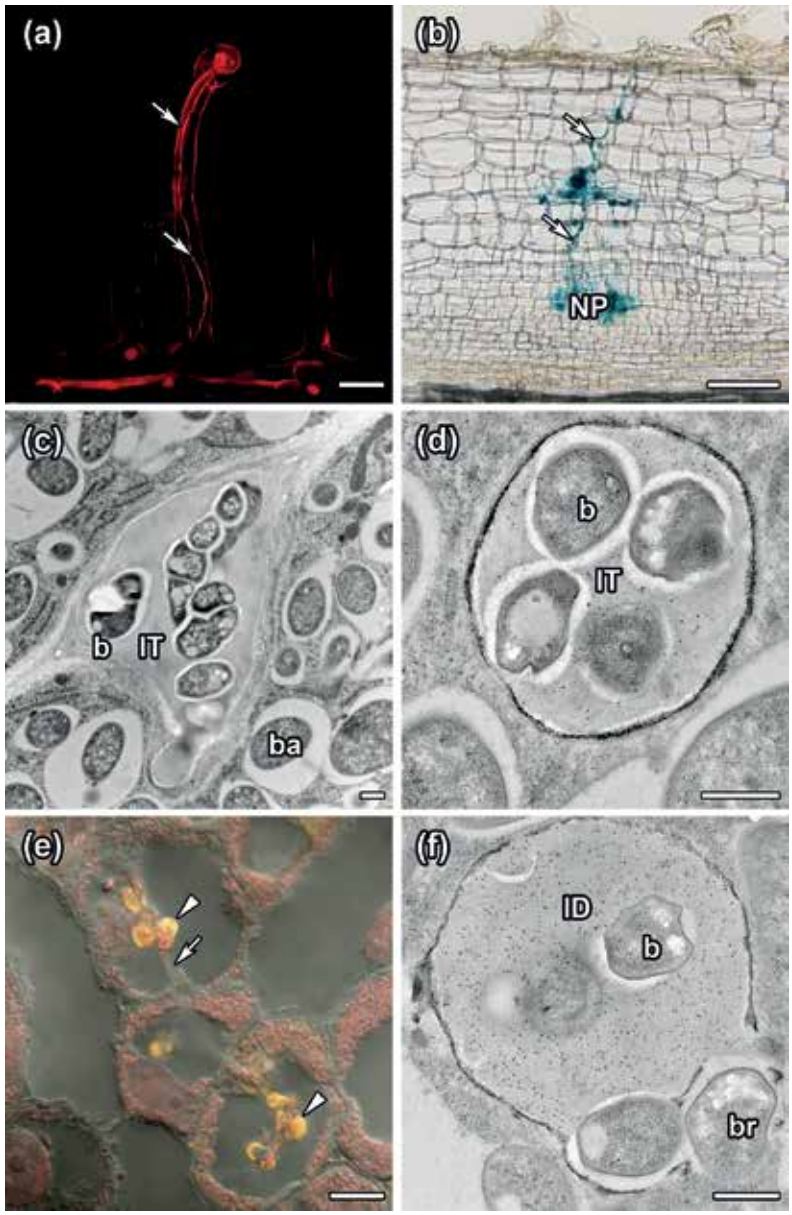

Figure 1. IT and infection droplet development in pea nodule. (a) IT inside a root hair, (b) IT colonizes a nodule primordium, (c) intercellular IT, (d) intracellular IT, (e) IT with infection droplets, and (f) infection droplet with bacterium being released. Arrows indicate infection thread (IT); arrowhead indicates infection droplet (ID). b, bacterium; ba, bacteroid, br, bacterium being released; NP, nodule primordium. (a) Bacteria visualized with propidium iodide, red channel; (b) bacteria visualized using reporter gene *gusA*; (d) matrix of IT is immune-gold labeled with antibody MAC265; (e) matrix of infection droplet is labeled with antibody MAC265, yellow channel; and (f) matrix of infection droplet is immune-gold labeled with antibody MAC265. (a, e) Confocal microscopy, (b) light microscopy, and (c, d, f) transmission electron microscopy. Scale bars (a) = 25 μm, (b) = 0.2 mm, (c, d, f) = 500 nm, and (e) = 10 μm. Images (a, e) courtesy of A.B. Kitaeva and (b) courtesy of V.A. Voroshilova.

2. Plant genetic control over the IT development

2.1. Identification of mutants impaired in IT growth

First abnormalities of IT growth were identified during the analysis of natural populations of red clover, the recessive homozygote $i_e i_e$. formed hypertrophied ITs [26]. Pioneering studies aimed at identification of pea symbiotic mutants after experimental mutagenesis allowed to identify the first mutants impaired in IT growth. Mutants in several independent genetic loci were characterized with interruption of IT growth at the early stages of nodule development, leading to the absence of nodules (Nod^- phenotype) [27–31] (**Figure 2a**, **b**). Further studies revealed pea symbiotic mutants blocked at the later stages of IT development, when nodules are formed, but they are ineffective (Fix^- phenotype) [32, 33]. The comprehensive genetic and phenotypic analysis revealed the significant amount of different genetic loci, controlling IT development in different crop legumes [34, 35]. In pea, 11 different loci, involved in genetic control over IT growth, were identified [36]. Moreover, based on performed phenotypic analysis, the existence of two genetic programs involved in nodule formation, infection and nodule organogenesis, and the coordination between the development of both programs were suggested [37].

However, the molecular products encoding by these loci have not been identified for a long time. The significant progress in identification of genes, controlling IT development, was achieved using two model legumes: *M. truncatula* and *L. japonicus,* and it is allowed to identify the sequences of some previously revealed loci in pea [6, 38–40].

2.2. Formation of growth chamber (pocket)

Root hair curling leads to entrapment of a single cell of rhizobia. Bacterium inside root curling actively divides, which leads to the formation of a microcolony, which in turn develops within the infection chamber (pocket), gradually increasing in size, and which is accompanied by a reorganization of the infection chamber. For *M. truncatula,* accumulation of the exocytosis marker Vesicle-Associated Membrane Protein 721e (MtVAMP721e) during the reorganization of infection chamber was observed [41]. Transport of vesicles to the membrane surrounding the infection chamber begins several hours after the end of the root hair curling. Accumulation of the infection-associated secreted protein MtENOD11 around the entrapped bacteria is probably related to the plasticity of the cell wall necessary for radial expansion and subsequent initiation of polar growth of the IT. Continuous deposition of new membranes and extracellular materials, including MtENOD11, from 10 to 20 hours after the root hair curling leads to a radial enlargement of the chamber and its transformation into a globular, IT-like compartment. Thus, the initiation of the IT should be considered as the tip growth of the expansion forming from an IT-like compartment [41].

2.3. Cytoskeleton rearrangements in a root hair

The initiation of IT growth is accompanied by a reorganization of the cytoskeleton elements and the movement of the nucleus in the root hair cell. It was shown that the microtubules

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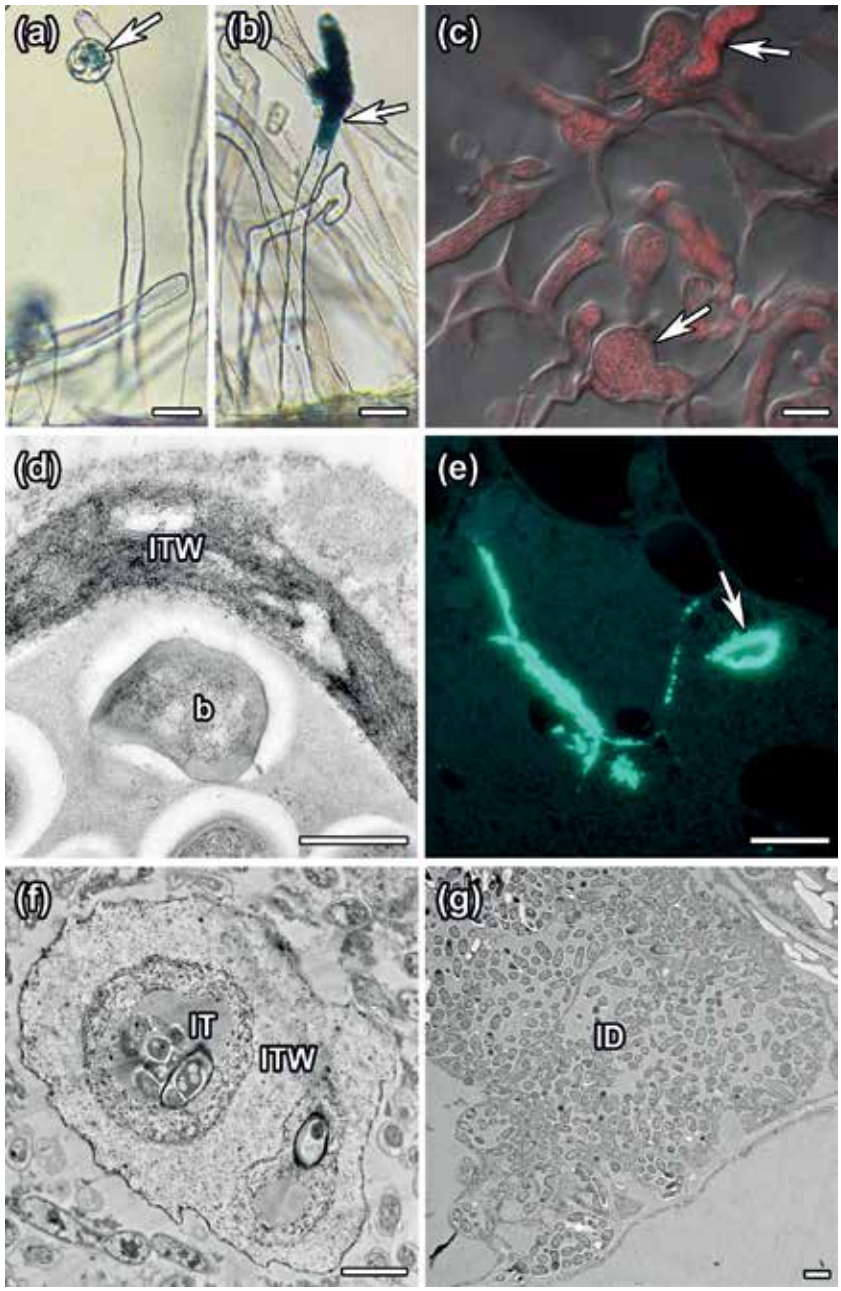

Figure 2. Mutant abnormalities in infection thread and infection droplet development in pea nodule. (a) Mutant in the gene *Pssym37*, IT is blocked in a root hair; (b) mutant in the gene *Pssym36*, IT is blocked in a root hair; (c, d) mutant in the gene *Pssym33*, "locked" thickened ITs without bacterial release; (e, f) mutant in the gene *Pssym42*, thickened walls around ITs, which are enriched with callose; (g) mutant in the gene *Pssym40*, hypertrophied infection droplet. (a, b) Bacteria visualized using reporter *gusA*; (c) bacteria visualized with propidium iodide, red channel; and (e) callose is visualized with aniline blue. (a, b) Light microscopy, (c) confocal microscopy, (e) fluorescence microscopy, and (d, f, g) transmission electron microscopy. Scale bars (a, b) = 30 μm, (c) = 10 μm, (d) = 500 nm, (e) = 20 μm, and (f, g) = 2 μm. Images (a, b) courtesy of V.A. Voroshilova and (c) courtesy of K.A. Ivanova.

form a dense network surrounding the growing IT and the connecting tip of the IT with the nucleus, whereas the longitudinal microtubules were parallel to the IT. The nucleus, with the growth of the IT, moved from the root hair curling to its base [19].

Changes in the location of the nucleus are also observed in the cells of the outer cortex that are underlying to the cells of the root hairs. In such cells, the nucleus occupies a central position, and the cytoplasm forms elongated strands oriented parallel to the direction of growth of the IT that penetrates and grows in these strands. Therefore, these cytoplasmic strands are called "preinfection threads" [42]. Preinfection threads were surrounded by longitudinal microtubules connecting the different ends of the cell [19]. In general, it is assumed that the microtubule network provides polar growth and serves as a template for the formation of an IT [43]. However, specific genes that control the reorganization of microtubules during the initiation and growth of the IT have not been identified.

The role of actin microfilaments in the development of an IT was studied using the analysis of mutant genes involved in the regulation of their functioning. These mutants were characterized by pleiotropic effects; in particular, along with defects in the nodule development, the development of trichomes was disrupted [44]. In *L. japonicus*, the gene *ACTIN-RELATED PROTEIN COMPONENT* (*LjARPC1*), which encodes the subunit ACTIN-RELATED PROTEIN2/3 (APR2/3) complex controlling the nucleation process of Y-shaped branched actin microfilaments, was identified [45]. Expression of this gene is observed in all organs of the plant. Mutants are characterized by a decrease in the number of microcolonies formed in curled root hairs, as well as a decrease in the number of ITs initiated, which were mostly aborted in the root hairs. Herewith, "empty" nodules without the ITs were formed. In the mutant *Ljarpc1*, differences in the organization of F-actin from the wild type were observed only in short root hairs. The transverse organization of actin microfilaments was more pronounced in them, and in mature root hairs, the actin microfilaments were located longitudinally, similarly to the wild type. The negligible abnormalities in F-actin can be explained that Y-like microfilaments represent a minor fraction of the actin cytoskeleton. However, abnormalities in this fraction of F-actin lead to significant disturbances in the development of IT, which may indicate that a network of Y-shaped actin microfilaments can participate in the initial selection of the site of initiation of IT and/or the subsequent management of endoplasmic microtubules, ensuring preservation of the growth polarity [45]. *L. japonicus* mutants *Ljpir1* (*121F-specific p53 inducible RNA*) and *Ljnap1* (*Nck-associated protein 1*) [22], as well as *M. truncatula* mutant *Mtrit-1* (*required for infection thread*) (*MtRit-1* is orthologous to the gene *LjNap1* [46]), were characterized by a similar phenotype. These genes encode the components of SCAR/WAVE (suppressor of the cAMP receptor defect/WASP family verpolin homologous protein) complex that activates the APR2/3 complex. In mutants, *Ljnap1* and *Ljpir1*, disorganization of the actin cytoskeleton, the formation of transversely oriented microfilaments in the root hairs, and the absence of reorganization of F-actin in response to inoculation (in particular, the accumulation of fine F-actin at the tip of the root hair) were observed. Also in these mutants, a decrease in the number of microcolonies in curled root hairs and a disintegration of ITs were observed, and only rare ITs reached the base of the root hair cell [22]. Thus, it is obvious that the reorganization of the actin cytoskeleton plays a leading role in the initiation and growth of the IT.

Later, another gene of *L. japonicus*, *SCAR-Nodulation (LjSCARN)* encoding a component of the SCAR/WAVE complex was identified [47]. Mutants in the *Ljscarn* gene were blocked at the stage of initiation of IT growth, after the formation of an infection chamber. In some hairs, the release of bacteria into the cytoplasm of the root hair cell was observed. Sometimes, the development of ITs was initiated, but they were aborted at the base of the root hair cell. Similarly, mutants in the genes *Ljnap1* and *Ljpir1* and mutants in the *Ljscarn* gene formed empty uninfected nodules. In contrast to the previously described mutants in the genes encoding the components of the SCAR/WAVE complex, all five mutations studied in the *Ljscarn* gene did not affect the development of trichomes. It was shown that the expression of *LjSCARN* is activated by the transcription factor LjNIN when it is bound to the *LjSCARN* promoter. Unlike the mutants *Ljarpc1*, *Ljnap1,* and *Ljpir1*, the mutations in the *Ljscarn* gene showed no disturbances in the organization of the actin cytoskeleton at the early stages of development, and it is likely that LjSCARN is needed at later stages of the reorganization of the cytoskeleton in the development of the IT [47].

2.4. The role of initial components of the Nod factor signaling pathway

Numerous studies have shown that the reception of Nod factors is important not only at the earliest stages of symbiosis development but also for the development of the infection process [40]. For example, *Mtlyk3* mutants showed early responses to the action of the Nod factor, but the development of the infection process was blocked [5, 48]. Mutants in the orthologous gene *Pssym37* was also characterized with the interruption of IT growth [6, 37] (**Figure 2a**).

In *M. truncatula*, the suppression of the level of expression of the gene *SYMBIOTIC REMORIN 1* (*MtSYMREM1*) led to the formation of abnormal nodules with a reduced meristem and an increase in the number of ITs that highly branched, formed "sac"-like structures, but aborted in the outer cortex. All this indicates the loss of the ability to polar growth by ITs. MtSYMREM1 interacts with MtNFP, MtLYK3, and MtDMI2, suggesting that MtSYMREM1 is a scaffold protein, which determines the spatial regulation of receptor complexes during nodule development [49].

In addition to the symbiosis-specific remorin MtSYMREM1, two flotillin-like proteins (FLOT2 and FLOT4) were detected in *M. truncatula* [50]. MtFLOT4 is predominantly localized at the tips of the root hairs inoculated with *S. meliloti*, which is possibly related to its role in the polar growth of the IT. Silencing of the *MtFLOT2* and *MtFLOT4* genes reduced the number of ITs, whereas the number of aborted threads was increased. It was suggested that MtFLOT2 and MtFLOT4 are involved in the primary invagination of the IT in the root hair cell, and MtFLOT4 is also necessary for the growth of the IT [50]. It was shown that in the root hairs, MtLYK3 and MtFLOT4 are localized independently in the absence of rhizobia, but are colocalized after inoculation, while their stabilization in the membrane is observed. It should be noted that later MtLYK3 was localized in the membrane of the IT, which indicates its possible role in the development of infection [51].

Deactivation of the gene *MtHMGR1*, encoding 3-hydroxy-3-glutaryl coenzyme A reductase 1, by RNA interference, led to disturbances in the development of the infection process [10].

In *L. japonicus*, LjCYCLOPS is a substrate that phosphorylates LjCCaMK. Analysis of a series of allelic mutants in *Ljcyclops* revealed that the mutants form nodule primordia, but no further development of nodules occurs. In the mutant *Ljcyclops-3*, the curling of the root hairs was colonized; however, ITs did not develop [14]. At the same time, mutants in orthologous genes *Mtipd3* [52, 53] and *Pssym33* [33, 53] formed nodules with ramified network of thicken IT, from which bacterial release does not occur (**Figure 2c**, **d**). However, a pea mutant, carrying the allele *Pssym33-3*, forms nodules in which infection droplets are formed [54] and bacterial release occasionally occurs [55]. LjCYCLOPS is also involved in the organogenesis of the nodule, being a transcription factor activating the *LjNIN* gene [56].

LjNIN (*Nodule inception*) was the first symbiotic gene whose nucleotide sequence was detected in legumes [57]. Mutants in this gene were characterized by the lack of initiation of cell divisions of the inner root cortex and pericycle, a characteristic reaction in response to inoculation by rhizobia. At the same time, curling of root hairs was observed in mutants, and their numbers were greatly increased, in comparison with the wild type. *LjNIN* encodes a transcription factor with a DNA-binding RWP-RK domain that is actively expressed not only in young but also in mature nodules [57]. In pea, an orthologous *PsSym35* gene was identified; three allelic mutants in this gene were characterized by intense excessive root hair curling and lack of cortical cell divisions [38, 58]. In *M. truncatula*, two mutants were identified: *Mtnin-1* and *Mtnin-2*, which were also characterized by excessive root hair curling and lack of division in the root cortex; although in the mutant *Mtnin-2* the development of ITs was occasionally initiated, however, they were aborted in the root hairs, indicating that *Mtnin-2* is a weak allele [59]. Thus, analysis of the *NIN* mutants obtained in various legume species showed that it occupies a leading position both in the development of infection and in the organogenesis of the nodule [59].

Genetic analysis revealed in *M. truncatula* two loci *NODULATION SIGNALING PATHWAY 1 and 2* (*MtNSP1* and *MtNSP2*), functioning after MtDMI3 [60, 61]. Mutants in these genes are characterized by the presence of deformations of the root hairs and the induction of calcium oscillations in response to inoculation with rhizobia, but cortical cell divisions and IT growth are completely absent [60–62]. These genes encode transcription factors belonging to the GRAS family and localized in the nucleus [63, 64]. Both genes are characterized by constitutive expression, the level of expression of *MtNSP1* is not altered by inoculation with rhizobia [63], and *MtNSP2* is increased [64]. In *L. japonicus*, the orthologous genes *LjNSP1* and *LjNSP2* were identified [65, 66]. The level of their expression increased after inoculation with rhizobia (although a slight decrease was observed by day 2 after inoculation) [66]. The *LjNSP2* gene was shown to be suppressed in mature nodules [65]. *PsSym7* is an ortholog of the *MtNSP2* gene [39, 64] and *Pssym34* is an ortholog of the *MtNSP1* gene [67]. It has been shown that MtNSP1 and MtNSP2 form a complex that is associated with the promoters of the early nodulin genes (in particular, the *MtENOD11* gene). *In vitro*, MtNSP1 binds to the *MtENOD11* promoter via the AATTT *cis*-element, but *in vivo* such an association requires the participation of MtNSP2 [68]. In MtNSP1, the LHRI and LHRII domains are involved in binding to DNA, and in MtNSP2, the LHRI domain is required for binding to MtNSP1. Moreover, the complexes MtNSP1 and MtNSP2 activate promoters *MtNIN* and *MtERN*, encoding the other transcriptional factors [68].

In *M. truncatula*, the transcription factor ERF REQUIRED FOR NODULATION 1 (MtERN1) belongs to the ERF family (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR) containing the highly conserved AP2 DNA-binding domain [69]. Mutations in the *Mtern1* gene lead to a block of infection after the formation of the microcolony of rhizobia in a curled root hair, although occasionally ITs are initiated, but they are aborted in the root hairs [69]. Mutants in the homologous gene *Mtern2* lead to the formation of nodules with signs of early senescence. The double mutant line *Mtern1-1 Mtern2-1* was completely incapable to initiate the development of infection and nodule organogenesis, indicating a functional redundancy of both transcription factors. At the early stages of infection, the functioning of both MtERN1 and MtERN2 is important, whereas at the late stages of development only MtERN1 functions [70]. In *L. japonicus*, *LjERN1* gene, an ortholog to the *MtERN1*, was identified; the ortholog of the *MtERN2* gene is absent [71]. Mutants of the *Ljern1* gene are characterized by a block at the stage of penetration of the IT into the root cortex and undeveloped nodules (bumps) are formed. In mutants, the frequency of the formation of ITs in the root hairs was reduced, and mutations influenced the frequency of root hair deformations, characterized by the formation of characteristic deformations in the form of balloons. It should be noted that in a mutant, which carries the weak allele of the gene, nodules with penetration of ITs were observed. The *LjERN1* gene was characterized by constitutive expression in the roots, and it was enhanced by inoculation with rhizobia. The *LjERN1* expression is induced by LjCYCLOPS and LjNSP2, as its level was reduced in the mutants in these genes. At the same time, in *Ljnin* mutant, the expression of *LjERN1* did not change, which indicates that LjERN1 and LjNIN can play a different role in the positive regulation of root hair deformations and the growth of the ITs [71]. In an another study, it was shown that LjCYCLOPS can bind to the *cis*-element of the *CYC-Response Element* in the *LjERN1* promoter region, which has a similar sequence to the *cis*-element in the *LjNIN* promoter. Activation of *LjERN1* by LjCYCLOPS was observed in the presence of an autoactive form of LjCCaMK^{314}, indicating a positive regulation of *LjERN1* by the LjCCaMK-LjCYCLOPS complex [72].

Recently, another component of the Nod factor activated signaling pathway, MtDELLA proteins, has been identified, which is involved in the activation of genes that control the infection and organogenesis of the nodule [16]. DELLA are known as transcriptional repressors, whose degradation by the 26s proteosome complex is induced by gibberellic acid [73]. Indeed, treatment of *M. truncatula* plants with gibberellic acid led to a decrease in the number of nodules and ITs. It was demonstrated that MtDELLA can be a component of a large complex, including MtDMI3-MtIPD3 and MtNSP2-MtNSP1 [16].

2.5. Cell wall remodeling

In addition to the reorganization of the cytoskeleton elements for the growth of the IT, the cell wall has to be re-modeled, as the IT moves along the root hair, enzymes for the synthesis of the cell wall, structural proteins, and components of the cell wall are constantly delivered to its tip. When the IT reaches the base of the root hair cell, the tip of the IT wall should fuse with the cell wall of the root hair. In addition, to promote the IT between the root hair cell and underlying subepidermal cell, local cell wall degradation in both cells must occur, followed by a new synthesis of the cell wall [23].

It was shown that one of the enzymes involved in rearrangement of the cell wall is pectate lyase [74]. In *L. japonicus*, a mutation in the gene *nodulation pectate lyase* (*Ljnpl*) leads to the blockage of infection in the root hairs; most often after the formation of an infection chamber, only rare ITs grow to the cell wall of the next cell. At the same time, white nodules formed on mutant roots, most of which were uninfected. The *LjNPL* gene was activated by the transcription factor LjNIN. Other enzymes involved in the degradation of cell wall elements during nodule development, such as pectin-methyl esterase, have also been identified [74].

A study of the growth of ITs *in vivo* revealed that the colonization of IT by rhizobia constitutes a combination of movement of rhizobia along the thread, the formation of gaps between short rows of rhizobial cells, and the subsequent division of these cells, which fill the gaps [75]. To move inside the IT, rhizobia use a mechanism of sliding mobility, which is characterized by a joint movement of the bacterial population. It is likely that rhizobial exopolysaccharides play an important role in providing this movement, because it has been shown that rhizobia in an IT are surrounded by an exopolysaccharide capsule [76]. The lumen of the IT is filled with a matrix containing plant extracellular glycoproteins [77]. It was suggested that in zone of the IT growth, its colonization by rhizobia depends on the transition of the matrix of the thread from the fluid gel state in which the rhizobia are able to move and divide into a solid state [23]. This transition is observed about 60 μm from the IT tip and is provided by cross-linking of root tyrosine residues of root nodule extensin as a result of the action of hydrogen peroxide [78]. It is likely that in the case of an ineffective infection, for example, as a result of mutations in the exopolysaccharide genes, the level of hydrogen peroxide increases, which leads to abnormal matrix solidification and abortion of the infection [23].

2.6. Bacterial colonization as the limiting stage of IT growth

Bacterial colonization appears to be the limiting stage in the IT growth [75]. At the beginning of growth, the IT contains rare bacteria, and there is a free space in the tip, which is probably due to the fact that the potential rate of lengthening of the IT is higher than the rate of progression and division of bacteria. Therefore, the growth of the IT is a discrete process in which the stage of rapid growth of the tip of the IT provided by the plant resulting in the formation of free space is replaced by the stage of its colonization as a result of sliding mobility and division of rhizobia [75]. It is noteworthy that the distance between the tip of the root hair and the rhizobia does not exceed 10 μm, which indicates a signal exchange between the symbionts. In such signaling molecules, one can assume both Nod factors [23] and low molecular weight exopolysaccharides [79].

2.7. The role of exopolysaccharides

Indeed, in *L. japonicus*, a gene recognizing exopolysaccharides was identified [80]. This receptor, which is a receptor-like kinase with 3 LysM-like domains, is encoded by the gene *Exopolysaccharide receptor 3* (*LjEpr3*). The mutants for the *Ljepr3* gene manifested a suppressor effect with respect to *Mesorhizobium loti* strain R7AexoU producing defective truncated exopolysaccharides, forming normal pink and white nodules. At the same time, in the wild-type plants after inoculation with a defective strain producing truncated

exopolysaccharides, small white nodules are formed. In the mutants in the gene *Ljepr3*, the number of ITs also increased when inoculated with the R7AexoU strain, and the infections that progressed through cracks in the epidermis were also observed. This indicates that the function of the LjEPR3 receptor is infection control, both through ITs and through epidermal cracks. Direct binding of exopolysaccharides to the receptor has been demonstrated. Activation of the components of the Nod factor signaling pathway is necessary to activate LjEPR3 [80]. Later, it was shown that LjEPR3 is necessary for the development of an IT not only in the epidermis but also during its growth in subsequent layers of the root in the direction of the nodule primordia, as mutants in the *Ljepr3* gene are characterized by impaired development of the IT at this stage [81].

2.8. The role of other genes in infection process

The active analysis of mutants in model legumes, conducted in recent years, reveals the new genes involved in the control of the development of the IT. In *L. japonicus*, the *LjCERBERUS* gene encoding E3 ubiquitin ligase containing a U-box domain and three WD-40 repeats at the C-terminal was identified [82]. Mutants in this gene form small uninfected nodules, and the infection process stops at the stage of formation of the microcolony (colonization of the infection chamber (pocket)). ITs occasionally developed in short root hairs, but they were aborted in the root hairs, not penetrating the underlying layers of the root cortex. The fact that the roots of the *Ljcerberus* mutant transformed with a construct containing $LjCCaMK^{T265D}$, encoding autoactive form of kinase, formed spontaneous nodules indicates that *LjCERBERUS* is not involved in the organogenesis of the nodules. Nevertheless, with the inoculation of rhizobia, the number of nodules increased significantly, although in such nodules, there was no bacterial release into the cytoplasm of plant cells. Thus, *LjCERBERUS* can play a role not only in the initiation and growth of the IT but also in coordinating the development of the infection process with the organogenesis of the nodule [82]. In *M. truncatula*, an ortholog to the *LjCERBERUS* gene, the *Lumpy Infections* gene (*MtLIN*) was identified [83, 84]. A study of the pattern of expression of this gene showed that in the early stages of nodule development, it is associated with the dividing cortical cells forming nodule primordia, at later times in the central zone of young nodules, and in mature nodules, its pattern was restricted to the infection zone. The obtained results are a good confirmation of the previously put forward hypothesis that the organogenesis of the nodule depends on the degree of development of the infection process [37, 84]. Previously, the role of E3 ligases has been shown in a manifestation of defense reactions; therefore, the possible function of MtLIN in nodule development is the fine regulation of defense responses, by maintaining the precise spatiotemporal activity of target proteins [84]. Later, with the use of the new *Mtlin-4* allele, which forms normal infection pockets, but does not initiate ITs, convincing evidence has been obtained that the initiation of an IT is necessary for the normal development of the nodule [85]. *Mtlin-4* forms nodules (by 60 days after inoculation) with a centrally located vascular bundle [85], like a lateral root, or an actinorhizal nodule [86]. Nodules of *Mtlin-4* were characterized by a modified pattern of cytokinin and auxin markers, indicating that early abortion of IT development leads to disturbances in the regulation of cytokinin and auxin signaling, which leads to an abnormal development of the nodule [85].

In *L. japonicus,* the gene *Nodule Specific RING Finger* (*LjnsRING*) was revealed using the methods of "reverse" genetics [87]. This gene encodes a potential nodule-specific E3 ubiquitin ligase with a RING-H2 domain with a zinc finger. Expression of *LjnsRING* was significantly activated in inoculated roots and nodules. In young and mature nodules, expression was associated with infected cells. Knocking down the gene with RNA-interference led to a serious disruption in the development of ITs, and they were blocked at the stage of penetration from the root hair cells into the cortical cells [87].

Participation in the development of an IT in *M. truncatula* was also shown for another group of E3 ligases, Seven in Absentia (SINA), which, in addition to the RING finger domain have an additional the SINA domain [88]. The increase in the level of transcription of the *MtSINA4* gene was shown during the development of nodules. To reveal the role of SINA proteins, heterologous expression in *M. truncatula* of the gene of *Arabidopsis thaliana SINAT5* and its mutant form *SINAT5DN,* having a dominant negative manifestation, was studied. In *M. truncatula* plants *35S::SINAT5DN*, the number of nodules was reduced, and some of them were ineffective. In such nodules, the infection threads were irregular in shape and formed outgrowths, and the number of bacteria was reduced [88].

In *M. truncatula*, the *Rhizobium-directed polar growth* (*MtRPG*) gene was detected, the *Mtrpg* mutation led to abnormalities in the development of infection chambers. They were formed with a delay, in addition the root hair curling was frequently incomplete and new growth sites were formed [89]. ITs were thickened and slowly growing. Thus, the root hairs of the *Mtrpg* mutant did not respond to colonization with rhizobia by changes in polar growth. At the roots of the mutant, uninfected nodules are formed, although occasionally the formation of pink nodules is possible, but with fewer infected cells. Expression of the *MtRPG* gene is significantly activated by inoculation of the roots with rhizobia, and it is observed in root hairs, in infected root hairs, in emerging primordia, and in developing and mature nodules in the infection zone. The *MtRPG* gene encodes a protein belonging to a new family of plant-specific proteins with a specific "PPR" (RPG-related proteins) domain and coiled-coil domain. It is suggested that MtRPG can be a transcriptional activator regulating genes involved in spatial subcellular reorganization leading to the deposition of cell wall material and membrane material at the sites of new polar growth in the curled tip of the root hair and IT [89].

An important role in the development of an IT was shown for small GTPases of the ROP family. In *L. japonicus*, LjROP6 interacts with the receptor to the Nod factor LjNFR5 in the plasma membrane [90]. After inoculation with *M. loti*, enhancement of *LjROP6* expression in the root hairs, root vascular bundles, nodule primordia, and young nodules was observed. In RNAi plants, the number of ITs growing to nodule primordia is significantly reduced, which indicates that LjROP6 is a positive regulator of IT formation [90]. It was later shown that LjROP6 also interacts with the heavy chain of clathrin CHC1 [91]. The gene of *L. japonicus Clathrin Heavy Chain 1* (*LjCHC1*) is constitutively expressed in all organs of the plant, and its expression is not activated by inoculation with rhizobia. In the genome of *L. japonicus*, there is a *LjCHC2* homologue, and it has been shown that LjROP6 can interact with both LjCHC1 and LjCHC2, exhibiting functional redundancy. Immunocalization revealed the localization of LjCHC1 and LjCHC2 in infection pockets and around ITs, and plasma membrane of plant

cells. Transgenic lines, in which the synthesis of the Hub domain is increased, that led to the disruption of the assembly of light and heavy clathrin chains, formed a reduced number of nodules. A similar effect was provided by knocking down the *LjCHC1* gene by RNA interference. The reduced number of nodules was caused by a decrease in the number of ITs and primordia. It is suggested that LjROP6 induces the transition of the CLC1 monomer from the cytoplasm to the plasma membrane, enhancing the endocytosis of the plasma membrane proteins, and clathrin may be involved in endocytosis of LjNFR5 [91].

In *M. truncatula*, a small GTPase MtROP10 is localized on the plasma membrane of the tips of the root hairs, regulating their growth [92]. Overexpression of MtROP10 resulted in the formation of several outgrowths at the tip of the root hair and in the formation of several microcolonies, but ITs from them did not develop. Also, aborted ITs forming sac-like structures in the root hairs were observed, only a few threads reached the cortical cells, and double threads in the root hair were observed. MtROP10 was localized in the plasma membrane of the root hair outgrowths induced by the action of Nod factors. It was shown that MtROP10 interacts with the intracellular domain of the MtNFP receptor. It is assumed that MtROP10 is temporarily activated in the root hairs by the action of Nod factors, which leads to an ectopic localization of MtROP10 in the plasma membrane at the tip of the root hairs, where it interacts with receptors to Nod factors in lipid rafts, causing changes in the polarity of the tip of the root hairs [92].

M. truncatula Cystathionine-β-synthase-like1 (*MtCBS1*) is also involved in the development of an IT [93]. Mutants in this gene formed inefficient small white nodules. The first abnormalities were observed at the stage of initiation of the IT growth from the infection pocket in the root hair, as a result of which increased in size or multiple microcolonies were formed. The number of ITs reaching the base of the root hair was significantly reduced, and they contained a small number of bacteria or were empty. In later stages of development, the mutant was characterized by an increased infection zone, with only a small number of infected cells, and some of the ITs were swollen. Expression of *MtCBS1* was first observed in the epidermis and in the layers of outer cortex, later with the cells through which the IT passes, and in the nodules in the infection zone and the meristem. Activation of the *MtCBS1* gene with the MtNIN transcriptional regulator was demonstrated. The protein MtCBS1 was localized in ITs and symbiosomes. Based on data on homologous proteins in *A. thaliana*, the function of MtCBS1 in the maturation of the IT wall was suggested [93].

It was shown that the gene of *M. truncatula Nuclear Factor YA1* (*MtNF-YA1*), encoding the transcription factor, for which participation in the development of symbiosis at late stages was shown [94], is also involved in the control of infection development [95]. Expression of this gene was activated already 6 hours after inoculation with rhizobia, and it was observed in curled root hairs, in root hairs with growing ITs, in cortical cells through which the IT passeds, and also in the infected cells. The mutant *Mtnf-ya1* was observed to form a reduced number of nodules with a greatly reduced level of nitrogen fixation. The growth of most ITs was arrested either at the stage of the microcolony, or in the root hair, while they formed sac-like swellings. In the case of the formation of the nodule, the ITs of the mutant were characterized by thinner wall, in comparison with the wild type. Thus, it was suggested that

the transcription factor MtNF-YA1 controls the development of the IT, including its walls [95]. Although the *MtNF-YA1* gene is activated already 6 hours after inoculation with rhizobia, the mutant *Mtnf-ya1* is blocked at a later stage of symbiotic development, compared with other mutants in genes activated by Nod factors [95]. This phenotype was shown to be associated with the presence in the genome of *M. truncatula* of the second *MtNF-YA2* gene exhibiting a redundant activity with the *MtNF-YA1* gene [96]. At the same time, there were differences in the expression of both genes. *MtNF-YA1* was activated in root hairs in plants treated with the Nod factors, and expression of *MtNF-YA2* was also observed in the cortex and the vascular system. Knocking down the *MtNF-YA2* gene with RNA interference in mutant plants *Mtnf-ya1* led to a more severe block in nodule development. Most infections were blocked in curled root hairs. A similar phenotype was observed when both genes were knocked down. Both transcriptional factors MtNF-YA1 and MtNF-YA2 have been shown to regulate the Nod factor-activating expression of *MtERN1* and *MtENOD11*. MtNF-YA1 can act synergistically with the MtNSP1-MtNSP2 complex, activating *MtERN1*. In turn, MtNIN regulates the expression of *MtNF-YA1*, but not *MtNF-YA2* [96].

M. truncatula mutants *Mtnip* (*numerous infections and polyphenolics*) were obtained which were characterized by the formation of small inefficient nodules. ITs were expanded, in comparison with the wild type, and also strongly ramified. The bacterial release was observed only in individual cells from increased infection droplets. In cells adjacent to cells with ITs, accumulation of polyphenolic compounds was observed [97]. Later, a strong allele *Mtlatd* (*lateral root organ-defective*) was identified. The mutant *Mtlatd* formed small white nodules, unable to fix nitrogen. Infection is limited at the stage of branching of the IT in the nodule primordia. As a pleiotropic effect of the mutation, the development of the lateral roots was limited [98]. It has been shown that the *MtLATD/MtNIP* gene encodes a potential NRT1 family (PTR) transporter transferring nitrate or other substances [99].

Mutants of *M. truncatula vapyrin* (*Mtvpy*) formed several small white nodules in which abnormal enlarged ITs were observed [100]. Most of the infections were blocked in the cells of the root hairs and never penetrated the cortical cells; the number of infections was increased, compared to the wild type. When plants were grown for a long time, single pink nodules formed on the roots, in which partial nitrogen fixation were observed. Expression of the *MtVpy* gene was significantly enhanced in the nodules, it encodes a protein with the N-terminal major sperm protein domain and ankyrin repeats at the C-terminal domain. The protein was localized both in the nucleus and in the cytoplasm. It was suggested that MtVPY can be associated with vesicular transport, involving in exocytotic polar growth of ITs [100].

The mutant *M. truncatula Mtapi* (*altered nodule primordium invasion*) was characterized by abnormalities at the early stages of IT development, when a large number of infections were blocked after the formation of microcolonies. Also, in the case of activation of the growth of ITs through the root hair cells, most of them were blocked in the layers of the cortex adjacent to the nodule primordium. As a result, abnormal infection structures of various shapes and sizes, containing rhizobia, were formed. The mutant *Mtapi* was characterized by a "leaky" phenotype, because rare nodules with abnormal ITs, but capable to fix atmospheric nitrogen, although with reduced efficiency, were formed. The molecular product of the gene was not detected [101].

In *L. japonicus*, several loci have been identified, mutations in which lead to abnormalities in the development of ITs [44, 102–104]. The mutant *Ljcrinkle* (*Ljsym79*) formed two types of nodules. In small white nodules, ITs were blocked at the stage of growth of the IT from the root hair cell to the root cortex cells. In pale pink nodules, infection progressed and infected cells were formed [44]. Four loci of *Infection-Thread Deficient* (*LjITD*) were identified, the mutations in which lead to the formation of small white nodules, and the *Ljitd2* mutation is allelic to the previously identified *Ljsym7* locus. In mutants, in all four loci, the majority of infections were blocked after the formation of infection pockets and single ITs developed, whereas the nodules remain uninfected. Only after prolonged cultivation, a few pale pink nodules were formed [103]. In the mutants at the loci *Ljsym82* (allelic *Ljsym6* [105]) and *Ljsym80,* small white nodules were also formed, in which the infection was blocked after the formation of the infection pocket or in the root hairs [104].

2.9. Formation of infection droplets

Infection droplet is unwalled outgrowth from IT, from which rhizobia are released into the host cell cytoplasm, being surrounded by symbiosome membrane [23]. A dense network of endoplasmic microtubules is observed around the infection droplet, which is probably related to the preparation of the bacterial release [106]. Little is known about the genetic control of infection droplet formation and functioning. In *M. truncatula,* a component of SNARE complex MtSYP132 was most abundant on the infection droplet membrane [107]. The mutant in the gene *Pssym40* is characterized by the formation of hypertrophic infection droplets [33] (**Figure 2g**). This gene is the ortholog of the *MtEFD* gene [108]. The *MtEFD* gene encodes a transcriptional factor, which negatively regulates cytokinin levels [109]. In mutants in the *Mtnip* gene (encoding putative nitrate transporter) [97] and *Ljnup133* gene (encoding a nucleoporin) [110], the infection droplets were also increased.

2.10. ITs in mature nodules

The development of ITs is not limited to infection of the nodule primordia. In temperate legumes, such as *P. sativum*, *Medicago sativa*, *Vicia faba*, and others, nodule meristem functions for a long time, and such nodules are called indeterminate. As a result of the meristematic activity, new cells constitutively leave meristem and can be infected. These cells form an infection zone into which ITs penetrate and grow, reaching meristematic cells. Longitudinal endoplasmic microtubules surround the IT and form a channel through which the IT grows [106]. One of the main components of the matrix of ITs and droplets is arabinogalactan-protein and extensins [76]. Defects in IT and droplet development in pea mutants in the genes *Pssym33* and *Pssym40* are accompanied with abnormal distribution of arabinogalactan-protein and extensins [111]. An important role in the maturation of the IT wall belongs to hydrogen peroxide [112, 113]. A possible role of ethylene in IT wall maturation was also suggested [114].

Several genes have been identified that control the development of IT in nodule. The mutant for the *Pssym33* pea gene forms the "locked" thickened ITs, from which the bacteria do not release into the cytoplasm of the plant cell [33]. This gene is the ortholog of the genes *MtIPD3* and *LjCYCLOPS* [53]. It is noteworthy that one of the alleles of this *Pssym33-3* gene leads to a leaky phenotype and the mutant forms two types of nodules: with and without rhizobia [33].

However, in the nodule without bacterial release in some cells, infection droplets still form [54] and bacterial release is observed [55]. The development of abnormal ITs leads to the activation of defense reactions manifested in their suberinization [115].

Abnormal ITs are also described in the nodules of mutants in *Pssym32* gene [32], but its nucleotide sequence is not detected. A mutant in the *Pssym42* gene, whose nucleotide sequence is not yet detected, is characterized by the formation of thickened walls around ITs [32, 116], which are enriched with callose [115] (**Figure 2e**, **f**).

In *L. japonicus,* the mutant *Ljalb1* (*aberrant localization of bacteria inside nodule*) formed two types of nodules: in small white nodules, hypertrophied ITs were formed, lacking bacterial release, and in pale pink nodules, infected cells were formed [117].

In *M. truncatula*, mutants impaired in the IT development in mature nodules were also identified, but they have not been described in detail [118].

2.11. Regulation of the number of ITs

During the nodule development, a significant amount of ITs are aborted [119] due to the autoregulation mechanism [120].

Disruption in the regulation of the number of ITs was observed in mutants in genes involved in pathways of signal transduction of phytohormones [121, 122]. In *M. truncatula*, the mutant *sickle*, insensitive to ethylene, formed 10 times as many nodules as the wild type [123]. In the mutant, unlike the wild type, numerous infections are successfully developed. Unlike the wild type, the *Mtsickle* mutant was insensitive to the treatment with ethylene and 1-aminocyclopropane-1-carboxylic acid (the precursor of ethylene biosynthesis), which indicates an abnormality in the transmission of the ethylene signal. *Mtsickle* carries a mutation in the gene *ETHYLENE-INSENSITIVE* (*MtEIN2*), which is a key component in the pathway of signal transduction activated by ethylene [124]. Synthesis of stress-activated proteins was reduced in the mutant in comparison with the wild type, which probably allows a much greater number of infections and nodules to develop [125]. In *L. japonicus*, there are two copies of the *LjEIN2* gene in the genome, and their knockdown also leads to an increase in the number of infections and nodules [126].

The *L. japonicus* mutant for the *HYPERINFECTED* (*LjHIT1*) gene has hyperinfection of the root, but most infections are aborted in the cortical cells without infecting them [127]. At the same time, there is no development of nodule primordia. Sometimes, there is a release of bacteria from ITs, resulting in the formation of large and flattened nodules, or nodules like those of the wild type. It was shown that *Ljhit1* is an allele of the gene *Lotus histidine kinase 1* (*LjLhk1*), which encodes a receptor for cytokinin. It was suggested that *LjLhk1* is not necessary for the development of infection [127]. It was previously shown that the expression of the *LjNIN* gene in cortical cells activates the synthesis of CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR)-RELATED (CLE) peptides that negatively regulate nodule formation [128]. The *Ljhit1* mutant does not activate expression of the *LjNIN* gene [127], which probably leads to a lack of activation of the synthesis of CLE peptides and, correspondingly, hyperinfection.

3. Conclusion

Using the genetic approach to study a set of pea mutants impaired at the early stages of nodule development, the existence of two independent but coordinated genetic programs, controlling infection thread growth and nodule organogenesis, accordingly, was suggested [37]. Since then, thanks to the achievements of molecular genetics of model legumes, sequences of many genes controlling the development of an infection thread have been identified. These genes encode different transcriptional factors, LysM receptor kinases, E3 ubiquitin ligases, SCAR/WAVE actin regulatory complex, nitrate transporter, remorins, flotillins, proteins involved in membrane biogenesis and traffic, and some other components. Despite the significant progress made in the study of genetic control over the infection thread development, many aspects are still insufficiently studied. Thus, genes that control the reorganization of tubulin cytoskeleton elements have not been identified. It is not enough known about the genes involved in the formation and functioning of infection droplets. Also, little is known about the genes involved in the control over the development of an infection thread in a mature nodule. The precise function and sequential functioning of many identified genes also still remains unclear, and the existence of parallel pathways involved in infection initiation and growth was suggested [129]. Moreover, some genes, like *MtNIN*, may have different roles in epidermis and root cortex [130]. The complexity of the genetic control over the development of the symbiotic nodule, including the growth of the infection thread, makes it necessary to conduct further research.

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Chapter 4

Non-nodular Endophytic Bacterial Symbiosis and the Nitrogen Fixation of *Gluconacetobacter diazotrophicus*

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Additional information is available at the end of the chapter

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Abstract

There is a need to reduce the negative polluting influence of mineral nitrogen fertilizers and to develop a more sustainable climate smart agriculture capable of meeting our future food security needs. Biological nitrogen fixation can have a role in this if it can be applied to the major food crop plants. Certain strains of the obligate nitrogen-fixing bacterial endophyte *Gluconacetobacter diazotrophicus* have the necessary attributes for this role. An 'extra-ordinary endophyte' this bacterium is one of relatively few that has mechanisms to cope with high levels of sucrose, an acidic pH, a wide range of oxygen environments, nitrogen fixation, as well as having respiratory chain attributes that make it a possible candidate eukaryote proto-mitochondria. Having a small genome relative to other endophytes, it is typical of facultative intracellular colonizers, with a life cycle that involves horizontal transfer to other high sucrose species via insects and potential vertical transfer through seeds. Every method used for demonstrating nitrogen fixation in rhizobia have been used to demonstrate nitrogen fixation in *G. diazotrophicus* both *in vitro* and *in planta*, and field trials demonstrate yield increases and the potential to reduce nitrogen fertilizer use, meeting both food security and climate smart agriculture needs.

Keywords: nitrogen fixation, *Gluconacetobacter diazotrophicus*, bioenergetic systems, cereals, non-nodular symbiosis, facultative colonization, intracellular colonization, endophyte, yield impact, food security, climate smart agriculture

1. Introduction

Sugarcane, *Saccharum officinarum*, is grown in many parts of the world for processing into cane sugar. In Brazil, a primary driver for growing sugarcane has been ethanol production for use as a sustainable substitute fuel for petrochemicals. For many years, and in deed for decades,

Brazilian sugarcane had been produced in the same regions with little use of nitrogen fertilizers, without any apparent loss in yield [1]. This led to speculation that the crop was benefiting from biological nitrogen fixation (BNF). In an experiment using labeled nitrogen, it was demonstrated that the sugarcane variety CB 47-89 derived around 60% of its nitrogen from a biologically fixed source [2]. Subsequent to this, studies confirmed that some varieties of Brazilian sugarcane were capable of obtaining 60–80% of their nitrogen requirements from BNF, highlighting the possibility that under the right conditions, it might be possible to dispense altogether with nitrogen fertilizers for these varieties [1, 3]. The bacteria thought to be responsible for the BNF was a new species, *Acetobacter diazotrophicus* [4] discovered in 1988 by Vladimir Cavalcante and Joanna Döbereiner in Alagoas, Brazil [5]; initially named *Saccharobacter nitrocaptans* and later renamed *Gluconacetobacter diazotrophicus* [6].

2. A review of the key aspects of the symbiosis of the endophyte *Gluconacetobacter diazotrophicus*

The nature of the symbiosis of the endophytic nitrogen-fixing bacteria *G. diazotrophicus* has increasingly become a subject of scientific inquiry because of its potential for reducing nitrogen fertilizer use in cereals and other major food crops, its extra-ordinary attributes and capabilities relative to other endophytes and nitrogen fixers, its life cycle and its ability to fix nitrogen under a range of circumstances.

2.1. Demonstrated impact of *G. diazotrophicus*

The ability of *G. diazotrophicus* to fix up to 80% of the sugarcane plants nitrogen requirements is significant in agriculture terms, not least if this capability could be transferred to other grass and cereal species. The drive to find a means of introducing BNF in non-legumes, particularly through the ability to transfer nodulation to non-leguminous cereal crops, had been an important focus of research since the 1970s [7]. The primary reason for this was the need to produce more climate smart, sustainable systems of agriculture that are less reliant on inorganic nitrogen fertilizers produced via the Haber Bosch process.

The 500 million tonnes of ammonia produced each year through this process in order to meet the needs for nitrogen fertilizer account for 1% of the world's energy usage and 3–5% of natural gas usage [8]. However, crops use only an estimated 30–50% of the nitrogen fertilizer applied to the soil. The remainder is lost, either to the atmosphere as nitrous oxide gas or into waterways as nitrate run-off. Nitrogen fertilizer use accounts for around 66% of UK agricultural nitrous oxide emissions contributing to climate change [9], while nitrate run-off contaminates drinking water, with 5% of the European population exposed to unsafe levels [10].

Despite the obvious need to find sustainable solutions for future more climate smart agriculture, it is now generally acknowledged that the promise of BNF through rhizobial-based root nodulation in non-leguminous plants has not been realized [7]. Unfortunately, it is also not currently considered possible in cereals without further years of genetic manipulation [11]. Alternative approaches however, based on the findings relating to *G. diazotrophicus* in Brazil

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in sugarcane offer some prospect for the development of non-legume crop symbiotic nitrogen fixation, not only to increase crop yields but also to potentially reduce nitrogen fertilizer use, and this prospect is now beginning to be realized [12].

Apart from fixing atmospheric nitrogen, diazotrophic bacteria such as *G. diazotrophicus*, can affect plant growth directly by the synthesis of phytohormones and vitamins, improved phosphate and nutrient uptake and enhanced stress resistance [13]. It has been demonstrated that strains of *G. diazotrophicus* differentially affected growth parameters of sugarcane, with some strains improving germination, tiller number and plant height relative to others [14] and there is also evidence that *G. diazotrophicus* improves tolerance to the sugarcane pathogen *Xanthomonas albilineans* as a result of production of bacteriocin; as well as reducing galling caused by root knot nematodes (*Meloidogyne incognita*) in bottle gourds and cotton [15]. *G. diazotrophicus* has also been shown to enhance photosynthetic capability and water use efficiency [16] and in sorghum increased chlorophyll and leaf nitrogen [17].

Inoculation of crop plants with *G. diazotrophicus* has been shown to increase crop yields in tomato [18], in sugar beet [19] and increased both the shoot and root dry weight of sorghum [20]. However, more significant yield enhancement has been demonstrated in recent independent field trial research utilizing proprietary NFix® technology (Patent Number: WO2016/016629) based on *G. diazotrophicus* of around 1 tonne per hectare in both maize and wheat (**Figures 1** and **2**) at any level of nitrogen fertilizer [12, 21].

These levels of plant yield improvement are somewhat surprising and suggest a close symbiotic relationship and multiple plant benefits from the association with *G. diazotrophicus*. Joanna Döbereiner even referred to *G diazotrophicus* as "this extra-ordinary endophyte" but perhaps even Döbereiner would be surprised by the level to which the bacteria she was jointly responsible for discovering [5], is truly extra-ordinary.

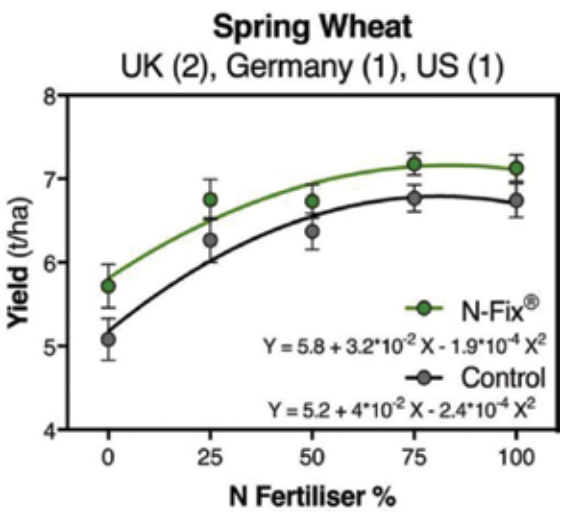

Figure 1. For spring wheat across sites (2015: UK, 2016: UK, 2017: Germany, US) and N levels, N-fix® inoculated seed increased yield by 7% (460 kg/ha) and demonstrated a potential to N-fertilizer savings of up to 61% with no reduction in yield [21].

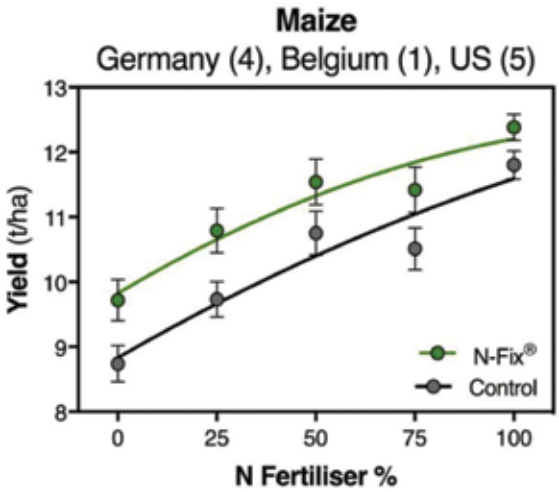

Figure 2. Combined data from 10 maize trials (2014: 4 Germany, 1 Belgium, 2015: 3 US, 2016: 2 US) demonstrated an overall increase in yield of 8% (830 kg/ha; **Figure 2**A). Estimation from second order polynomial fit, predicts that N-fix® can replace 27% of the nitrogen fertilizer inputs without yield penalty [21].

2.2. *G. diazotrophicus*: an "extra-ordinary endophyte"

G. diazotrophicus is a Gram-negative, non-spore forming, non-nodule producing, endophytic nitrogen-fixing bacterium. This bacterium belongs to the phylum Proteobacteria, the class Alpha-Proteobacteria, the order Rhodospirillales, the family Acetobacteraceae (Acetic acid bacteria; AAB), within the genus of *Gluconacetobacter* [22]. Such a phylogeny does not suggest anything particularly remarkable about the species—*G. diazotrophicus*. However, there are a number of key attributes that distinguish this bacterium from others and point to the reasons why it is able to achieve the types and levels of impact demonstrated in **Figures 1** and **2**, when colonizing crop plants. Among these attributes *G. diazotrophicus* has the ability to cope with high sucrose concentrations, low oxygen and pH levels and the ability to intracellularly colonize and fix nitrogen in a wide range of crop plants [12, 23, 24].

The availability of water is essential for the functioning of living systems and relatively few bacteria can survive and reproduce at water activity levels below 0.90 aw [25, 26]. The presence of solutes such as, salts or sugars can create an osmotically stressful environment for bacteria and relatively few species have mechanisms that allow cell multiplication under extreme conditions of <0.70 aw [26, 27]. Plant sap generally has water activity values between 0.99 and 0.96 aw (and pH 4.4–8.0); levels that are able to support a phylogenetically diverse groups of micro-organisms, including plant pathogens, plant and insect bacterial and fungal endosymbionts [27].

G. diazotrophicus is one of the relatively few bacteria capable of being cultured at very high sucrose concentrations (876 mM sucrose [28]; 30% [29]) and can tolerate a water activity level of 0.892 aw [26]. This is perhaps not surprising given its host plant, sugarcane and other high sucrose content host plants from which it has been isolated (**Table 1**), but for *G. diazotrophicus* to tolerate sucrose-induced stress, it has to have the mechanisms with which to cope. In general for bacteria, a number of osmotolerant mechanisms exist and most of these exist in *G. diazotrophicus*,

Plant family	Host plant	References
Amacariaceae	Mango	[146]
Amaranthaceae	Beet root	[146, 147]
Apiaceae	Carrot	[146, 147]
Arecaceae	Oil Palm	[148]
Brassicaceae	Radish	[147]
Bromeliaceae	Pineapple	[149]
Cactaceae	Forage cactus	[150]
Convolvulaceae	Sweet potato	[151]
Euphorbiaceae	Cassava	[146]
Musaceae	Banana	[94]
Myrtaceae	Guava	[146]
Poaceae	Cereals and grasses	[5, 90, 112, 137, 151–155]
Rubiaceae	Coffee	[94] [156]
Solanaceae	Tomato	[157]
Theaceae	Tea	[94]

Table 1. The natural host range of *G. diazotrophicus* is restricted to 19 plant species representing 15 plant families.

but also in bacteria that do not live with such high levels of sucrose. Therefore, additional mechanisms that protect *G. diazotrophicus* specifically against high sugar concentrations may also act in this species [30].

G. diazotrophicus lacks a sucrose transport system and depends on the secretion of a constitutively expressed levansucrase (LsdA), a fructosyltransferase exoenzyme with sucrose hydrolytic activity, in order to utilize plant sucrose [31, 32, 33]. Levan is implicated in sucrose tolerance in *G. diazotrophicus*. A levansucrase defective mutant of *G. diazotrophicus* demonstrated a significant decreased tolerance to sucrose compared to the wild type [33]. Osmotic pressure is regulated in many bacteria by the movement of potassium ions in to and out of the cell [34]. In *G. diazotrophicus* sucrose tolerance is, at least partially, achieved through genes encoding for the KupA protein [27]. Interestingly, however, this gene is considered only a secondary low affinity potassium transporter for bacteria generally and certainly has not been implicated in the regulation of osmotic stress [35]. Hence, this high-affinity potassium transport role of the KupA protein by which *G. diazotrophicus* regulates osmotic stress in high sucrose concentrations, is different from other bacterial species [27]. *G. diazotrophicus* seems to have a larger number of isoforms of enzymatic systems involved in osmotolerance [30].

High sucrose concentrations occur in a range of environments that may be associated with bacterial endosymbionts. In addition to the sap of the host plant other sites of high sucrose include floral nectar, plant fruits and fruit juices as well as the guts of sugar-feeding insects and the rhizosphere [27]. Studies of bacterial-insect symbiosis have demonstrated that the AAB

are capable of establishing symbiotic relationships with insects that rely on a sugar-based diet [36]. The AAB form symbiotic associations within the mid-gut of insect species representing a diverse range of Orders namely Diptera, Hymenoptera, Hemiptera and Homoptera. This insect habitat is characterized by the presence of sucrose or other diet related sugars, low oxygen concentrations and a low pH. Symbiotic associations of species of *Gluconacetobacter* have been found in fruit flies, *Drosophila melanogaster*; bees, *Aphis mellifera* and for *G. diazotrophicus*, within the gut of the sugarcane mealybug, *Saccharicoccus sacchari* [36].

While the insect gut may suit the ability of *G. diazotrophicus* to tolerate sucrose rich environments, as an aerobe, the oxygen levels in the guts of many insects may be less suitable, varying as they do from aerobic to completely anoxic [37]. However, the presence of *Gluconacetobacter* species within insect guts and of *G. diazotrophicus* in *S. sacchari*, would suggest some ability to cope with a range of oxygen environments. In a genomic analysis of 14 AAB to assess traits associated with insect symbiosis, the presence and distribution of the oxygen-reacting systems of the electron transport chain (terminal oxidases) were studied [38]. It was found that the operons of both cytochrome bo3 (CyoA-D) and bd (CydAB) ubiquinol oxidase, which have a high affinity for oxygen, were present in the genomes of all of the AAB studied, including *G. diazotrophicus*. The high oxygen affinity cytochrome bd oxidases are typically expressed by enterobacteria, intracellularly colonizing animal cells (e.g. *Brucellar suis*; [39]), which have oxygen concentrations lower than those found in the extracellular environment. Although, AAB are typically considered aerobes the capacity to live in low oxygen concentrations conferred through the ubiquinol oxidases enables endosymbionts such as *G. diazotrophicus* to survive in a range of environments, including the micro-oxic environment of the insect gut [37]. Phylogenetic comparisons demonstrate that these terminal oxidases were present in the common ancestor of AAB, thereby constituting an ancestral character [38]. In addition, the presence of reactive oxygen species (ROS) detoxifying genes in *G. diazotrophicus*, have a high similarity to related enzymes from phylogenetically distant symbiontic organisms [40]. This could be an indication that nitrogen fixation is an ancient process in *G. diazotrophicus* and was probably acquired before the adaptation to the endophytic lifestyle [30]. An obligate symbionts lifestyle necessitates a close metabolic association with its host plant. *G. diazotrophicus* antioxidant catalase genes that act to reduce the toxicity of oxygen during nitrogen fixation [40] are related phylogenetically to distant organisms that are normally isolated from plant leaves with the ability to promote the growth of various plant seedlings [41, 42]. The enzyme pyruvate decarboxylases (PDC) are rare and found in bacteria that are strongly plant associated, in which the environment contains ethanol and a low pH [43]. Their rarity suggests that the PDCs have a significant and specific metabolic role in these environments. PDCs are expressed in plants as part of the pathway of fermentation converting sugars into cellular energy under conditions of low pH caused by oxygen stress, when normal aerobic energy metabolism is not possible, for example, root water logging [44]. In *G. diazotrophicus*, PDC expression is regulated and is not constitutively expressed and it is possible that the expression of *G. diazotrophicus* PDC is also pH or oxygen dependent. It is conceivable that *G. diazotrophicus* PDC could perform a role outside the bacterial cell in support of plant cell metabolism under oxygen stress and in doing so would further deepen the symbiotic relationship between the plant and the bacterium to the point where *G. diazotrophicus* could almost be considered a "plant organelle" [43].

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The study of bioenergetic systems associated with terminal oxidases, and the ability to fix nitrogen and function under a wide range of oxygen concentrations has also raised the prospect of *G. diazotrophicus* having been associated in evolutionary time scales with a key eukaryote cell organelle—the mitochondria. It has been postulated that proto-mitochondria 'bacteria' were adapted to different levels of environmental oxygen of the anoxic proterozoic oceans [45], exploiting also the terminal oxidases of facultatively anaerobic bacteria to obtain bioenergy [46].

It is logical to argue that the mitochondrial systems that generate most cellular bioenergy must define the minimal bioenergetic capacity of proto-mitochondria. Ubiquinol in the mitochondrial respiratory chain produces most bioenergy in eukaryotic cells and shows strong similarity with that of aerobic proteobacteria [47, 48]. On this basis the maximum number of bioenergetic systems carrying out the oxidation of ubiquinol includes the bc1 complex, cytochrome c, cbb3, aa3, bo and bd as well as nitrogen metabolism since nitrogen compounds can function as electron acceptors for the oxidation of dehydrogenases [49, 50].

Analysis of all of the available genomes of the Alpha-proteobacteria and using a model based upon the pathways of differential loss of the six bioenergetic systems leading to the reduced subset of current mitochondria, concluded that those subsets lacking the cbb3-type oxidases probably represents the closest match for the bioenergetic capacity of the distal ancestors of mitochondria [49]. Alpha-Proteobacteria lacking the cbb3 type oxidase is typified by methylotrophs and the genus *Gluconacetobacter*.

2.2.1. G. diazotrophicus in comparison with other bacterial endophytes

Bacterial genomes vary a great deal in size ranging from 0.16 megabases (Mb) in *Carsonella ruddii* [51] to approximately 9.7 Mb in *Burkholderia xenovorans* [52]. Among the nitrogen-fixing endophytes the rhizobia are the most well studied, and soybean a key leguminous crop. In a systematic comparative genomic analysis of soybean micro-symbionts and other rhizobia sampled from a range of ecological zones, it was found that the average genome size of *Bradyrhizobium* strains was 9.8 ± 0.87 Mb which was significantly ($P < 0.001$) larger than that of nine *Sinorhizobium* genomes—6.6 ± 0.30 Mb [53]. Similarly the genome size of 48 strains of *Sinorhizobium* varied between species and strains from 6.2 to 7.8 Mb [54]. The key requirement in assessing these differences among the rhizobia has been the need to gain an understanding of the types of genome essential for nodulation and nitrogen fixation. In trying to define these core characteristics, the genome size of 14 strains of the Rhizobiales ranged between 4.9 Mb, exemplified by *Mesorhizobium* species, up to 9.1 Mb in *Bradyrhizobium japonicum* [55].

The intracellular environment is the main factor that correlates to genome size in bacteria [56, 57]. An analysis of 350 bacterial species genomes comparing the nature of their association with their host (early, advanced and extreme stages of adaptation) demonstrated a decreasing genome size with increasing levels of host adaptation [56]. Bacteria in an early facultative intracellular stage of adaptation tend to have a median genome size ca. 3.1 Mb, advanced obligate intracellular stages a median genome size ca 1.3 Mb and an extreme obligate intracellular mutualist, a median genome size ca. 0.7 Mb.

For plant endosymbionts a comparison of genome sizes of nine bacteria (*Burkholderia phytofirmans* PsJN, A*zospirillum* sp. B510, Klebsiella pneumoniae 342, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619, *Pseudomonas stutzeri* A1501, *Enterobacter* sp. 638, *Azoarcus* sp. BH72, *Gluconacetobacter diazotrophicus* Pa15) with differing lifestyles exhibited a range in size from 7.6 to 3.9 Mb (**Table 2**), with *G. diazotrophicus* having the smallest genome ([58]; 3.9 Mb [30]). The genome size of 3.9 Mb places *G. diazotrophicus* firmly in the facultative intracellular colonizer category [56]; an intracellular colonization capability that was first demonstrated in 2006 [24]. Certain strains of *G. diazotrophicus* are capable under the right conditions to intracellularly colonize a range of crop species and this ability has subsequently been demonstrated for a range of other bacteria and host plants [59–63].

Facultative intracellular symbionts are characterized by their adaptive flexibility which is reflected in the relatively greater number of mobile genetic elements compared with obligate intracellular symbionts [56]. *G. diazotrophicus* has 4–5 times more mobile elements than other endophytes, for example, 109 transposases [30, 58], reflecting a high degree of adaptive flexibility. Such flexibility is needed to overcome constraints that include the ability to attach to host cells, entering the cytoplasm, multiplying, exiting and being transmitted to new host individuals without being recognized by the host immune system [56].

Genetic diversity and adaptive flexibility is also achieved though bacterial plasmids with genes controlling important functions such as nitrogen fixation, sulfur utilization and hydrocarbon degradation. Nitrogen-fixing genes can be conserved in chromosomal DNA and within plasmids [64]. The symbiotic bacterium of genus Rhizobium carry high molecular weight plasmids (90–350 Å~ 106) and in *R. leguminosarum* plasmids have a role in nodule formation, symbiosis as well as carrying nitrogen fixation (nif) genes [65]. Plasmids occur in *G. diazotrophicus* but their number and size varies between strains, with for example *G. diazotrophicus* UAP8070 and UAP5665 each having three plasmids of 93, 22 and 22 kb in size [66], PR2 has two plasmids one particularly large at 170 kb and a smaller one at 24 kb [66], whereas Pal5 has two plasmids of 38.8 and 16.6 kb, [30] and strain UAP5541 has no plasmids at all [66–68].

Genes responsible for nitrogen fixation in *G. diazotrophicus* are located on the chromosome [30, 66]. However, plasmid genes will have other key roles and it has been speculated that for *G. diazotrophicus* they contribute to an improved fitness of the colonized host plant or the insect symbiosis for the bacterium [66]. Strain differences in *G. diazotrophicus* are complex with a mix of highly conserved regions and highly variable groups of genes [30]. A considerable number of coding sequences on 20 genomic islands across a range of 19 strains of *G. diazotrophicus* encode genes involved in processes that could confer intra-specific differences such as, responses to oxidative stress, proteases, biosynthesis of antimicrobial agents, amino acid metabolism and secondary metabolites, as well a large number of transport systems and transcriptional regulators [30]. Strain differences in *G. diazotrophicus* have been observed for a range of key attributes including expression of cell wall degrading enzymes [69], intracellular colonization [24], responses to nitrates [70, 71], siderophore production [72], as well as bacterocin production [73].

The presence and expression of nitrogen-fixing nif genes, are key to the ability of *G. diazotrophicus* to fix nitrogen. In 2000, a major and unique 30.5-kb cluster of nif and associated genes of *G. diazotrophicus*, was sequenced and analyzed [30, 74]. This cluster represented the largest

Endophyte functions	Range	Gd value	Implications
Motility and Chemotaxis	- or +	+	MCP, a transmembrane sensor protein permits *G. diazotrophicus* to detect concentrations of molecules while Che proteins enable orientation and movement.
Type IV pilli & flagella	9–88	9	
Methyl accepting proteins	12–73	12	
Che-protein response regulators			
Plant polymer	26–68	35	GHs facilitate plant entry, sugar metabolism, bacterial cell wall metabolism, and host-microbe interaction [158]. *G. diazotrophicus* has specific cell wall degrading enzymes [69].
Degradation (PPD)	23–63	23	
Glycoside hydrolases (GH)			
% putatively PPD			
Detoxification	8–21	12	Endophyte survival requires the ability to detoxify or manage movement of xenobiotics using efflux pumps. *G. diazotrophicus* has poor survival in the rhizosphere [89, 92, 93].
Antioxidative enzymes	209–681	209	
Efflux pumps			
Fe uptake	6–22	22	Biologically available Fe is limited in plants and endophytes. Uptake of ferric siderophore complexes is achieved via TonB-dependent receptors [159]. Endophytes with large numbers of these receptors may compete with plants or fungi for iron acquisition.
Ton-B dependent receptors			
Degradation	0–16	0	*G. diazotrophicus* is at the extreme low end of the ability to degrade complex plant metabolites.
Dioxygenases			
Transporters	510–1196	510	*G. diazotrophicus* has a relatively high number of transporter genes enabling transport of nutrients and excretion of toxins. Low numbers of the ABC family of transporters, porin genes and the lack of putrescine transporters perhaps suggests poor rhizosphere competence.
Total number	105–183	131	
No./Mbp genome	95–126	95	
No. transporter types	3–53	7	
Porin	142–477	142	
ABC transporters	- or +	-	
Putrescine			
Secretion systems	- or +	+	*G. diazotrophicus* in common with many other endophytes has available key secretion systems
Type I & IV	- or +	-	
Type II, III, Va, Vb, VI			
Signaling	87–272	87	Complexity of signaling systems correlates with the genome size, phylogeny, ecology and metabolic activities of the bacteria [160]. Bacteria living in diverse habitats encode more ECF sigma factors than in stable niches [161].
Two component systems	65–142	96	
Bacterial IQ	2–17	3	
ECF Sigma factors			

Table 2. Compiled from the survey and analysis of nine endophytes: *Burkholderia phytofirmans* PsJN, *Azospirillum* sp. B510, *Klebsiella pneumoniae* 342, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619, *Pseudomonas stuzeri* A1501, *Enterobacter* sp. 638, Azoarcus sp. BH72, Gluconacetobacter diazotrophicus Pa15, with particular reference to G. Diazotrophicus (Gd) [58].

single grouping of genes required for nitrogenase structure and function, found in any diazotroph at that time [74]. Interestingly, the overall arrangement of genes was similar to the nif-fix cluster in *Azospirillum brasilense*, while the individual gene products most closely resembled those in species of Rhizobiaceaeor, proteobacteria comprising multiple subgroups that can both enhance or hinder plant development [75]. The individual *G. diazotrophicus* gene products are generally similar to those found in other groups of proteobacteria, with 17 gene products being most like those in members of the Rhizobiaceae and 9 gene products being most closely related to *Rhodobacter capsulatus* proteins. NifU and NifS were most similar to the gene products of *Azotobacter* species [74].

2.3. Life cycle of *G. diazotrophicus*

As a Gram-negative bacteria, *G. diazotrophicus* has no spore or resting stage; it reproduces asexually through binary fission. *G. diazotrophicus* is also an obligate endophyte [23], which means it is a bacterium requiring internal as opposed to external plant tissues to complete its life cycle. *G. diazotrophicus* primarily inhabits intercellular apoplastic spaces, the xylem and the xylem parenchyma [76, 77]. However, studies using β-glucuronidase (GUS)-labeled *G. diazotrophicus*, demonstrate that this bacterium is also capable of intracellular colonization within membrane-bound vesicles in its host plant [24]. Some strains of *G. diazotrophicus* have this intracellular colonization capability in common with a number of other bacteria, for example a phylotype related to *G. diazotrophicus* in *Pinus flexilis* (limber pine) and *Picea engelmannii* (Engelmann spruce) [62] and *Methylobacterium extorquens* in *Pinus sylvestris* [78].

The symbiosome is the unifying feature of all endosymbiosis [79]. The symbiosome is created by the engulfment of the microorganism by a plant-derived membrane in a manner that resembles phagocytosis in animal cells [80]. In legume symbiosomes, bacteriods are enclosed within such a plant-derived membrane. The challenge for any other nitrogen-fixing endosymbiont is first to establish intracellularity within living plant cells and within symbiosome-like structures. All carbon and nitrogen sources and oxygen must cross the symbiosome and bacteriod membranes making them crucial to the establishment and maintenance of symbiosis [81].

The UAP5541 strain of *G. diazotrophicus* is known to constitutively produce three hydrolytic enzymes such as endoglucanase, endopolymethylgalacturonase and endoxyloglucanase that facilitate bacterial penetration of plant cell walls [69]. After cell wall penetration, when *G. diazotrophicus* is present at the surface of the plasma membrane, uptake into vesicles may be triggered by sucrose-induced endocytosis [82]. *G. diazotrophicus* is known to produce large amounts of IAA. At low concentrations, IAA can function as a reciprocal signaling molecule in bacterial-plant interactions [83]. Once intracellular, the enzymes enable *G. diazotrophicus* to colonize cell walls, intercellular spaces and to be transmitted cytoplasmically to daughter cells in actively dividing plant cells thereby spreading systemically throughout the roots and shoots [84]. The plant will not be passive in this process of colonization by the endophyte; plants have evolved molecular mechanisms to deal with challenges imposed by colonizing bacteria [85]. In sugarcane a number of genes have been found to be differentially expressed in the presence of bacteria [86]. The shr5 gene was differentially expressed after inoculation of sugarcane with *G. diazotrophicus* and other nitrogen-fixing bacteria [87]. This gene encodes a protein involved in plant signal transduction during establishment of plant-endophyte interactions. Down regulation of shr5

was evident when the plants were colonized by *G. diazotrophicus*. This suggests that the initial steps of endophytic colonization are actively monitored and possibly enhanced or diminished by the plant [88].

Obligate endophytes such as *G. diazotrophicus* are thought to spread from plant generation to plant generation via seeds, vegetative propagation, dead plant material and possibly by insect sap feeders [89].

2.3.1. Horizontal transmission of G. diazotrophicus

G. diazotrophicus is a non-invasive, obligate, endophytic species [90]. Hence, its ability to survive outside its plant hosts is likely to be poor and its infection capability will be low [91]. There is certainly little evidence of its survival in soil [89, 92, 93]. In host range studies, *G. diazotrophicus* has only been isolated from the rhizosphere of plants in two cases, in banana [94] and rice [95] (**Table 1**). Studies involving immunocapture and PCR have failed to find *G. diazotrophicus* in soil collected between rows of sugarcane plants grown in the field (Santos et al., unpublished data; source [93]). When PCR was used, fragments of the same size as those from *G. diazotrophicus* genomic DNA were detected in soil samples from sugarcane fields, however, the bacterium could not be re-isolated from micro-propagated sugarcane plants used as a trapping host [92]. *G. diazotrophicus* has been isolated from arbuscular mycorhizal fungi (AMF) associated with sweet potato and sweet sorghum [96] and sorghum [17] but survival of *G. diazotrophicus* in soil appears to be limited. Populations of *G. diazotrophicus* residing in plant debris could, following release into the soil, potentially gain entry into a new host plant through the roots, tips and cells of the root cap and meristem, at areas of lateral root emergence and through root hairs [77, 97, 98]. This process would be facilitated by the release from the bacteria of their hydrolytic enzymes in the presence of root exudates containing suitable sugars. Within the stems of host plants, specifically sugarcane, the bacterium is capable of entering at breaks caused by the separation of plantlets into individuals [77].

The ability of *G. diazotrophicus* to survive in the soil long enough to multiply and find a potential host plant is probably limited given its lack of putrescine transporters, because of restricted carbon availability (as sucrose/glucose), and competition from free-living soil bacteria. Hence, the *G. diazotrophicus* must have a means of horizontal transmission that does not rely solely on soil-mediated transfer.

Surveys have indicated that the *G. diazotrophicus*, although present at all sites, in all parts of the sugarcane plant and in all trash samples examined, it was not present in samples taken from associated forage grasses, cereals or weed species within the sugarcane fields [99]. *G. diazotrophicus* has only been found to occur naturally in a total of 19 plant species, mainly crops, across 15 plant families including, Poaceae, Convolvulaceae, Rubiaceae and Bromeliaceae (**Table 1**). Given the bacterium thrives in an intercellular environment rich in sucrose which it uses as a carbon source the number of candidate host species for natural colonization is low. However, despite difficulties in achieving colonization [100], *G. diazotrophicus* has been intentionally inoculated into cotton, calabash (*Lagenaria siceraria*) [15], maize [101] sugarcane, wheat, rice, oilseed rape, tomato, white clover [24, 102], sugar beet, common beans [103] *Arabidopsis* [24] and sorghum [104].

Another potential means of horizontal transmission is through the uptake and distribution via plant feeding insects. The symbiotic association of AAB with insects has been reviewed [36] and the genus of *Gluconacetobacter* has been identified in the guts of fruit flies (*G.* '*munehiro*' [105] and *G. europaeus* [106]) and honeybees (*Gluconacetobacter* sequences [107] and *Gluconacetobacter* clone sequences [108]), while in sugarcane *G. diazotrophicus* has been isolated from the gut of the pink sugarcane mealybug (*Saccharicoccus sacchari*) [70, 109–111] a plant sap-sucking insect. This would suggest that horizontal transmission of *G. diazotrophicus* is possible through sap-sucking insect vectors, such as the pink sugarcane mealybug.

> *The insects might become colonized during sap-feeding and then re-inoculate the bacteria to stems of other plants. It has been suggested that G. diazotrophicus is imbibed from sugarcane by S. sacchari and the population within the insect is a subset of the sugarcane population [70]. Alternatively, G. diazotrophicus may be an autochthonous microbiota of mealybugs associated with sugarcane [109]. An investigation of the frequency of strains of G. diazotrophicus isolated from cane internodes and sugarcane mealybugs in Cuba indicated a higher frequency of isolation from the plant than from the insects [110]. This would suggest that the primary host of G. diazotrophicus is the plant rather than the insect: the latter acting only as a transmission vector. It may also imply that the insects do not provide the optimal conditions for multiplication or survival of the G. diazotrophicus [110]. If the strains differ due to whether they are isolated from the plant or the insect host, the function of the insect as a transmission vector [109] would be unlikely. Given that G. diazotrophicus was recovered from mealybugs in 1 out of 20 insect colonies associated with plants from 11 varieties growing in 4 localities; if G. diazotrophicus were an autochthonous microbiota of mealy bug then the recovery of G. diazotrophicus from the insect would be more frequent [110].*

Successful transmission of bacterial endophytes by insects depends on host and cultivar preferences of the vector and on the vector inoculation efficiency and how rapidly the insect can effectively transmit the bacterium to another host plant. From the limited information available, the vector inoculation efficiency is at best 5%, which would imply a low chance of successful insect transmission. This low figure is supported by the natural plant host range of *G. diazotrophicus* (see **Table 1**), which is restricted to 19 plant species. In addition, the important role of the host and cultivar preferences is supported by surveys in sugarcane that have indicated that the *G. diazotrophicus*, although present at all sites, in all parts of the sugarcane plant examined, the bacteria was not present other plant species within the sugarcane fields [99].

Horizontal transmission of *G. diazotrophicus* has most likely occurred through vegetative propagation of crops (particularly sugarcane) with interspecies transmission potentially having occurred via vesicular-arbuscular-mycorrhizal fungi [17, 112], or more likely, sap-feeding insects. *G. diazotrophicus* has been isolated from *Saccharicoccus sacchari*, the sugarcane mealybug [70, 109]—which has a host range including many species of grasses (including sorghum, rice and miscanthus as well as sugarcane) and pineapple (CABI Invasive Species Compendium; http//www.cabi.org), which through horizontal transmission, could explain the presence of *G. diazotrophicus* in these plant species (**Table 1**.).

2.3.2. Vertical transmission

Plant endophytes may be vertically transmitted through plant seeds either endophytically or epiphytically. Bacteria have been isolated from the seed of a diverse range of plant species [112]. Genomic adaptation of bacterial endophytes for a symbiotic life cycle may include strategies for vertical transmission via the seed at the expense of competitiveness and ability to survive in most environments outside the plant. The rich diversity of bacteria in the seed

of *Miscanthus* indicated the bacteria are not only able to avoid plant defenses, but potentially have a more active role, acting primarily during germination and seedling establishment [113]. *G. diazotrophicus* has not been isolated from the seeds of its host sugarcane [66]. However, the intracellular capability of some strains of *G. diazotrophicus* means they have the potential for vertical transmission through intracellular colonization of the seed [24]. Certainly, the ability of *G. diazotrophicus* to fix nitrogen and produce plant growth hormones may aid initial seedling establishment and growth but there is little recorded evidence to date of vertical transmission for *G. diazotrophicus*. Vertical transmission has been demonstrated in seeds of OSR at ca. 15% and seed treated and field grown Barley of 1–3%, but the presence of *G. diazotrophicus* in S1 wheat seed from colonized plants, either in the laboratory or under field conditions, has not been possible (unpublished data Azotic Technologies Ltd.).

2.3.3. Nitrogen fixation in G. diazotrophicus

Although most often associated with rhizobial symbiosis in the root nodules of legumes, BNF occurs in species of more than 100 genera distributed among several of the major phylogenetic divisions of prokaryotes [114, 115]. The principles are the same, whichever bacteria and wherever it may be located in the plant. BNF is simply a process by which atmospheric dinitrogen (N_2) is reduced into two molecules of ammonia (NH_3) by the enzyme nitrogenase with 8H+, 8e− and 16 Mg ATP [116]. The process in *G. diazotrophicus* is catalyzed by nitrogenase which is a molybdenum-dependent system that consists of two proteins, dinitrogenase reductase (Fe protein containing the ATP-binding sites) and dinitrogenase (MoFe protein containing the substrate binding sites) [117–119]. Both of these proteins are irreversibly inactivated by oxygen but with dinitrogenase reductase being the more sensitive of the two. However, because nitrogen fixation is a very energy demanding process, it requires oxygen for aerobic respiration for ATP synthesis. This creates what is known as the "O_2 Paradox" [120] whereby nitrogen-fixing bacteria need to respire to generate the energy for nitrogen fixation, while minimizing O_2 to enable the nitrogenase to function.

Rhizobia manage the O_2 paradox by creating a micro-aerobic environment within a root nodule (providing a barrier to O_2 diffusion) that involves a specific O_2-delivering leghemoglobin combined with a highly efficient respiratory pathway. The large energy demands for fixing nitrogen are generated through respiration utilizing the extremely high O_2 affinity cyt cbb3 terminal oxidases [88, 121]. Interestingly, *G. diazotrophicus* lacks the cytochrome cbb3 that allows respiration at very low levels of oxygen [122] in rhizobia, and does not fix nitrogen within nodules or have the benefit O_2 delivery by leghemoglobin. However, in *G. diazotrophicus* a number of other factors appear to be involved in providing the necessary protection; sucrose, the colony structure and the extrapolysaccharide levan, detoxification of reactive oxygen species as well as control of oxygen through its respiratory pathway.

Firstly, sucrose: *G. diazotrophicus* has no sucrose transport system and in high sucrose concentration environments of around 10% the sucrose has a positive effect on nitrogenase activity protecting nitrogenase against inhibition by oxygen [123]. Secondly, the fructo-oligosaccharide levan; this enables an unusual feature of *G. diazotrophicus*, namely its ability to fix nitrogen in colonies grown on both semi-solid and solid media [124–126]. This is achieved because of the levan mucilage in culture, is capable of limiting oxygen diffusion. It does this to the extent of enabling *G. diazotrophicus* to fix nitrogen even when the pO_2 is not much lower than tropospheric levels [127].

In addition, the levan also increases tolerance to reactive oxygen species (ROS) that may be increased under conditions of high respiration rates causing oxidative stress [40, 128, 129]. There is some evidence for a nitrogenase protection mechanism in fluctuating levels of oxygen [126], possibly involving a putative FeSII Shethna protein, which forms a complex with the nitrogenase during sudden increases in oxygen pressure. This process renders the enzyme temporarily inactive but protected from oxygen damage, similar to the situation in the species, *Azotobacter vinelandii* [130]. However, it has been suggested that other FeSII proteins, rather than Shethna proteins represent more appropriate candidates for this role [30, 131].

One of the remarkable features of *G. diazotrophicus* is its respiratory system whereby its extremely high respiratory rates are among the highest ever reported for aerobic bacteria [132, 133] underpinning *G. diazotrophicus*'s candidature in evolutionary terms, as a potential proto-mitochondrion [49]. Glucose provides the principle energy source to meet the high-energy demand associated with the conversion of dinitrogen by nitrogenase [134, 135] via the pyrroloquinoline quinone-linked glucose dehydrogenase in the periplasmic membrane.

G. diazotrophicus is able to change its electron transport chain composition during nitrogen fixation. In well-aerated cultures, cytochrome a1 and cytochrome bb are expressed as the main terminal oxidase, whereas when nitrogen fixation is repressed, cytochrome a1 diminishes dramatically concomitantly with the appearance of cytochrome bd [132]. Oxidase activities are also much higher in membrane preparations obtained from cultures under nitrogen-fixing conditions than in those from cultures under non-nitrogen-fixing conditions.

The combination of the sucrose environment in natural host plants (**Table 1**), the barrier formed by the extrapolysaccharide levan and the enhanced tolerance this provides to ROS, the very high respiration rates and the ability of *G. diazotrophicus* to change its electron transport pathway during nitrogen fixation plus the extra energy provided by the pyrroloquinoline quinone-linked glucose dehydrogenase, provides all of the conditions necessary for effective nitrogen fixation in this bacterium.

The methodology for determining nitrogen fixation by endophytic bacteria is now well established and every method used to determine nitrogen fixation in rhizobia root nodules has been used to demonstrate nitrogen fixation in crop plants by *G. diazotrophicus* [12]. These techniques include chlorophyll levels and leaf percentage nitrogen [17], nitrogenase activity measured through an acetylene reduction assay (ARA) [136, 137], nif gene mutant studies [138], labeled nitrogen 15 N2 studies [137–138], enhanced photosynthetic rates [16] and plant growth and yield benefits [12, 19, 139, 140].

There are two key characteristics of *G. diazotrophicus* with regard to its nitrogen-fixing capability: (i) its ability to excrete almost half of the fixed nitrogen as ammonium which is potentially available to plants [141, 142] and (ii) its lack of a nitrate reductase protein which suggests that the ability of *G. diazotrophicus* to fix nitrogen is independent of the amount of nitrate in its environment [124]. With regard to the latter, laboratory studies have indicated that nitrogenase activity was not inhibited or repressed by nitrates [141] and was only partially inhibited by ammonia [23, 141, 143]—which is consistent with the possibility of having a feedback mechanism for ammonium—the form in which nitrogen may be excreted by the

bacterium [93, 141], but not nitrate for which there may be no nitrogen reductase feedback mechanism. Studies with different sugarcane varieties comparing ammonia versus nitrate sources of nitrogen have demonstrated their effects (using both ARA and bacterial counts) to be plant variety dependent, but with ammonia having a greater negative impact on nitrogen fixation than nitrate, and the reverse true of counts of colonized bacteria [144, 145]. Growth of *G. diazotrophicus* in culture was not affected by nitrate but was reduced in sugarcane plants treated in the field with high levels of nitrate fertilizers [68]. **Figures 1** and **2** clearly demonstrate that the *G. diazotrophicus* treated maize and wheat crops generated higher yields relative to the controls, irrespective of levels of nitrogen fertilizer applied.

3. Conclusions

G. diazotrophicus is an extra-ordinary nitrogen-fixing endophyte; a bacterium with important ancestral attributes, the significance and value of which are increasingly becoming apparent as research to facilitate its use in climate smart agriculture is undertaken. Typical of a facultative intracellular symbiont, *G. diazotrophicus* retains genetic flexibility through its genome and plasmids and can respire under a wide range of oxygen concentrations suitable for both an intracellular plant and insect habitat. With a respiratory system that enables extremely high respiratory rates, as well as large groups of genes associated with nitrogenase structure and function and a range of mechanisms that protect the nitrogenase from oxygen, the bacterium combines these factors to ensure symbiotic nitrogen fixation *in planta*. A highly adaptive obligate endophyte, with different strains demonstrating a range of attributes, including both inter- and intracellular colonization capability, *G. diazotrophicus* has the potential to reduce nitrogen fertilizer use while maintaining crop yields.

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Conflict of interest

The author declares a role in the development of the proprietary NFix® formulation cited above in this publication and its commercial utilization but no other competing or conflict of interests exist.

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Chapter 5

Bacterial Leaf Nodule Symbiosis in Flowering Plants

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Additional information is available at the end of the chapter

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Abstract

Bacterial leaf nodule symbiosis within angiosperms is a less known phenomenon compared to the well-documented legume root-*Rhizobium* symbiosis and certainly deserved much more scientific attention. Leaf nodules associated with bacteria was first recognized in *Pavetta* (Rubiaceae) in early twentieth century. Further survey added other members of Rubiaceae, Primulaceae, Dioscoreaceae, and Styracaceae to the short list of plants with specialized bacteria-containing structure in aerial part of plants. The actual role of the bacteria has been questioned by several researchers, mostly due to the problems associated with the identities of these unculturable bacteria. Many progresses have been achieved provided with molecular phylogenetic analysis and also genomic data of the bacteria. Recent evidence from genomic sequences showed the symbiotic bacteria may serve as a defense role in Primulaceae and Rubiaceae, and may increase stress tolerance in Dioscoreaceae. In this article, we reviewed the current knowledge of the bacterial leaf nodule symbiosis in angiosperm. Future research and applications were also discussed.

Keywords: bacterium, convergent evolution, coevolution, endophyte, leaf gland, leaf nodule, symbiosis

1. Introduction

Symbiosis is a long-term and close relationship of two or more biological species that live together for at least part of their life cycle. An endosymbiont is an organism that lives within another, that is, forming endosymbiosis, either intercellular or intracellular [1, 2]. Endosymbionts can be transmitted either vertically (from parent to offspring) or horizontally (from other individuals or environment) [3]. Symbiotic relationships can be obligate or facultative, the former means that one or both symbionts cannot survive without each other. In some cases, the symbiotic relationship provides extra benefits for surviving but is not absolutely necessary to each other, which is known as mutualistic symbiosis.

In plants, various intimate relationships may occur with microbes, which may be friendly, antagonistic, or dynamic [4]. Most of the plant-microbe interaction occurs on surface of the plant body at either underground portion (i.e. rhizosphere) or aerial portion (i.e. phyllosphere), while some interaction occurs within the plant tissues and forms endosymbiosis. The endosymbiotic microbes in plants are also called endophytes, which are often bacte ria or fungi. Though the endophytes are ubiquitous in plants, most of these plant-endophyte relationships are not well understood [5]. Most endophytes in plant are without clear function, and only a few are known to be beneficial or harmful to their hosts [6]. In some cases, host plants develop a special structure for harboring the beneficial endophytes. For instance, legumes form root nodule, a specialized structure to house the symbiotic nitrogen-fixing rhizobia. The well-known *Azolla*-cyanobacteria association is another example that the *Azolla* species form a chamber in their leaf, which is often full of nitrogen-fixing cyanobacteria [7, 8]. However, the mutualistic symbiosis with obligate and vertically transmitted is rare in plants. So far, the only two known cases are the *Nostoc-Azolla* association [9], and the bacterial leaf nodule or leaf gland symbiosis [10]. The latter will be the main focus of this article.

Bacterial leaf nodule symbiosis, like legume root-*Rhizobium* nodule, consists of a specialized structure of a nodule, or sometimes referred as a "leaf gland", with bacterial fluid inside a swollen part of the leaves. In angiosperm, the leaf nodules have been discovered in three genera of Myrsinoideae, Primulaceae (*Amblyanthopsis*, *Amblyanthus*, and *Ardisia*), three genera of Rubiaceae (*Pavetta*, *Psychotria*, and *Sericanthe*), *Dioscorea* (Dioscoreaceae), and *Styrax* (Styraceae) (**Figure 1**) [10, 11]. Among these cases, the bacterial leaf nodule symbiosis has been verified microscopically, except in *Amblyanthopsis* and *Amblyanthus* species [10]. In the swollen part of the leaf of *Dioscorea*, the symbiotic bacteria do not invade and digest the plant tissue but are maintaining in the chamber by the host plant, so the term "leaf gland"

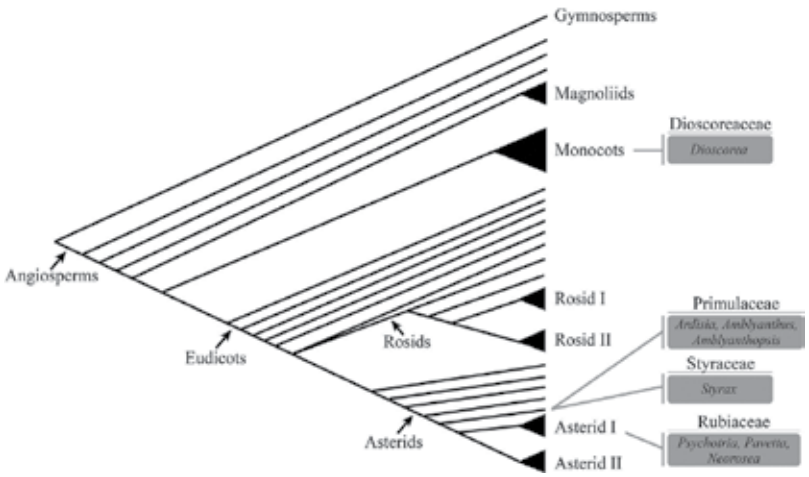

Figure 1. Angiosperm phylogeny modified from APG website [80]. The families and genera with leaf nodulate species are shown next to the corresponding clade.

is used rather than "leaf nodule" in strict sense. In *Styrax*, the bacteria are associating with glandular-trichome-like structure on the shoots and leaves, so the term "leaf gland" is also used in this case.

To date, there are about 530 species reported to have bacterial leaf nodule, which represents about 0.2% of all flowering plants. Nodulated species are mostly distributed through the tropical and subtropical regions in the Old World. The nodulated species of Primulaceae were restricted to tropical and subtropical Asia. The nodulated *Pavetta* has a broader distribution through the tropical and subtropical Africa and Asia. Most nodulated *Psychotria*, *Sericanthe*, and *Dioscorea* are endemic to Africa. In contrast, the recently documented *Styrax camporum* is the only nodulated species that endemic to the New World, Brazil, and South America.

2. The symbionts and the role of the symbionts

2.1. Identity and specificity of the symbionts

The identification of the bacteria of leaf nodule symbiosis is long to be a tempting and controversial question ever since the discovery of these plant-bacteria associations around 1900s [10]. Though many researchers tried to culture and identify the symbionts from the nodulated host plants, many of these isolated bacteria were not congruence with previous morphological observation and were often assigned to different genera (reviewed in [10]). Direct morphological observation of the symbiotic bacteria in all leaf nodulate species is rod-shaped or ovoid- to rod-shaped, Gram-negative, and without flagella [10, 11]. Thus, those isolated/cultured bacteria might not be the true endophytes in the leaf nodule, but contaminants during cultivation.

In the past two decades, much effort had been made in understanding the true identity of the symbionts and the evolution of the leaf nodule symbiosis relationships, through molecular identification. Based on the 16S rRNA sequences analyses, the bacterial symbionts were successfully identified in many nodulate species, and all belong to Gram-negative beta-proteobacteria. The symbionts of the nodulate species of *Ardisia*, *Pavetta*, *Psychotria*, and *Serricanthe* were identified as *Burkholderia* species, while the symbiont in the *Dioscorea sansibarensis* was identified as a novel genus and species, *Orrella dioscoreae* [12–16]. Molecular analyses also demonstrated that the phyllosperic endophyte community of all nodulate species is composed of only one specific bacterial species, which are congruence with the morphological observation. However, the symbionts identity and specificity of the *Styrax camporum* leaf glands remains obscure [11].

2.2. Phylogenetics of the symbionts

It is interesting that the symbionts in nodulate *Ardisia* and nodulated rubiaceous plants all belong to the genus *Burkholderia*. *Burkholderia* is an ecologically diverse genus, including both plant and animal pathogens, animal-, plant-, or fungus-associated species, and many free-living species from environment [17]. Phylogenetic studies showed monophyly of the

leaf nodule symbiotic bacteria identified from *Ardisia* species, which suggested a single origin of the leaf nodule symbiosis to *Ardisia* in *Burkholderia* clade [12, 18]. However, the relationships in symbiotic bacteria of nodulated rubiaceous species are much more complicated. The identified symbionts do not form a monophyletic clade corresponding to their host plant genera. The clade consists of symbiotic bacteria from all nodulated rubiaceous hosts that also includes the endophytes identified from non-nodulated *Psychotria* and another non-nodulated rubiaceous genus, *Globulostylis* and some environmental species [14, 15, 19–23] (also see Section 3.3). Other non-nodulated *Burkholderia* species in rubiaceous species together with some plant-associated beneficial and environmental (PBE) species form a monophyletic group. The currently known phylogenetic relationships of *Burkholderia* are showed in **Figure 2**.

The isolated symbiont from the leaf gland of *D. sansibarensis* was assigned to *Orrella dioscoreae*, which belongs to the family Alcaligenaceae in the order Burkholderiales of beta-proteobacteria [16]. The genus name, *Orrella*, is to honor M. Young Orr, who first described the leaf glands of *D. sansibarensis*. Strains isolated from different *D. sansibarensis* populations show limited phylogenetic and phenotypic variation, suggesting the bacteria-plant association in this plant is probably very specific.

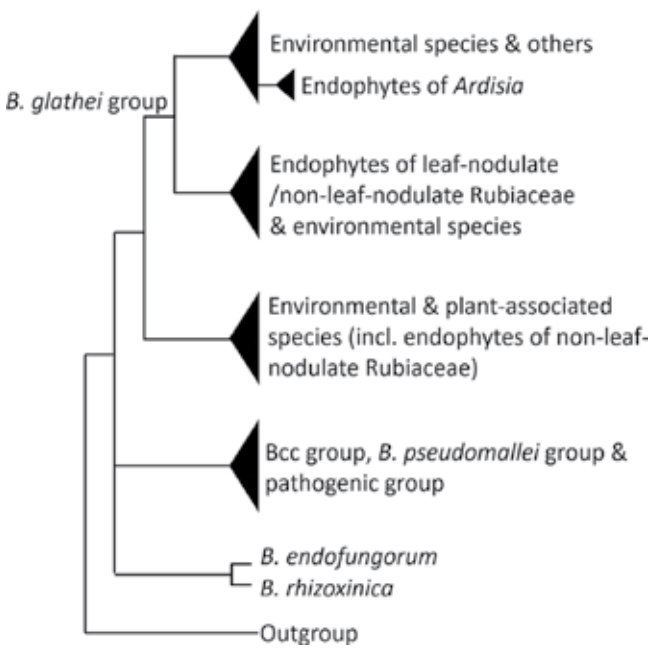

Figure 2. Phylogeny of the bacteria genus *Burkholderia* (modified from [20, 81]). Clades are summarized as triangles. The endophytic bacteria, or symbionts, of nodulate *Ardisia* are a monophyletic group, which embed in a clade consists of mostly environmental species and some symbionts of fungi and insects. The leaf-nodulate *Burkholderia* of the three Rubiaceae genera are not monophyly, respectively, which are mixed together with some endophytes of non-nodulate Rubiaceae, environmental species, and some symbionts of fungi, insects, and plants. Bcc group, *Burkholderia cepacia* complex.

2.3. The role of the symbionts

It has been long speculated for the role of the leaf nodule bacteria and if it is mutualistic. From an evolutionary point of view, it is reasonable to expect a mutualistic relationship in the vertically transmitted symbiosis like the case of the leaf nodule bacteria and their host plants [24]. In a mutualistic symbiosis, the host plants provide a shelter and metabolites for the endophytes. On the other hand, the endophytes may benefit host plants in various form, such as nutrients synthesis, growth regulators synthesis, stress resistance, and defensive metabolites production.

The first proposed function of the leaf nodule or gland symbionts was nitrogen fixation [25, 26], which was widely known from the root nodule association between rhizobia and legumes. However, all in planta studies so far showed negative results on nitrogen fixation, either by using the $^{15}N_2$ method or acetylene reduction test (reviewed in [10, 27]). Moreover, all authors claimed the leaf nodule endophyte can fix nitrogen based on the isolated bacteria that differs from the ones according to molecular identification. The nitrogen-fixing hypothesis was thus mostly ruled out in *Ardisia*, *Dioscorea*, and rubiaceous plants since the late twentieth century [10]. The lack of nitrogen-fixing-related genes in the symbionts genomic analyses in the currently sequenced genomes also showed disagreement of the hypothesis [19, 28, 29].

In *Ardisia* and *Psychotria*, evidence showed that if the associated bacteria were lost (or decrease to a limit amount, see below), the shoot tip would loss normal function, degenerated to callus (called "cripple" symptom or phenotype), and eventually died within a few years [25, 26, 30–32]. The symptom suggests that the endosymbionts may be responsible for plant normal growth and development, probably by producing hormonal substances. After the hypothesis was proposed, many plant hormones were specified as candidate [10, 25]. Among the various plant hormones, only cytokinin(s), or cytokinin-like substance, was better supported (reviewed in [10, 27]). However, until now there is no direct evidence that the leaf nodulate endosymbionts can produce cytokinin or cytokinin-like substance. In fact, all the evidence supporting the cytokinin-producing hypothesis was obtained by detecting high cytokinin concentration of leaf nodule and nearby tissue, or by the extraordinary need of cytokinin of the plant tissue. Moreover, none of the plant hormone producing genes could be found in all the symbiont genomes sequenced so far [19, 28, 29].

The cripple symptom of leaf nodulate plants were believed to be bacteria free because there are no bacteria in leaf nodule under microscope and no nodule formed on the abnormal leaf [25]. Crippled plants can be grown from seeds which occurred (1) naturally, (2) by either heat treatments, or (3) by antibiotics treatments. The crippled plants were widely used as bacteria-free plants in re-infection experiments and functional analyses. However, the crippled plants were reported to revert to normal state sometimes after a period of time, without additional treatment [27]. Because of the natural recovery of the cripple syndrome, some workers emphasized that crippled plants should not be used for re-infection experiment controls [31, 33]. It is thus reasonable to speculate that the crippled plants are actually bacteria-less rather than bacteria-free. If these plants are not completely bacteria-free, it is interesting to exam the conditions and mechanism how the cripple symptom occur and revert.

Defensive mechanism is another hypothesis for the role of leaf nodule symbiosis to hosts in *Ardisia* and rubiaceous plants. Neal and colleagues first reported the leaf toxicity of *Ardisia crenata* to insect herbivores, whereas the toxicity was not found in another leaf nodulated species, *Ardisia crispa* [34]. In contrast to the evidence in *Ardisia*, the chemical defensive hypothesis was better supported in rubiaceous species. A possible linkage between leaf toxicity and endophyte in two non-nodulate genera, *Fadogia* and *Vangueria*, were first revealed in Rubiaceae [35]. Later, the correlation between leaf toxicity and the presence of leaf endophyte was found both in nodulate and some non-nodulate rubiaceous plants [23, 36]. These results suggested that the nodulating endophyte and non-nodulating endophytes may play a similar role on synthesizing defensive chemicals. The defensive role of the symbionts is also supported by the recent genomic analyses (see Section 3.2 for *A. crenata* and Section 4.2 for rubiaceous plants). If the symbionts do serve a defensive role to its host, then it makes sense that removing nodules has no significant effect to next generation seedling growth in *Ardisia* [37]. Nevertheless, the defensive hypothesis has no explanation to the cripple symptom, which was believed causing by losing symbionts. It remains possible that the symbionts in *Ardisia* and *Psychotria* regulate the plant growth and development through unknown regulators, maybe through hormone, or by non-coding RNAs that regulating plant growth-related genes.

In *D. sansibarensis*, the functions of the symbionts were speculated to be beneficial and involved nitrogen fixing at first [38]. Other researchers considered the symbionts as parasites due to the associated bacteria is not always observed in the acumens [39, 40]. However, cultivation experiments showed the host plants grew slowly and looked fragile when the symbiotic bacteria are absent in their leaf glands, while the plants turned vigorous after the bacteria re-infection [32]. This result suggested that the symbiotic bacteria are beneficial to the host and the association is indeed mutualism. Nitrogen fixation in *D. sansibarensis* had not been detected, as in *Ardisia* and rubiaceous plants [10, 32]. However, the genomic analysis suggested that increasing stress tolerance should be the main function of the symbiotic bacteria in *Dioscorea* [16] (see Section 5).

The function of the bacterial symbiosis in *S. camporum* is still unclear. The *S. camporum* extract showed antioxidant and cytotoxic activities, which is a potential source for chemopreventive effect against carcinogenesis [41, 42]. Inspired by other leaf nodule symbiosis, the leaf glandular symbiont in *S. camporum* may also serve a defensive role, although some alternative hypotheses such as nitrogen fixation, plant regulatory hormone synthesis, and stress resistance also cannot be ruled out.

3. Leaf nodule symbiosis in Primulaceae

3.1. The occurrence, initiation, and development

Leaf nodule was found in three woody genera of Primulaceae such as *Ardisia*, *Amblyanthus*, and *Amblyanthopsis* in Myrsinoideae, in which it was formerly recognized as Myrsinaceae. The

genus *Ardisia* contains about 500 species all over the world. Many of them are economically used as ornamental plants and sources of traditional herb medicine. The leaf nodulated *Ardisia* are classified as subgenus *Crispardisia*, consisting of about 70 species, mostly in the Old World tropical and subtropical regions [43–45]. Both the genus *Amblyanthus* and *Amblyanthopsis* contain four species and are only found in Assam. Two species of *Amblyanthus* and three species of *Amblyanthopsis* have leaf nodules, however, none of them have been examined for bacterial symbiosis. The relationship of the three Myrsinaceae genera is still unclear. The leaf nodules in the three Myrsinaceae genera are ellipsoid or dotted structures that localizing on the margins of the leaves (**Figure 3A**, **C**). To be precise, the nodules are on the incisions of the crenation or, less commonly, forming tips of the dentation.

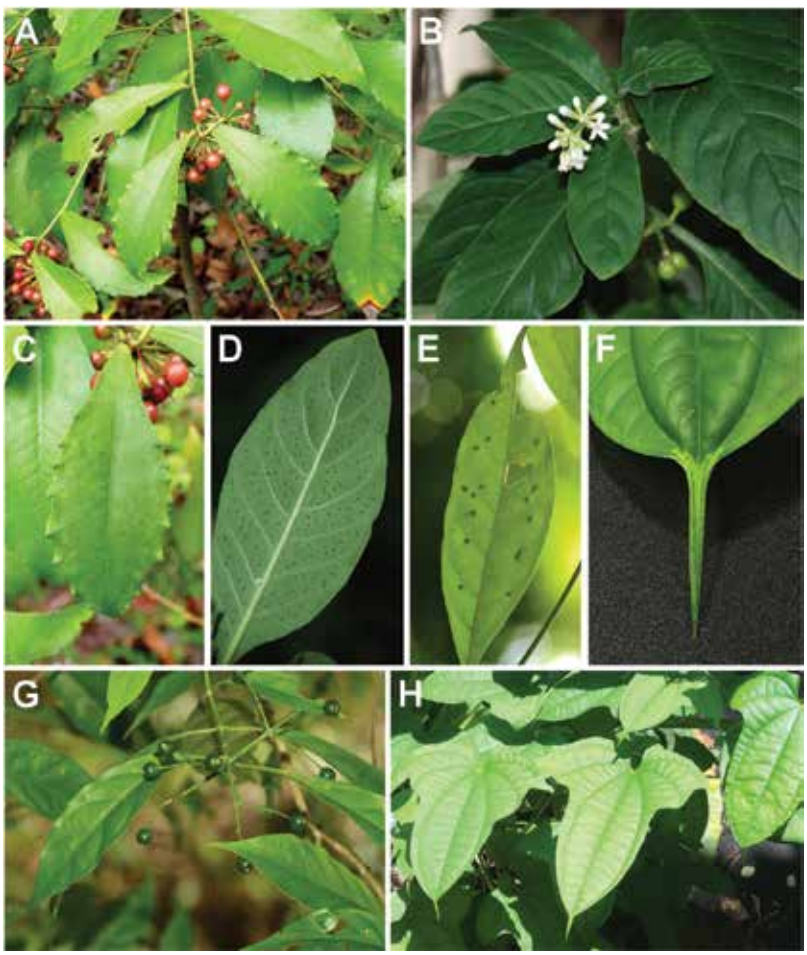

Figure 3. Examples of leaf-nodulated species. (A), (C) *Ardisia cornudentata* Mez (Primulaceae). The leaf nodules are located marginally, forming the tips of the dentation. (B), (D) *Psychotria kirkii* Hiern. (Rubiaceae). The leaf nodules are randomly distributed on the leaf lamina. (E), (G) *Pavetta* sp. (Rubiaceae). The leaf nodules are scarcely distributed on the leaf lamina. (F), (H) *Dioscorea sansibarensis* Pax. (Dioscoreaceae). The leaf apex is swollen and forms a gland.

The most well-known nodulate Myrsinaceae plant is *A. crenata*, or coral berry, which is widely cultivated for ornamental uses. The first description of the bacterial leaf nodule and most of leaf nodule symbiosis studies were demonstrated in *A. crenata* [27]. It is worth noted that *A. crenata* was previously misused as *Ardisia crispa* (Thunb.) A. DC., and most authors in studies before 1990 referred *A. crenata* using the name *A. crispa* [46].

Miehe was the first to describe the swollen structure on leaf margin of *A. crenata* as bacterial nodule [47]. In *Ardisia*, the symbiotic bacteria are observed not only in the leaf nodules but also in the shoot buds [26, 48–50]. The structure and development of the leaf nodules have been described in details [26, 48, 51, 52]. The structure and developmental processes are briefly introduced as below. The shoot bud of *Ardisia* contains a closed chamber forming by two to three tightly convoluted young leaves. The enclosed chamber is full of mucilage that is secreted by the trichomes on both sides of the young leaves. The symbiotic bacteria are harbored within the chamber and supported with the nutrient-rich mucilage. The leaf primordium is immersed in the mucilage until the bud opened. As the leaf initiates and develops, the primordium grows and elongates inward to form a small chamber with some bacterial mucilage. As the leaf matured, some early forming or "precocious" stomata-like pores (or premature hydathode pores referred by some authors) on the leaf margin open and trap some bacterial mucilage to form the nodules in a lysischizogenous manner. At the final stage of nodule maturation, a distinct and sharp boundary of the external vascular sheath and the internal bacterial region can be clearly observed. The surrounding vascular bundle of the nodule indicates that the symbionts could exchange substances with their hosts, and the symbionts probably could produce and translocate certain substances that are beneficial to the host plants. Many of the bacteria in the mature nodule of *A. crenata* were observed to be pleomorphic, as well as in *A. kusukusensis* (**Figure 4**) [18, 47, 52].

In leaf nodulate *Ardisia*, the symbionts were also observed within reproductive tissues and seeds [26, 48, 49]. Based on the distribution of the symbionts on the plant body, the relationship

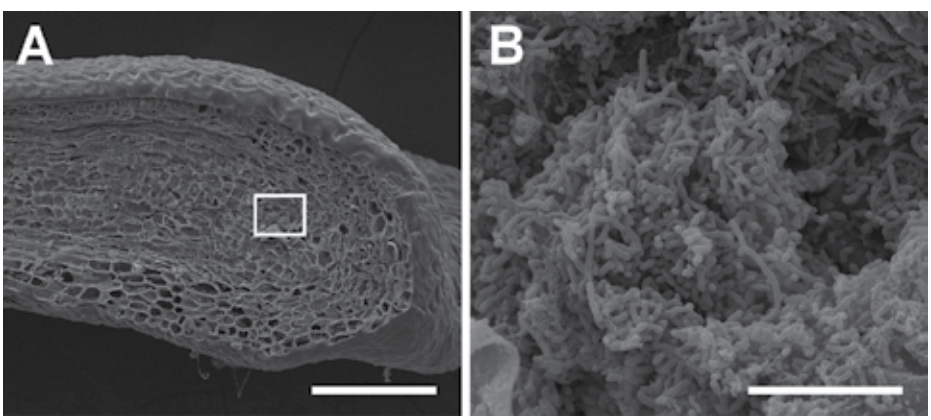

Figure 4. Scanning electron micrographs of a leaf nodule of *Ardisia kusukusensis* Hayata. (A) Cross section of a mature leaf nodule. (B) Rod-shaped endosymbiotic bacteria in the leaf nodule, enlarged from the square region in (A). Bar = 200 μm in (A) and 10 μm in (B). Photos provided by Chuan Ku.

between the symbionts and their *Ardisia* host was speculated to be cyclic, from generations to generations [10]. The inflorescence primordium of *Ardisia* is protected by a small protoleaf, which is functionally a protective bract. The bract is later rolled-up to form a chamber-like structure just as in the vegetative bud. The inflorescence primordium is immersed in the chamber filling with bacterial mucilage secreted by trichomes on the adaxial surface of the bract. In the early stage of flower development, the calyx develops and forms a new compartment that encloses the rest of flower primordium, and traps some bacterial mucilage inside. The bacterial mucilage then flows into the embryo sac of each ovule and eventually be incorporated into seeds afterwards. The embryo is thus localized in the seed cavity, filling with the bacterial mucilage that is secreted from the trichome on adaxial surface of the cotyledons. When seed begins to germinate, the first true leaf bends backward and roll inward, enclosing the primordium and some bacterial mucilage. Thus, the first shoot bud of the seedling forms and a new life cycle continues.

Interestingly, Gram-negative bacteria were also observed inside the ovary of *Myrsine laetevirens* (also in Primulaceae), a neotropical dioecious tree [53]. The flowers of *M. laetevirens* develop in a similar pattern as in *Ardisia*, and the bacterial mucilage is observed in every stage as pistillate flower development, including the micropyles of ovules. However, the bacteria are absent in staminate flowers, though the mucilage-secreting trichomes is observed. Although the mechanism of bacteria transmission to the embryo sacs is similar with that in *Ardisia*, leaf nodules are absent in *M. laetevirens*. It seems the bacteria are also harbored in the buds in *M. laetevirens*, but it remains unclear whether the bacteria are also present in the leaf or other tissues. In contrast to the non-nodulating rubiaceous plants, the plant-endophyte association of non-nodulating Myrsinaceae plants received much less attention. It is interesting to comprehensively exam whether the associated bacteria are common in non-nodulating Myrsinaceae plants. The identity of the *M. laetevirens* endosymbiotic bacteria and the relationship with other symbiotic bacteria in Myrsinaceae plants are unresolved.

3.2. The origin, phylogeny, and genomics

The *Ardisia* phylogeny showed that the nodulated species form a well-supported monophyletic group, which suggested that the leaf nodule symbiosis only occurred once in *Ardisia*, corresponding to the subgenus *Crispardisia* [12, 18]. Together with the symbionts phylogeny, the origin of the *Ardisia-Burkholderia* association probably evolved only once, both in *Ardisia* and in *Burkholderia*. The estimated origin time of the leaf nodule was about 5 Mya [54]. Cophylogenetic analyses showed weak evidence for *Ardisia-Burkholderia* co-speciation. At least two events of host switching, or horizontal gene transferring, have been postulated based on the comparisons of the bacteria-host phylogenies [12, 18].

The genome of the *Cadidatus* Burkholderia crenata (using "*Cadidatus*" here referring the bacterium is yet to be cultured, abbreviate to "*Ca.*") was sequenced recently [29]. The genome size was estimated of 2.85 Mb with one chromosome and two plasmids, based on genome assembling of the next genomic sequencing. However, the result is incongruence with the estimation made by our unpublished data [55]. The estimated genome size and composition

of the *Ca*. *B. crenata* and *Ca*. B. polysticta by the aforementioned researchers were both around 4.7 Mb with two chromosomes and two large plasmids, based on gel electrophoresis methods. Even if the true genome of *Ca*. *B. crenata* size is around 4.7 Mb, it is smaller compared to the free-living *Burkholderia*. The reduced genome size and low coding capacity suggest that *Ca. B. crenata* have adapted to a symbiotic life form. The genomic analysis further indicated that *Ca. B. crenata* has lost many essential genes, which should be a result of reductive evolution. Genomic analysis of these bacteria did neither identify nitrogen-fixing-related genes, nor the plant hormone-related genes. However, the incongruent genome size estimation between the assembled genome and gel electrophoresis based estimates suggest the completeness genomic sequences can be improved, and more leaf nodule symbionts genomes of other *Ardisia* would be helpful to draw concrete conclusion.

Nonetheless, two gene clusters related to polyketide and non-ribosomal peptide synthesis were found on the plasmids. The gene clusters have lower GC content and are flanked with transposable elements, suggesting a recent acquisition via horizontal gene transfer. Further studies showed that one of the gene clusters may be correlated with the synthesis of FR900359, a cyclic depsipeptide with potential biomedical application. This result suggested that the symbionts of *A. crenata* may in fact serve a pathogen-defense role for the host.

4. Leaf nodule symbiosis in Rubiaceae

4.1. The occurrence, initiation, and development

The bacterial leaf nodule occurs in three genera of Rubiaceae, that is, *Psychotria*, *Pavetta*, and *Sericanthe*. These three genera belong to different tribes that have no close phylogenetic affinity within Rubiaceae [56]. *Psychotria* belongs to subfamily Rubioideae, while *Pavetta* and *Sericanthe* belong to different tribes of subfamily Dialypetalanthoideae. The shape and distribution of the nodules on the leaves are divergent among genera and species. In general, the leaf nodules of *Psychotria* and *Pavetta* are punctate to ellipsoid scattered, rarely shortly linear (**Figure 3B**, **D**, **E**, **G**), while the nodules of *Sericanthe* are punctate to linear or branched along the mid-vein or scattered on leaves.

The genus *Psychotria* (syn. *Apomuria*) contains about 1850 trees, shrubs, subshrub, or liana species, distributing through tropical and subtropical regions. The *Psychotria* species with leaf nodule (about 80 species) are only found in Africa, mostly in southeastern part and surrounding islands.

The genus *Pavetta* comprises about 400 species of trees, shrubs, or subshrubs, distributing in Africa, tropical Asia, Australia, and Pacific islands. *Pavetta* contains about 350 species with leaf nodule, which is the largest number among the leaf nodulate genus. The leaf nodulate *Pavetta* species are found around the entire geographic range of the genus.

The African genus *Sericanthe* is composed mostly of shrubs, with about 21 species in southern and western Africa [57]. The genus *Sericanthe* was formerly referred to the genus *Neorosea*,

which was separated from the genus *Tricalysia* (see detail in [58]). Leaf nodules have been discovered in about 13 species of *Sericanthe*. The leaf nodules of *Sericanthe* are only visible on the abaxial side of the leaves.

The bacterial leaf nodules in Rubiaceae were first described as bacterial nodule and studied in 1902 [59]. In Rubiaceae, bacterial leaf nodule symbiosis was hypothesized to be obligate and cyclic in *Psychotria* and *Pavetta* [10]. In other words, the associated bacteria and the host plants cannot survive without each other, and the symbionts are retained in the host plant in all stages of its lifecycle. The symbiotic bacteria of the leaf nodulate rubiaceous plants are maintained in the mucilage secreted from dendroid colleters, a type of multicellular secretory trichome, in both apical and lateral buds [48, 60–62]. The nature and development of the leaf nodulate rubiaceous plants are briefly introduced below.

In the bud of *Psychotria* shoot apex, each pair of young leaves develops in a chamber formed by two pairs of stipules. The chamber is filled with mucilage that secreted by the branched colleters on the adaxial side of the stipules. The symbionts are maintained in the chamber and nurtured by the mucilage. During the leaf maturation, the bacteria enter the leaf tissue through precocious stomata on the abaxial side of young leaf and the sub-stomatal chamber begins to develop into a leaf nodule. As in *Ardisia*, the floral development in *Psychotria* is initiated from the mucilage-filled shoot bud [10]. The inflorescence primordium is enclosed by the chamber formed by circulate bracts with colleters adaxially. As each floret development, evidence shows that some mucilage is enclosed by the developing carpels and then the bacteria are eventually housed in the ovary. However, the detail mechanism of how the symbionts transferred to the embryos remains unclear in *Psychotria*. It was speculated that the bacteria may enter the embryo sac at the pollination stage where the bacteria are pushed into the embryo when the pollen tube penetrates micropyle.

In *Pavetta*, the symbionts are postulated to be maintained in shoot apex, leaf nodule, ovules, and seeds, but the complete life cycle of the bacterial symbiosis is not yet described [48]. The mechanism of bacteria maintenance in shoot buds and inflorescence primordium is similar to the case of *Psychotria*, also the nodule development in leaves. In *Pavetta*, the inflorescence buds are developed in the chamber formed by the circular stipules and immersed in the bacterial mucilage that secreted by colleters. As the floret develops, some bacterial mucilage is enclosed in the ovary and the bacteria are maintained by the mucilage secreted by the aril-like tissue at the base of each ovule. The details of how bacteria enter the embryo sac in *Pavetta* has not been observed and the same speculation as in *Psychotria* was made. The bacteria are also found in the mucilage around the cotyledons of the embryo in *Pavetta* seeds, which is failed to observe in *Psychotria*. However, the associated bacteria are found in the seedling of *Ps. kirkii*, suggesting the bacteria may retain in elsewhere of the seed rather than around the embryo [10].

Study of nodule structure and development of *Sericanthe* was only demonstrated in the species *S. andongensis* [63]. Mature nodules of *S. andongensis* are linear, and are localized on both sides of the petiole and mid-vein. The bud structure and nodule initiation of *S. andongensis* is similar to those of *Psychotria* and *Pavetta*. It is noteworthy that the "pseudonodules", leaf nodules without bacteria inside, were observed in *S. andongensis*, as well as in *Ardisia* and

Pavetta species [25, 48, 63]. The results suggested the nodule in *Ardisia* and rubiaceous species could initiate the nodule development spontaneously rather than induced by the symbionts. Alternatively, the symbionts in this "pseudonodule" were present, but dead afterwards, or even may be digested by the host plants [27]. The complete life cycle of the leaf nodule symbiosis in *Sericanthe* is also unclear. It is not known whether the symbionts are present in the ovaries and/or the seeds. Thus, whether the symbiosis is cyclic in *Sericanthe* as that in *Pavetta* and *Psychotria* remains a question.

The structure and development of leaf nodule and the mechanisms of maintaining bacteria in shoot bud of the three genera in Rubiaceae are highly similar, which is a obvious result of convergent evolution. However, the opening the precocious stomata for bacterial infection of the leaves and the shape and distribution of the leaf nodules are different among the three genera. The stomata of leaves open adaxially in the process of nodule formation in *Pavetta* but open abaxially in *Psychotria* and *Sericanthe*. The shape and distribution pattern are variable between species, while a general pattern is described as above.

It is important to note that nodulation is not required in endophytic growth of bacteria in plants. Bacterial leaf endophytes are also found in non-nodulated Rubiaceae [64, 65], as well as in many angiosperms (such as in *Vitis* [66]). It is interesting that the endophytic *Burkholderia* was now known being widespread in the leaves of five non-nodulated rubiaceous genera, which are all in the tribe Vanguerieae of Rubiaceae [20, 21, 23]. None of these host plants showed an external sign of infection. The leaf endophytic *Burkholderia* was also found in non-nodulated *Psychotria* species [22]. The preference of the *Burkholderia* species forming a leaf endophyte association with rubiaceous plants is still a mystery, but definitely a key to understand the origin of the leaf nodule symbiosis.

4.2. The origin, phylogeny, and genomics

The ages of the origin of the leaf nodule evolution in *Psychotria*, *Pavetta*, and *Sericanthe* were estimated at about 9, 4, and 3 Mya, respectively [54, 67]. In *Psychotria*, the phylogenetic analyses showed ambiguous results by different authors that the leaf nodule evolved once or twice within the genus, and at least one secondary lost event was detected [22, 68]. Non-nodulated *Psychotria* forms an independent monophyletic clade in the genus, which is separated from the nodulated clade [22]. However, not all members in the clade harbors bacteria in their leaves, suggesting the non-nodulated *Psychotria-Burkholderia* association may be an unstable relationship between generations and/or individuals. In *Sericanthe*, the leaf nodule symbiosis may have a single origin, in spite of the phylogeny based on plastid genetic markers is poorly resolved [14]. A representative phylogeny of the members in *Pavetta* is not available so far, thus the origin and evolution in *Pavetta* is still unclear.

Horizontal gene transfer events occurred frequently between the leaf nodule symbionts of Rubiaceae. Both evidence of population genetics and whole genome study support the frequent genetic exchange hypothesis [19, 69]. However, the mechanism that how the symbiont exchanges their gene from the cyclic symbiosis system and how the association changes their partner is unknown.

The genome sizes of the sequenced symbiotic *Burkholderia* species from seven *Psychotria* species and a *Pavetta* species are around 2.4–6.2 Mb, which are relatively small in comparison of free-living plant-associated *Burkholderia* [19, 28]. All of these bacterial genomes contain large proportion of pseudogenes and transposable elements, referred to "eroded genomes". Both the genomic size and composition indicate that the leaf nodule symbioses of rubiaceous plants are at an early stage of transition from free-living to host-restricted lifestyle. The genomic features are common in some recently evolved and vertically transmitted symbionts, such as the obligate cyanobiont of *Azolla filiculoides*, the bacterial symbiont *Serratia symbiotica* of pea aphids (*Acyrthosiphon pisum*) and conifer aphids (*Cinara tujafilina*) [9, 70, 71]. The essential housekeeping genes are mostly intact in the sequenced leaf nodule symbiont genomes despite of genome reduction, suggesting that these symbionts may not be dependent on the host for essential housekeeping functions.

The results of genomics, transcriptomics, and proteomics analyses revealed the capability of kirkamide synthesis in *Ca. B. kirkii*, the symbiont of *Ps. kirkii* [28, 72]. Kirkamide is a kind of C_7N aminocyclitol, which is a cytotoxin to insects and aquatic arthropods [73]. These results suggested that the leaf nodule symbionts may serve a defensive function to the host plants. Further studies sequenced genomes of the leaf nodule symbionts from seven *Psychotria* species and a *Pavetta* species, also showed the presence of these putative genes involved in kirkiamide biosynthesis pathway [19]. However, many genes are not intact, that is, as pseudogenes, in the leaf nodule symbionts genomes, indicating that producing kirkamide might not be necessary for the host plants. These kirkamide synthetic genes are unique to the rubiaceous leaf nodule symbionts in comparison to the related *Burkholderia* species associated with other plants. Interestingly, the gene cluster is often located on a plasmid of the symbiotic bacteria, and sometimes flanked by transposon-like fragments, suggesting that these genes may be acquired from horizontal gene transfer [19]. With the current understanding from the genomic studies of the rubiaceous leaf nodule symbionts, the reasons for the seemly obligate relationship still could not been readily answered.

5. Leaf gland symbiosis in Dioscoreaceae

The only case in monocots that bearing species with bacterial leaf gland symbiosis is found in Dioscoreaceae. This family is representing by the genus *Dioscorea*, the true yams, which comprises about 90% species of the family. Only one species, *Dioscorea sansibarensis*, was reported to have bacterial glands on leaf apexes [10]. The Zanzibar yam, *D. sansibarensis* (syn. *Dioscorea macroura*), is a fast-growing vine that native to tropical Africa and Madagascar, and it is widely introduced and cultivated in many regions all over the world. *Dioscorea sansibarensis*, like most true yams, produces perennial underground tubers and aerial bulbils, which is the main reproductive organs of the species. Leaves of *D sansibarensis* are large and roughly heart-shaped, with a conspicuously caudate apex or acumen (**Figure 3F**, **H**). Orr was the first who discovered the acumens are in fact full of bacteria and should be regarded as bacterial leaf glands [38].

The bacterial symbiosis of *D. sansibarensis* is not obligate because the plants can survive without the symbionts, and it is likely the host plants acquire the symbionts from environment, and the symbionts can also survive without the host plants for at least part of their leaf cycle [32]. Thus, it is not surprised that the associated bacteria in *Dioscorea* are so far the only leaf-nodule-associated species that can be cultured in ex situ condition. According to the microscopic view of the bacteria, the symbionts are non-motile, non-spore forming, ovoid-rod, Gram-negative bacteria [10, 16, 32]. Although several studies claimed they successfully cultured the symbiotic bacteria from *Dioscorea* in the past century [10], the true identity is not revealed until 2016 (i.e. *Orella dioscoreae*, see Section 2.2). Surprisingly, the symbiont samples from various localities have been shown to be the same bacterial species [16]. It suggested that somehow the specificity still retains to a certain degree between the symbionts and *D. sansibarensis.*

The initiation and development of the bacterial symbiosis of the leaf glands has been studied in details in *D. sansibarensis* [32, 38–40]. The symbiosis first initiates during the development of the leaf acumen, which is apparently thicker than the leaf laminar in mature leaf. In the early stage of leaf growth and expansion, the margins of the acumen are swollen and bending inward to form an enclosed channel. The cavity becomes flask shaped as the two flanges develop. The central portion of the cavity then elevates to the level of the epidermis, separating the channel into two cavities. Secretory trichomes are also developing in the cavity and fill the whole space at this stage. The two cavities remain open to the external environment until the last stages of leaf maturation. In the maturation stage, the closed lumen is occupied by the mucilage secreted by the trichomes and the rapidly multiplied bacteria. Interestingly, the glandular acumen of *D. sansibarensis* is developed even the symbiotic bacteria are not present, but the glands are not as swollen as the bacteria-infected glands. Also, no mucilage is produced in uninfected glands and the trichomes in the gland lumen tend to degenerate. The results indicated that the development of glandular acumen is not induced by the symbiont, while the maintenance of the mucilage production and trichomes activity does require the cues from the bacteria. However, several questions remain obscure, for example, do the bacteria vertically transmitted in *D. sansibarensis*, if so, through seeds or bulbils? Also, do the plants acquire the bacteria from the environment in each generation, if so, how do the bacteria live with the facultative strategies in the dynamic environment?

In contrast to the leaf nodule symbiosis in other families, the bacteria-host relationship in Dioscoreaceae seems not so intimately associated. In Primulaceae and Rubiaceae, the bacteria are both harbored at the shoot apex and can be transmitted to the next generation through seeds; and, the symbiotic bacteria pass through the cuticle and invade plant tissue in some degree. However, neither the symbiont in Dioscoreaceae is found at the shoot apex, nor any invasion to host plant tissue is observed. Moreover, the symbiosis in Primulaceae and Rubiaceae are regard as obligate, while the association in Dioscoreaceae seems to be facultative. Therefore, the symbiosis of Dioscoreaceae was suggested to be a more primitive form of symbiosis than the leaf nodule symbioses in Primulaceae and Rubiaceae [32].

Dioscorea sansibarensis is by far the only known species with leaf gland in *Dioscorea*. There may be more *Dioscorea* species bearing the bacterial leaf gland symbiosis, such as *D. cochleari-apiculata*

and *D. dodecaneura* [10]. However, to our knowledge, no other bacterial leaf gland symbiotic species was formally reported except *D. sansibarensis*.

The genome of the symbiont of *D. sansibarensis*, *O. dioscoreae,* has been sequenced recently, which is about 5 Mb in size and is composed of a single chromosome without plasmid [16]. Based on the sequence data, the nitrogen-fixing-related genes are not found in the symbiont genome, so does the plant hormone gene associated with auxin or cytokinin biosynthesis or metabolisms. However, an ethylene signaling modulating gene, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, was identified in the genome. Some plant-associated bacteria can help plants to increase stress tolerance by producing the ACC deaminase, which can decrease the level of "stress ethylene" that inhibit plants growth [74, 75]. The discovery of the gene of ACC deaminase in the genome of *O. dioscoreae* provides clues for further study on the function of the symbiosis relationship to plants. Furthermore, genomic analysis showed similar features to many facultative anaerobic, free-living bacteria, and little effects of the interaction with host plants on the bacterial genome, suggesting the symbiosis association may be very young or facultative. It makes sense because the symbiotic bacteria of *D. sansibarensis* are epiphytes of phyllosphere in at least part of their life cycle.

6. Leaf gland symbiosis in Styracaceae

The leaf nodule or gland symbiosis has been known restricted in Primulaceae, Rubiaceae, and Dioscoreaceae for over a century. Until 2014, a newly found leaf-nodulated taxa was reported in *Styrax camporum* [11]. *Styrax*, known as storax or snowbell, is a small genus containing about 130 species of large shrub or small trees in the family Styracaceae. *Styrax* is mostly found to warm temperate to tropical regions in eastern and southeastern Asia and South America [76]. Stellate or peltate trichomes are common in *Styrax* species, while the glandular trichomes are rarely observed [76]. The glandular structure, sometimes refers to trichome, of *S. camporum* was found on young shoots and mature leaves, producing sticky secretion [11]. Unlike other cases of leaf nodule or gland which form swollen structures of part of leaf blade, the leaf gland of *S. camporum* is a glandular trichome-like structure with a nonsecretory short stalk and a secretory glandular body. The mature gland body is composed of a single layer of secretory cells around the axis. The actively secreting glands are distributed on top of leaf primordia and mature leaves. As the leaves maturation, the glands dry up and fall off except those on the petiole and leaf margin. Mature glands are turgid, irregular, pale yellow, and secreting mucilage, while the glands turn dark brown and shrink when senesces. The secreted mucilage covers the leaf primordia and young leaves at the shoot apex. Bacteria are observed immersed in the mucilage of the gland surface and intercellular space of the gland body. The associated bacteria are rod to ovoid shape with capsule. The sieve elements were observed in the stalk of the glands, which suggesting the transportation of some substances from the gland toward other tissues.

Unfortunately, little is known about the newly discovered leaf gland symbiosis in *Styrax*. The complete life cycle and nature of the bacterial symbiosis in *Styrax* remain obscure, as well as

the symbiont itself. For instance, does the bacterial leaf gland symbiosis also occur in other *Styrax* species? If so, does the associated bacteria specify to particular host species? Does the bacterial gland also occur on the reproductive tissue? If so, how do the glands develop on the reproductive tissue? Can the associated bacteria transmit to the seeds and seedling, as in *Ardisia* and *Psychotria*? If so, how? What is the main function of the symbiont serve to the host plants? In all, the identity and specificity of the associated bacteria and the function to the host plants are important issues, which are easy to achieve nowadays by the modern genome sequencing.

7. Conclusion, application, and future research

The leaf nodule symbiosis is the only case of the cyclic plant-microbe symbiosis with specialized structure in flowering plants. However, there are lots of knowledge gaps to be filled for such unique associations of plants and the symbiotic bacteria. The leaf nodule symbiosis is probably all cyclic in the examined species, except of *Dioscorea*, while only weak evidence was found in *Sericanthe* and *Styrax*. It is important to verify if it is true cyclic symbiosis in these cases, from the evolutionary aspects, and better our understanding on plant-microbe interactions. Three factors are necessary to confirm the presence of cyclic leaf nodule symbiosis. First, the symbiont should be able to maintain in the shoot apex through the nutrient-rich mucilage secreting from the specialized trichomes. Yet, having an enclosed chamber in the shoot apex is not necessary. Second, the symbionts could infect the young leaf and form mature leaf nodules or glands during the leaf development. Third, the symbionts have to enter the carpel and embryo, to form the symbiont-containing seeds. Despite much effort have been done by previous researchers, many details of the life cycle of these leaf nodule symbioses are still unclear, especially about the mechanisms of transferring the bacteria between different life stages of the host plant. The morphology of leaf nodules is usually diverse among species within the genus, except for the rather simple cases in *Dioscorea* and *Styrax*. The initiation and development of the leaf nodule symbiosis, however, are only observed in one or few species of each genus, leading to the questions on whether they are consistent between different species with different nodule morphologies.

Many symbiont genomes have been sequenced recently, but the interaction between the symbionts and host plants at molecular level is not well demonstrated. For instance, the mechanism of how the host plants prevent from the symbionts invasion and restricting the symbionts in the nodules or glands are unclear. The direct evidence of how the symbionts benefit to the host plants is also absent, and it is undoubtedly one of the most important knowledge for understanding the ecological evolution and agricultural application for the plants with the leaf nodule symbiosis.

In the leaf nodule symbiosis, the symbiotic bacteria retain in the plants cyclically as a permanently partner, which is a potential system for improving crops through genetic engineering and manipulating. In application, to design a plant-bacteria cyclic symbiosis system would be very useful for delivering the growth promoters, extra nutrients, pesticides, and so on, and the leaf nodule is even not necessary for the system. Several *Dioscorea* species are important

agricultural crops in tropical regions. The crop is threatened by various insect pests, fungi, viruses, and nematodes [77, 78]. The bacterial symbiosis in *Dioscorea* is a potential copartner for improving the crop against the pathogens. In *D. sansibarensis*, the bacteria are culturable, which means to modify bacterial genome or to insert particular gene fragments are feasible. For instance, inserting the pesticide synthetic genes to against the pathogens and herbivores may elevate the yield and lower the cost of pesticide using by farmers. In addition, it could also lower the environmental concerns often raised by the GMO crops since the crop itself does not contain a modified genome. In *Dioscorea*, the bacterial symbiosis is so far only found in *D. sansibarensis*, but it is possible that the same association will be found in other *Dioscorea* crop species or the bacteria could be used to infect other crop species.

In *A. crenata*, one of the peptide with biomedical application was found in fact contributed by the leaf nodule symbionts [29]. To the authors experience, *A. crenata* grows slowly and the growth condition is somewhat demanding. If the symbiont could be isolated and cultured in the future, synthesizing and purifying the peptide will be much faster and space-efficient. Even if the symbiont could not be cultured after all, the gene cluster can be inserted to another operable and culturable bacteria. The results might be able to benefit other *Ardisia* species since many of them are important Chinese medicinal plants [79].

The genomics of the leaf nodule symbionts have been studied in most genera, except of *Sericanthe* and *Styrax*. However, many questions are awaiting to be answered with the symbiont genomic sequences. One of the most interesting questions is the hypothetical "obligate symbiosis" relationships in *Ardisia* and rubiaceous plants. The symbionts genomes give no explanation for the dependence of the symbionts from the host plants. Clearly, there are more to be explored with the genomic or metabolomics data.

The cause of the leaf nodule initiation and development, as well as the maintenance of the symbionts in the shoot bud is poorly known at molecular level so far. In *Dioscorea*, the leaf gland seems being able to develop spontaneous without the symbionts cue. However, the cues of the leaf nodule development in other nodulate plants are poorly known. In rubiaceous nodulate plants, the "pseudonodule" has been observed, but the formation of "pseudonodule" may be caused by fading out of the symbionts. To compare the genomics or transcriptomics data of nodulated *Psychotria* and non-nodulated *Psychotria* is a possible method to find out the candidate genes that regulating the leaf nodule development and the mucilage-secreting trichomes, which are important determinants of the cyclic leaf nodule symbiosis. Taken these together, we shall be able to shed light on the intriguing phenomenon of symbiosis between the plants and the bacteria lived intimately in their leaves.

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Chapter 6

Potential of Rhizobia in Improving Nitrogen Fixation and Yields of Legumes

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Additional information is available at the end of the chapter

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Abstract

Strong demand for food requires specific efforts by researchers involved in the agricultural sector to develop means for sufficient production. While, agriculture today faces challenges such as soil fertility loss, climate change and increased attacks of pathogens and pests. The production of sufficient quantities in a sustainable and healthy farming system is based on environmentally friendly approaches such as the use of biofertilizers, biopesticides and the return of crop residues. The multiplicity of beneficial effects of soil microorganisms, particularly plant growth promotion (PGP), highlights the need to further strengthen the research and its use in modern agriculture. Rhizobia are considered as PGP comes in symbiosis with legumes taking advantage of nutrients from plant root exudates. When interacting with legumes, rhizobia help in increased plant growth through enriching nutrients by nitrogen fixation, solubilizing phosphates and producing phytohormones, and rhizobia can increase plants' protection by influencing the production of metabolites, improve plant defense by triggering systemic resistance induced against pests and pathogens. In addition, rhizobia contain useful variations to tolerate abiotic stresses such as extreme temperatures, pH, salinity and drought. The search for rhizobium tolerant strains is expected to improve plant growth and yield, even under a combination of constraints. This chapter summarizes the use of rhizobia in agriculture and its benefits.

Keywords: rhizobia, PGP, biocontrol, induced resistance, stress tolerance

1. Introduction

Agricultural productivity is significantly affected by nitrogen and phosphorus deficiencies, which are essential for plant growth. In addition, it is related to the physical and biological

properties of the soil, pest and disease attacks and abiotic stresses. For sustainable agriculture, it would be interesting to carry out an efficient management of nitrogen in the environment. This usually involves the use of microorganisms biologically fixing nitrogen that is used directly by the plant and is, therefore, less susceptible to volatilization, denitrification and leaching. In agricultural settings, perhaps 80% of this biologically fixed N comes from symbiosis involving leguminous plants and one of the Rhizobia species [1]. Legumes are able to establish a symbiotic *interaction* with soil bacteria termed *Rhizobia. These bacteria in association with legumes can* fix atmospheric N and through this feature, they are introduced into agricultural systems to improve soil fertility, plant growth and limit the use of chemical fertilizers [2]. However, the anticipated benefits of the nitrogen fixing bacteria may be positive or negative depending on rhizobium species and its interaction with the environment [3]. Isolation and selection of rhizobia stress-tolerant strains may enhance the plant growth through nodulation and nitrogen fixation ability of plants under stress conditions [4]. Selection of effective *Rhizobium* strains is the most critical aspect to have maximum benefits from this technology [5]. Regardless of their functions in direct plant growth promotion, rhizobia can act by protecting

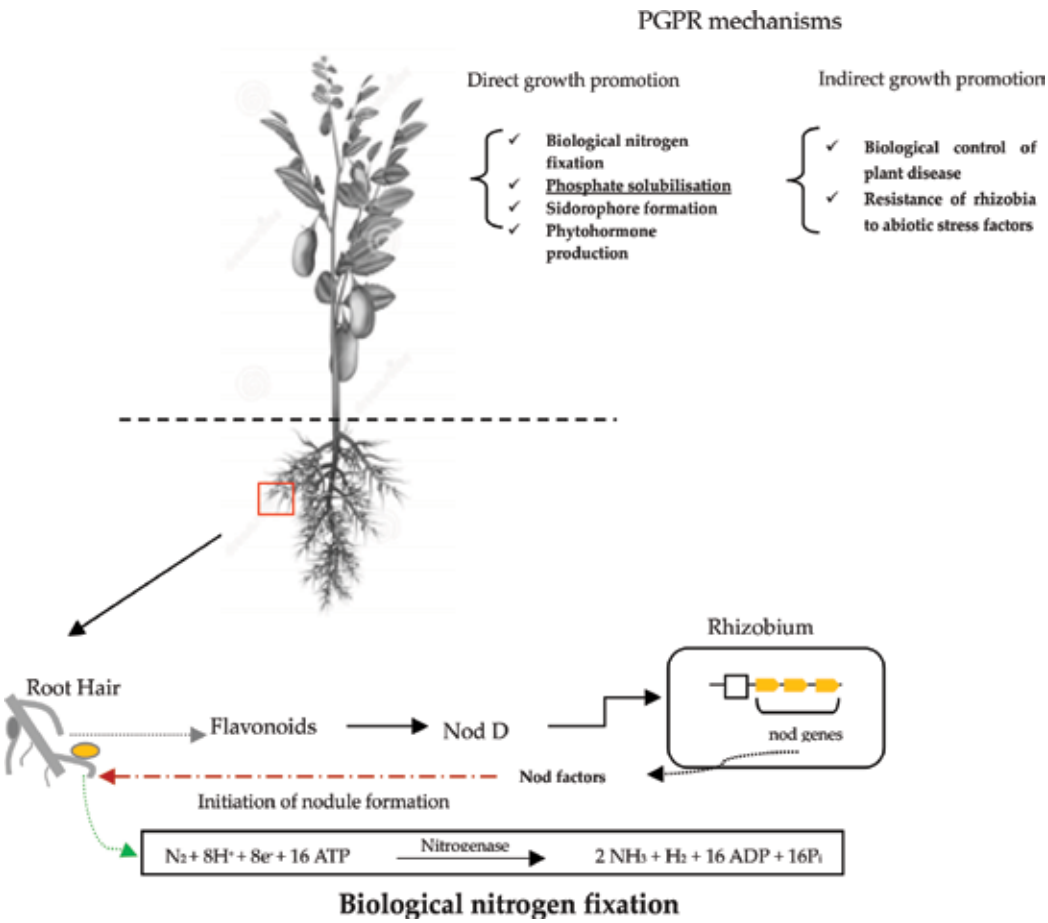

Figure 1. Schematic overview of the nodulation process and plant growth promotions by rhizobia.

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host plant against pests and diseases. Different mechanisms can be involved in plant protection by rhizobium inoculation such as competition for nutrients, antibiosis or induced resistance in host plant. This chapter presents an overview highlighting the enhancement of plant growth by rhizobia. Different mechanisms of plant growth promotion by rhizobia were described. Rhizobia can act directly by facilitating plant nutrients acquisition or influencing plant hormone levels or indirectly by attenuating the inhibitory effects of pathogens (**Figure 1**).

2. Plant growth promotion by rhizobia inoculation

Rhizobia can enhance plant growth promotions by both direct and indirect ways. Several mechanisms are involved in the plant growth promotions by rhizobia, different mechanisms involved are discussed as follows.

2.1. Direct plant growth promotions

2.1.1. Biological nitrogen fixation

Nitrogen is a vital element for plant growth; it is required for synthesis of macromolecules such as amino acids, nucleic acids and chlorophyll. In agriculture, fertilization with nitrogen products is practiced to increase the production yield of food [6, 7]. About 78% of the atmospheric air is N, this gaseous substance cannot be used in this form by most living organisms until it has been fixed, that is, reduced (combined with hydrogen) to ammonia. Biological nitrogen fixation (BNF) accounts for about 60% of nitrogen used in agriculture. Significant growth in fertilizer-N usage has occurred in both developed and developing countries [8]. The requirements for fertilizer-N are predicted to increase further in the future [9]; however, the use of high doses of fertilizers has a negative and unpredictable impact on the environment and contaminates the soil, water and natural areas. These effects are considered a threat to human and animal health affecting the quality of life. In addition, developing countries must use cheaper and environmentally friendly alternative methods. Legumes are BNF capable and meet their own needs. The use of legume crops substantially reduces the N requirement from external sources [10]. For more than 100 years, BNF has commanded the attention of scientists concerned with plant mineral nutrition, and it has been exploited extensively in agricultural practice [11]. However, its efficiency varies, and depends on the host genotype, rhizobial efficiency, soil conditions and climatic factors [8]. Currently, the use of microorganisms capable of fixing atmospheric nitrogen is of great practical importance because it makes it possible to bridge the limits of chemical fertilization, which has resulted in unacceptable levels of water pollution [12]. In addition to pollution problems, especially in the water supply, the application of chemical fertilizers is carried out in excess, which becomes very expensive for farmers, whereas the biological fixation of nitrogen through microorganisms can be adapted to the needs of the plant [12]. In legume-*Rhizobium* symbiosis, rhizobia induce nodules formation on the roots of leguminous plants. In this process, N_2 which is chemically inert and makes up approximately 80% of the volume present in the Earth's atmosphere is reduced to ammonia by the bacterial enzyme nitrogenase. The nitrogenase enzymes are irreversibly damaged by exposure to atmospheric levels of oxygen. To protect the nitrogenase from the negative effects

of oxygen, the plants provide a microaerobic environment to ensure the proper functioning of the nitrogenase. In addition, plants exude carbohydrates to support the metabolism of bacterial endosymbionts. In return, bacteria through symbiotic fixation of atmospheric nitrogen provide forms of nitrogen used by the plant for the synthesis of organic nitrogen compounds to meet its nutritional needs. Most of the N added naturally to soils is from biological fixation, that is, symbiotic or nonsymbiotic in nature. BNF is an efficient source of nitrogen [8]. It has been estimated that about 100 Tg N, valued at $US 40 billion, is required annually for the production of the world's grain and oilseed crops [13], 20% comes from biological nitrogen fixation, and 26% from soil sources could also have originated from pasture or crop legume residues. The other sources are mainly from lightning discharges, burning of fossil fuels and forest and from the emission of magmatic gases. This N is added to soils as nitrate and ammonium in precipitation. If N-fertilizer derived from fossil fuels rises in price, the enhancement of BNF in agriculture will become more important. It has been reported that throughout the world, several areas of land have been degraded, and there is a need for reflection to develop new methods to stop land destruction and to institute a serious reversal of land degradation. Among the alternatives, the BNF can be used in land remediation. Legumes are well known for their ability to fertilize soils through their symbiotic relationship with specific nitrogen-fixing bacteria known as rhizobia, a name that portrays root and stem nodulating bacteria. There are approximately 700 genera and about 13,000 species of legumes, only a portion of which shown to have the ability to fix atmospheric nitrogen [12]. Soil fertilization is carried out in part by the BNF, each year, half of the amount of nitrogen fixed by microorganisms is provided by a 100 legumes in association with rhizobial strains [14]. Legumes are very important both nutritionally and agriculturally because they are very rich in protein, and are responsible for soil fertilization through symbiotic nitrogen fixation in association with rhizobia. The annual N-value of legume symbioses is about 70 million tons [15]. The accumulation of proteins in plants and the enrichment of soil in N result from the fixation of atmospheric nitrogen. Growing plants on soils that are low in mineral nitrogen often limits the growth of these plants, so the yields are affected. The need for nitrogen has meant that symbiotic relationships are evolving between plants and a variety of nitrogen-fixing organisms [16]. Nitrogen input to soils by the BNF is considered a renewable source of N for agriculture [8]. The quantities of N supplied per year and per hectare vary from 200 to 300 kg, such impressive quantities are sufficient to ensure a good yield [8, 17].

2.1.2. Phosphate solubilization

Phosphorus (P) is the most limiting element for plant growth after nitrogen. There are several forms that are inorganic (bound, fixed or labile) and organic (bound), and the concentration depends on the source. The concentration ranges from 140 ppm in carbonate rocks to over 1000 ppm in volcanic materials [18]. The majority of P applied as fertilizer enters into the immobile pools through precipitation reaction with highly reactive Al^{3+} and Fe^{3+} in acidic soils, and with Ca^{2+} in calcareous soils [19, 20]. The availability of phosphorus for plants is influenced by several conditions such as soil pH, aeration, temperature, texture and organic matter, extent of root systems of plants and secretions of root exudates and microbes. Soil microorganisms play a key role in soil P dynamics and subsequent availability of P to plants [10]. Although chemical fertilizer supplies plants with P requirements, excessive application of

P fertilizers is costly for the farmer and harmful to the environment. The content of phosphorus in plants varies from 0.2 to 0.8% dry weight, but only 0.1% of this phosphorus is available to plants [21]. The main source of P for the plant remains in the soil solution. The P content values of agricultural soil solutions are generally very low and remain unsuitable for the needs of the host plant. With the ability to solubilize phosphate, the microbial system can compensate for the amounts of P required for growth of the host plant [22]. Several rhizobia species may solubilize phosphorus, including *R. leguminosarum*, *R. meliloti*, *M. mediterraneum*, *Bradyrhizobium* sp. and *B. japonicum* [23]. These bacteria solubilize phosphorus by the production of low molecular weight organic acids that act on inorganic phosphorus. A large number of strains of Rhizobium were able to solubilize phosphorous in liquid culture [24]. The importance of this ability to solubilize phosphorus in plant growth by some rhizobia has been demonstrated in chickpeas and barley [25].

2.1.3. Siderophore formation

Iron is considered an essential micronutrient of plants and is present in the soil with a significantly different distribution. Iron can be present in different forms, either in divalent (ferrous or Fe^{2+}) or trivalent (ferric or Fe^{3+}) states. Soil pH and Eh (redox potential) and the availability of other minerals determine the state of iron in the soil [26]. In aerobic environments, iron exists as insoluble hydroxides and oxyhydroxides, which are not available to plants and microbes [27]. In general, bacteria have the ability to synthesize siderophores, low molecular weight compounds capable of sequestering Fe^{3+}. These siderophores have a high affinity for Fe^{3+}, making iron available to plants. Siderophores are soluble in water and exist in extracellular and intracellular environments. Fe^{3+} ions are reduced to Fe^{2+} and released into cells by Gram-positive and -negative rhizobacteria. This reduction leads to the destruction/recycling of siderophores [27]. Siderophores can also form a stable complex with heavy metals such as Al, Cd, Cu, and so on and with radionuclides including U and NP [28]. Thus, plant inoculation by siderophore-producing bacteria protects them from stress caused by heavy metals and helps them absorb iron. Several rhizobial species nodulating various legumes are known for their production of siderophores [29].

2.1.4. Phytohormone production

Substances that stimulate plant growth at low concentrations, less than or equal to micromolar concentrations are called phytohormones. These molecules include indole-3-acetic acid (IAA) (auxin), cytokinins, gibberellins and abscisic acid.

*Indole-3-acetic acid (IAA):*IAA is the most advanced phytohormone that enhances root growth resulting in accelerated growth and plant development. IAA is involved in cell division, differentiation and vascular beam formation and plays a key role in nodule formation. Several of the isolated rhizosphere bacteria have been shown to produce IAA. IAA production in rhizobia is via indole-3-pyruvic acid and the indole-3-aldehyde acetic pathway. Inoculation of vetch roots with certain strains of *R. leguminosarum* bv. viciae shows a 60-fold increase in IAA in nodules [30]. One of the highest productions of IAA has been described by Mishra et al. [31] with the inoculation of *B. japonicum*-SB1 with *B. thuringiensis*-KR1. Co-inoculation of *Pseudomonas* with *R. galegae* bv. orientalis has shown that it produces AIA that has contributed to

increased nodule numbers, root and root growth and nitrogen content. Environmental (acidic pH, osmatic and matrix stress and carbon limitation) and genetic stressors (auxin biosynthetic genes and expression mode) influence the biosynthesis of AIA [32].

Cytokinins: Cytokinin stimulates plant cell division and in some cases, root development and the formation of absorbent hairs [33]. Most rhizospheric microorganisms have been reported to release cytokinins [34]. *Rhizobium* strains are also capable of producing cytokinins [35].

Gibberellins: Gibberellins are considered as plant hormones ensuring the lengthening of the stems and the expansion of the leaves. Some types of dwarfism have been attributed to gibberellin deficiency, but this has no effect on the roots. Many plant growth promoting bacteria are reported to produce gibberellins [36], including *Rhizobium* and *S. meliloti* [37].

Abscisic acid: Several constraints such as low temperatures and lack of water increase the production of abscisic acid. Biosynthesis is regulated indirectly by the production of carotenoids. Unlike auxin, the movement of abscisic acid in plants has no polarity and the transport of abscisic acid can occur in both phloem and xylem tissues [38]. It has been reported that abscisic acid stimulates stomatal closure, inhibits shoot growth without affecting or even promoting root growth, inducing seeds to store proteins and dormant, inducing gene transcription of proteinase inhibitors, and thereby, providing a defense against pathogens and gibberellins [39]. *Rhizobium* sp. and *B. japonicum* produced abscisic acid [36, 37].

2.2. Indirect plant growth promotions

2.2.1. Biological control of plant disease

In addition to their plant growth promoting effects, *Rhizobium* spp. have been increasingly associated with disease suppressive effects in the recent literature [40, 41]. Improvements in plant health are mediated by two different ecological mechanisms: (1) antagonism of pest and pathogens and (2) stimulation of plant host defenses.

2.2.2. Antagonistic effects of rhizobia to pathogens and pest

Antagonism of pest and pathogen populations by *Rhizobium* spp. takes several forms wherein species are pathogens of fungi, bacteria, nematodes and/or parasitic plants. There is evidence that a strain of *Bradyrhizobium japonicum* can cause up to a 75% decrease in sporulation of *Phytophthora megasperma*, 65% in *Pythium ultimum*, 47% in *Fusarium oxysporum* and 35% in *Ascochyta imperfecta* [42]. These findings suggest that only one bacterial strain will control a population of a multitude of pathogenic strains, thus potentially providing bioprotection for the host plant. It is clear from these findings that rhizobia show great potential for use against plant diseases, and therefore, deserve more attention in future studies of cropping systems.

Several studies on the mode of action of *Rhizobium* spp. have shown that the growth inhibition of plant pathogens is ensured by the production of toxic compounds. Early work has allowed the characterization of antimicrobial activities related to extracellular compounds of *Rhizobium* spp. such as trifolitoxin [43] indicating that antibiosis may be part of their reported biocontrol efficacy. Mabrouk et al. [44, 45] have recently demonstrated that the beneficial effect on growth and N-fixation efficiency in pea is evident for some *Rhizobium* isolates.

In addition to pea nodulation, inoculation with rhizobia significantly protect pea against parasitic plant (*O. crenata*) infection. Induced resistance in inoculated peas was characterized by reduction in seed germination of broomrape, radicle growth, parasite attachment to pea roots and finally tuber growth blockage on host roots. These observations have been attributed to the lignification and accumulation of toxic substances in pea roots following inoculation by rhizobial strains [44, 45].

2.2.3. Induction of plant defense by rhizobia against pests and diseases

Rhizobium populations may also promote plant health by stimulating the plant host. The presence of *Rhizobium* spp. would in this case indirectly stimulate the plant to activate its defense mechanisms when challenged with a pathogen through the production of plant defense compounds (phenolics, flavonoids or other phytoalexins, in particular). Induced resistance against Orobanche in peas inoculated with some rhizobial strains was found to be associated with significant changes in levels of the defense enzymes such as peroxidase, polyphenoloxidase and oxidative lipoxygenase (Lox), and in the accumulation toxins derived, including phenolic acids and pisatin and pea phytoalexin. These modifications were attributed to the activation of defense genes following inoculation of pea plants with rhizobia [44–45, 47, 48]. The work of Arfaoui et al. [50] identified some isolates of *Rhizobium* spp. activating the defense in chickpeas against *Fusarium oxysporum* f. sp. in reducing the severity of the disease developed in the host plant. They showed that inoculation of chickpea plants with Rhizobium strains, a few days before the attack by Fusarium oxysporum f.sp. ciceris, allows the reduction of the incidence of wilting resulting from the significant increase in the activities of several defense-related enzymes such as peroxidases and polyphenoloxidases, resulting in the accumulation of phenolic compounds and the expression of genes related to phenylpropanoid defense [51, 52]. Induced resistance by the bacteria of the rhizosphere has been described against several pathogens such as viruses, bacteria and fungi in several species of plants. However, induction mechanisms and metabolites involved in the induction of plant defense are highly variable depending on the bacterial strain and pathosystems. Several studies have shown that salicylic acid produced by bacteria can induce resistance in many plant species. Several studies have shown that lipopolysaccharides (LPS) of rhizobia are involved in triggering induced systemic resistance (ISR). Some authors have shown that the elicitation/triggering of ISR in potato against the Globodera pallida cyst nematode results from LPS of *R. etli* [53, 54]. In pea, systemic resistance induced by *O. crenata* infection was triggered by heat-killed cells and purified LPS of *Rhizobium leguminosarum* [46, 48, 49].

2.2.4. Resistance of rhizobia to abiotic stress factors

In the Rhizobium-legume symbiosis, which is a N2-fixing system, the physiological state of the host plant is a determining factor in the process of atmospheric nitrogen fixation. Therefore, limiting agents do not allow the tolerant and competitive rhizobium strains to express its full nitrogen-binding capacity, which affects the vigor of the host legume. In Tunisia, several factors may limit the symbiotic nitrogen fixation, particularly drought, especially since Tunisia is located in semiarid, arid and Saharan climatic zones where annual rainfall ranges from 100 to 300 mm [55]. Drought affected the crop yields of pulses in Tunisia, which led farmers to

abandon this crop in some areas. In addition to drought, legume crops are affected by salinity, soil pH, nutrient deficiency, mineral toxicity, extreme temperatures, diseases and pests [44].

2.2.5. Soil salinity

Salinity is considered a limiting factor in nodulation and nitrogen fixation in legume-Rhizobium associations, which can adversely affect the yield of legume crops [56]. Rhizobia can tolerate high concentrations unlike legume plants. The growth of certain strains is inhibited by 100 mM NaCl [57, 58], whereas other strains such as *R. meliloti* and *R. fredii* support saline concentrations greater than 300 mM [59, 60]. Therefore, in saline soils, the multiplication of these strains will not be affected in the rhizosphere of the plant host. The accumulation of K ions with several ranges of low molecular weight organic solutes is involved in the osmoadaptation of most microorganisms, in order to balance the osmotic pressure of the growth medium and to maintain the turgor pressure and allowing the cell extension [61].

2.2.6. Water deficiency and drought

Water deficiency is a major limiting factor of symbiotic nitrogen fixation in many arid regions of the Mediterranean basin. One of the immediate responses of rhizobia to water deficiency concerns the morphological changes [62, 63]. Water stress allows the reduction of legume root infection by rhizobia, hence the reduction of nodulation. In addition, the water deficit also restricts the development and function of nodules [59, 64]. The development of effective nodules in desert soils highlights that some strains can tolerate extreme conditions in soils with limited moisture levels [65, 66, 67].

2.2.7. High temperature and heat stress

In temperate regions, the free life and symbiotic life of rhizobia is affected [68]. The optimal temperature range for growth of rhizobial strains varies from 28 to 31°C. Some rhizobial strains cannot grow at 38°C, while others that survive heat stress can lose their nodulation power due to alteration of compounds involved in the infective process such as plasmid hardening or alterations of cellular polysaccharides [68]. Nodules formed at high soil temperature (35–40°C) are usually ineffective formation; however, some strains of rhizobia, such as R. *leguminosarum bv. phaseoli*, were heat-tolerant and formed effective symbioses with their host plants [69, 70]. These associations will be of great interest for cultivation in arid climates.

2.2.8. Acid soils and soil acidification

Acid soils constrain agricultural production in worldwide [71], with the scope of the problem likely to increase as the result of acid rain, long-term N fertilization and legume N_2 fixation. Legumes are particularly affected, acidity limiting both survival and persistence of nodule bacteria in soil, and the process of nodulation itself [72]. The absence of nodules has been noted in legumes grown in acidic soils, particularly in soils with a pH below 5. The susceptibility of certain rhizobial strains to these conditions is a cause of inhibition of nodule formation [73–75]. Nodules are absent even when a viable population of Rhizobium can be demonstrated [76, 77]. Some researchers have observed that nodulation of *P. sativum* was 10 times more sensitive to acidity than rhizobial multiplication or plant growth [78]. Recent reports indicated

that by selection of acid-soil tolerance in both symbiotic partners [79, 80], annual medics such as *Medicago murex* can be grown symbiotically on soils as acidic as pH 4.3 [81]. Meanwhile, the genetic control of acid tolerance in Sinorhizobium is becoming increasingly understood [82]. The establishment of legume symbioses requires the interaction of specific recognition signal molecules produced by both bacterial and plant partners [83]. It has been shown that pH affects the exchange or recognition of these signal molecules by both plant and bacterial partners in both the medic symbiosis [84] and the clover symbiosis [84, 85].

3. Conclusions

Rhizobia produce multiple beneficial effects on plant growth stimulation, host defense against disease and survival under stress with many other unknown benefits. This chapter describes the potential of rhizobia for the promotion of plant growth and highlights the different mechanisms of growth stimulation and the spectrum of resistance available against various abiotic stresses in several crops. In sustainable agriculture, the biological fixation of nitrogen is an important process, particularly in the legume farming system. To benefit from leguminous crops, it would be interesting to select symbiotic pairs adapted to severe conditions and to fix considerable quantities of nitrogen. The importance of the *Rhizobium*-legume interaction is not limited to their symbiotic nitrogen fixation activity or several other activities in the soil, possibly improving soil fertility and plant growth, but some strains of rhizobia can be used to protect plants against attack by pests and pathogens. However, further studies on the precise mode of action and adaptation to the different ecophysiological conditions of these microorganisms may help to maximize the benefits of rhizobia for improving plant growth and health.

Conflict of interest

Authors confirm there are no conflicts of interest.

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Chapter 7

Dye-Linked Flavin-Containing Dehydrogenase from Bacteria Related to Plant

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Additional information is available at the end of the chapter

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Abstract

The so-called dye-linked dehydrogenases catalyze the oxidation of various biomolecules in the presence of an artificial electron acceptor, in which several unique compounds related to plants as substrates such as opine(s) and L-hydroxyproline are contained. Opines including nopaline and octopine are produced from nutrients of plant by pathogenic agrobacteria species in a crown gall tumor and subsequently degraded for their nutrients by (hypothetical) opine dehydrogenase(s) (OpnDH). The homologous proteins of *Pseudomonas putida* and *Bradyrhizobium japonicum* (isozymes 1 and 2) function as nopaline- and octopine-specific dye-linked dehydrogenases, to yield α-ketoglutarate + L-arginine and pyruvate + L-arginine, respectively. L-Hydroxyproline is detected in such hydroxyproline-rich glycoprotein of plant cell walls. In the degradation pathway of bacteria, D-hydroxyproline dehydrogenase (HypDH) catalyzes the dehydrogenation reaction of *cis*-4-hydroxy-D-proline, and is classified into two types: homomeric and heteromeric enzymes. Both OpnDH and heteromeric HypDH commonly consist of three different subunits ($\alpha\beta\gamma$), in which 2 FAD, 1 FMN, [2Fe-2S] and [4Fe-4S] clusters are contained as prosthetic groups. In D-amino acid dehydrogenase superfamily, these enzymes are physiologically related to L-proline dehydrogenase from archaea and hydrogen cyanide synthase from bacteria, whereas isozyme 2 of OpnDH from *B. japonicum* and other OpnDHs had appeared by convergent evolution.

Keywords: dye-linked dehydrogenase, opine, L-hydroxyproline, molecular evolution

1. Introduction

Oxidoreductases catalyze the reversible electron-transfer from many compounds such as amino acids, alcohols, sugars, and amines, and are classified into three main groups, based on available

electron acceptor(s): oxidases, oxygenases and dehydrogenases/reductases. FAD- and/or FMN-dependent dehydrogenases, one of subgroups in dehydrogenases/reductases, play a variety of important roles in the generation of energy and in the biosynthesis and metabolism of biomolecules [1, 2]. These enzymes are frequently called as "dye-linked dehydrogenases", because of utilization of artificial dyes such as 2,6-dichloroindophenol (Cl2Ind) and ferricyanide instead of the natural acceptor(s). Furthermore, most of them are frequently associated with cell or organelle membranes and are unstable in solution, which has made their expression in host cells, purification, preservation, and characterization very difficult. Since dye-linked dehydrogenation activity for several unique compounds related to plants as substrates including opine(s) and L-hydroxyproline had been reported in bacterial cell-free extract from 1950s to 1980s, those genetic and molecular information have only recently been elucidated [3–6].

2. Opine dehydrogenase

2.1. Opine concept

Plant tumors known as crown gall are incited by pathogenic, soil-inhabiting *Agrobacterium* species including *Agrobacterium tumefaciens*. During the infections [7], the expression of the virulence genes (*vir*) within the tumor-inducing (Ti) plasmid is first specifically induced by phenolic compounds, including acetosyringone, which are released from the wounded plant cells (① in **Figure 1**). Next, the products expressed by the *virD* locus promote fragmentation of so-called T-DNA region (②) and subsequently transfer to the nuclei of host plants (③). T-DNA region integrated into a host chromosome contains several genes including synthase(s) of phytohormones such as auxin and cytokinin (④), and the genetic expressions direct the production of phytohormones such as auxin and cytokinin, which can lead to uncontrolled cell proliferation, producing a crown gall tumor (⑤). The (integrated) T-DNA region also encodes genes for the synthesis of unique compounds, opines (⑥). The opines produced and secreted by the neoplastic plant cells are utilizable as nutrient sources by the agrobacteria that induced the tumor (⑦). The genes for the utilization of the opines by the bacteria are also encoded by the Ti plasmid, but they are located outside the T-DNA region (⑧) (i.e., the "opine concept").

2.2. What's opine?

Opines have been structurally classified into several groups, among which two groups have a common secondary amine dicarboxylic acid structure. One group has been categorized as the N^2-(L-D-carboxyethyl) derivatives of L-arginine (octopine), L-ornithine (octopinic acid), L-lysine (lysopine), L-histidine (histopine), L-methionine (methiopine), and L-phenylalanine (phenylalaninopine). The second group has been categorized as the N^2-(1,3-D-dicarboxypropyl) derivatives of L-arginine (nopaline), L-ornithine (nopalinic acid (ornaline)), L-leucine (leucinopine), and L-asparagine (succinamopine). Of these, octopine (N^2-(L-D-carboxyethyl) derivatives of L-arginine) and nopaline (N^2-(1,3-D-dicarboxypropyl) derivatives of L-arginine) are

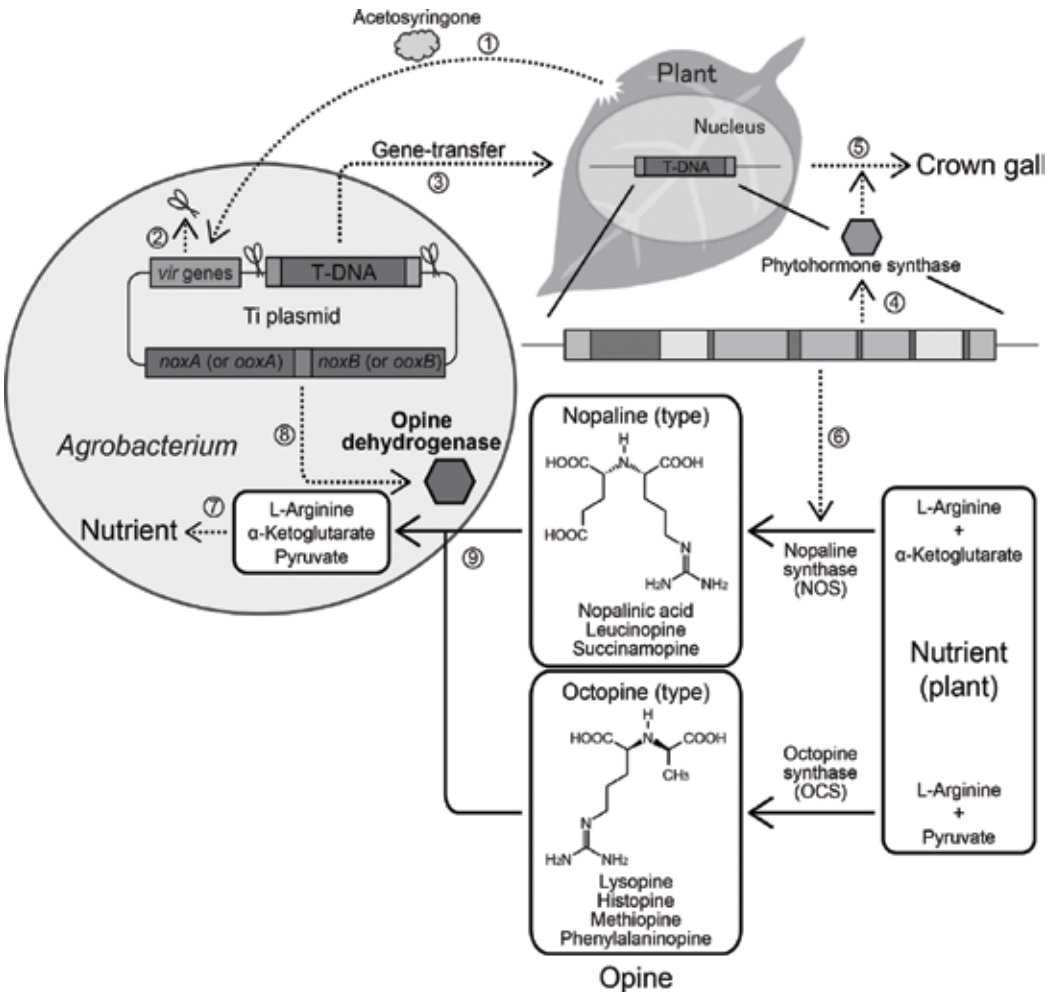


Figure 1. Opine concept by *Agrobacterium*.

well studied, and synthesized by NAD(P)H-dependent soluble dehydrogenases: octopine synthase (OCS; [8]) and nopaline synthase (NOS; [9]) (⑥ in **Figure 1**). These enzymes catalyze the reductive condensation of pyruvate (for octopine) or α-ketoglutarate (for nopaline) with L-arginine. Although these reactions may be reversible *in vitro*, the frequent use of the term "synthase" rather than "dehydrogenase" has emphasized the importance of "biosynthesis", but not "degradation".

2.3. Opine assimilation by *Agrobacterium*

The octopine catabolic (*occ*) operons of octopine-type Ti plasmids consist of at least 15 genes that include ABC-type (opine) permease (encoded by *occQMPJ*) and ornithine cyclodeaminase

(*ocd*) [10] (**Figure 2**). On the other hand, nopaline-metabolizing genes including *nos* are encoded within the nopaline catabolic (*noc*) region in the pTiC58 plasmid [11]. Both the *occ* operon and *noc* region also contain several genes suspected of being involved in opine metabolism such as *ooxA* (pTi_037), *ooxB* (pTi_038), *noxA* (Atu6019), and *noxB* (Atu6021) (**Figure 2A**). In both opines, the first step of degradation is the reverse of biosynthesis, i.e., oxidative cleavage to L-arginine and pyruvate or α-ketoglutarate (⑨ in **Figure 1**): L-arginine may subsequently be metabolized to L-proline via L-ornithine through two sequential enzyme reactions by arginase (encoding *arc*) and ornithine cyclodeaminase. More than 20 years ago, it was reported that when *noxB-noxA* and *ooxB-ooxA* were transcriptionally fused with the vector promoter and expressed in *Escherichia coli* cells, cofactor-independent oxidase activities for nopaline and octopine were observed in the membrane fraction [12]. However, these activities were directly assayed using the *E. coli* lysate with lysozyme (without centrifugation). Until now, there has been no clear evidence to show that these genes (proteins) function as opine oxidases.

2.4. Nopaline dehydrogenase from *Pseudomonas putida*

2.4.1. Successful preparation of recombinant protein

The homologous *ooxB-ooxA* and *noxB-noxA* genes exist as a gene cluster together with putative ABC-type transporter gene(s) on the genomes and/or plasmids of non-*Agrobacterium* bacterial species, although the mechanisms underlying opine catabolism are unclear. Among them, PP_4457 and PP_4456 (genes) from *Pseudomonas putida* KT2440 correspond to OoxB (40% of identity) and NoxB (40%), and OoxA (41%) and NoxA (48%), respectively (referred to as OdhB and OdhA, respectively) (**Figure 2A**). The *odhB-odhA* operon, in which the $(His)_6$-tag sequence

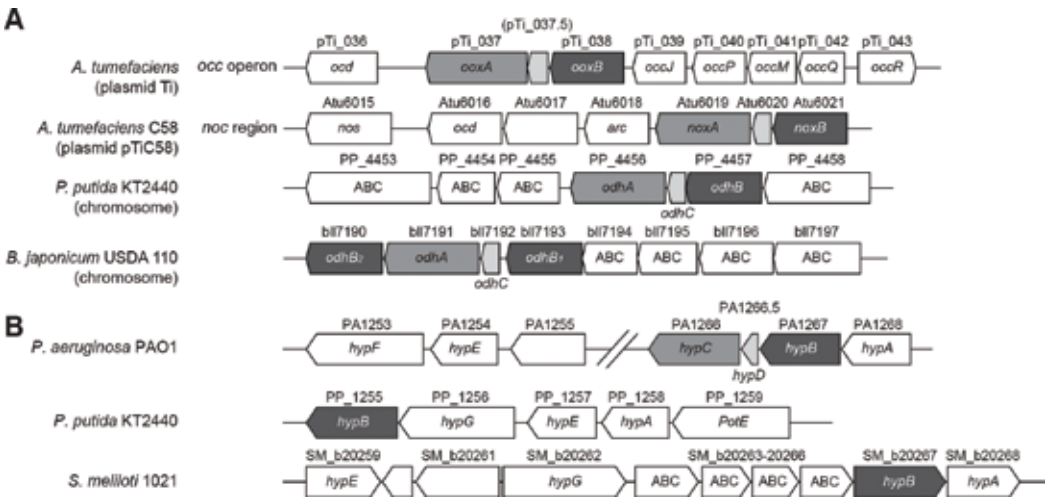
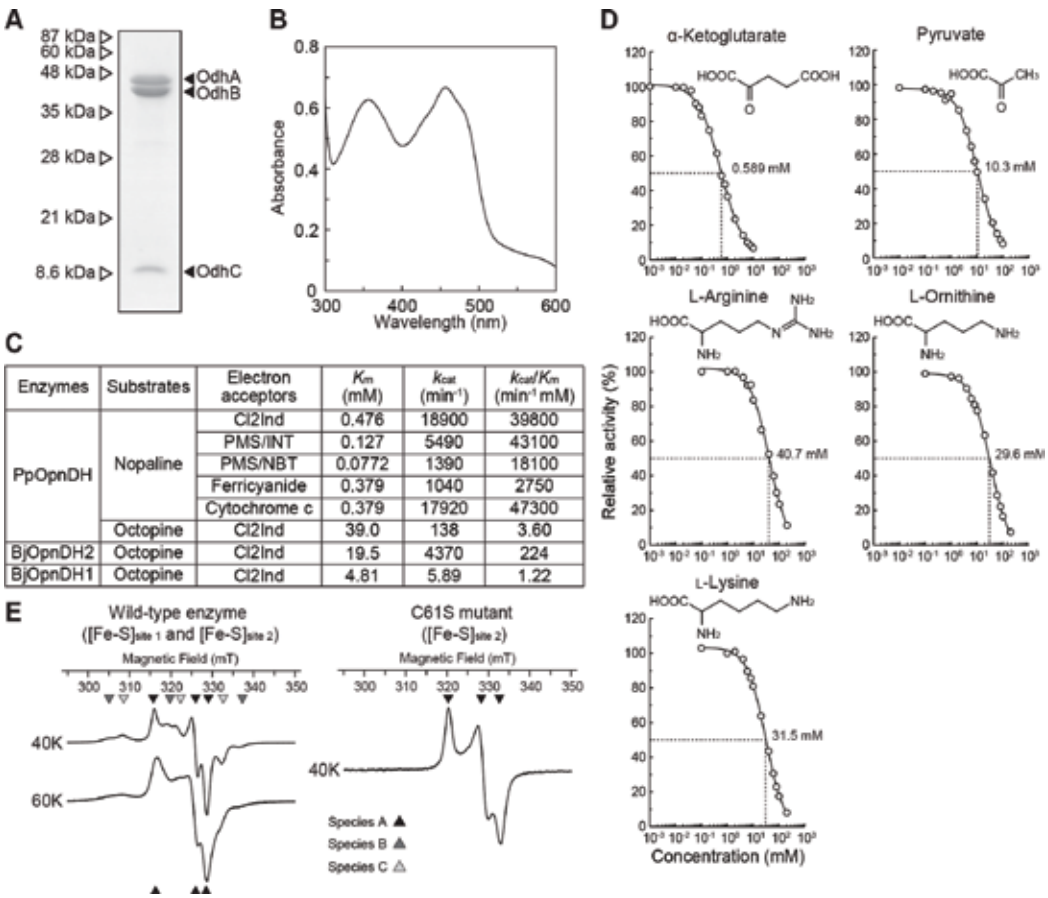


Figure 2. Schematic gene clusters related to opine (A) and L-Hyp metabolism (B).

was attached at the N-terminal of the *odhB* gene, expressed in *P. putida* cell (but not *E. coli*) [13, 14], and purified using immobilized metal (Ni^{2+}) affinity chromatography (**Figure 3A**). A buffer system containing Tween-20 was absolutely necessary for this procedure, indicating tight binding to the cytoplasmic membrane. The purified recombinant protein consisted of three major distinct bands with molecular masses of 46, 42, and 9 kDa, respectively, among which the two formers expectedly corresponded to OdhA and OdhB, respectively. Interestingly, N-terminal sequence of the latter was identical with that of a provisional open-reading frame between *odhA* and *odhB* genes (referred to as *odhC* gene): the 3′ part of *odhB* and *odhC* was slightly overlapped by the 5′ part of *odhC* and *odhA*, respectively. Thus, this protein indeed consisted of α-, β-, and γ-subunits encoded by *odhA*, *odhB*, and *odhC* genes, respectively (referred to as PpOdhABC).



Enzymes	Substrates	Electron acceptors	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM)
PpOpnDH	Nopaline	Cl2Ind	0.476	18900	39800
		PMS/INT	0.127	5490	43100
		PMS/NBT	0.0772	1390	18100
		Ferricyanide	0.379	1040	2750
		Cytochrome c	0.379	17920	47300
	Octopine	Cl2Ind	39.0	138	3.60
BjOpnDH2	Octopine	Cl2Ind	19.5	4370	224
BjOpnDH1	Octopine	Cl2Ind	4.81	5.89	1.22

Figure 3. Characterization of opine dehydrogenase. An SDS-PAGE analysis (A) and absorption spectra (B) of purified recombinant PpOdhABC. (C) Kinetic parameters. (D) Inhibition study by α-keto acids and L-amino acids. (E) EPR spectra of wild-type and C61S (in γ-subunit) of BjOpnDH2.

2.4.2. Prosthetic group(s)

The purified protein was orange-brown, and the absorption spectrum showed the characteristics of a typical flavoprotein (maxima at approximately 350 and 450 nm; **Figure 3A**): the flavin compounds were identified as FAD and FMN by HPLC. Alternatively, each subunit of heteromeric PpOdhABC was also purified and functionally characterized. Only FAD was extracted from (almost) inactive OdhA, OdhB, OdhAB (co-expression of OdhA+B), OdhBC, and OdhAC, while FAD and FMN were only extracted from active OdhABC; the molar ratio of FAD:FMN was 1.9:1.0. Therefore, it was concluded that PpOdhABC contained 2 FAD (α- and β-subunits) and 1 FMN (between the α- and β-subunits) within the structural unit of $\alpha\beta\gamma$ (**Figure 4A**). On the other hand, as described below in detail, the OdhC and OdhA proteins contain two different types of [Fe-S]-binding motifs, in which [4Fe-4S] and [2Fe-2S] clusters might be bound, respectively (see Section 2.6).

2.4.3. Characterization as a dye-linked opine dehydrogenase

When Cl2Ind was used as an artificial electron acceptor, the catalytic efficiency (k_{cat}/K_m) value of PpOdhABC with nopaline was 11,000-fold higher than that with octopine. *p*-Iodonitrotetrazolium violet (INT) or nitroblue tetrazolium (NBT) together with phenazine methosulfate (PMS) (electron-transfer intermediate), ferricyanide, and horse heart cytochrome *c* (but not $NAD(P)^+$ or oxygen) were additional artificial electron acceptors. Since other opines were not available commercially, more detailed substrate specificity was alternatively estimated by an inhibition study (**Figure 4D**). The IC_{50} value for α-ketoglutarate was ~17-fold higher than that for pyruvate, thereby confirming the preference for nopaline over octopine. On the other hand, among several L-amino acids, significant inhibition was only observed in basic L-arginine, L-ornithine, and L-lysine. These results indicated that PpOdhABC recognized the *N*-substituted glutamic acid moiety of nopaline and nopalinic acid (**Figure 1**), also found in crown gall tumor tissues, and may also be the active substrate.

2.5. Octopine dehydrogenase from *Bradyrhizobium japonicum*

Bradyrhizobium japonicum is a nitrogen-fixing bacteria found in the roots of a soybean plant, *Glycine max*. Interestingly, the homologous gene cluster with *odhB-odhC-odhA* from *P. putida* contained an additional *odhB* gene, in the order of *odhB*$_1$*-odhC-odhA-odhB*$_2$ (**Figure 2A**) In order to estimate the subunit assembly of this protein, $(His)_6$-tagged $OdhB_1$ or $OdhB_2$ was co-expressed together with S-tagged OdhA and OdhC in *E. coli* cells and purified using Ni^{2+}-affinity chromatography. A western blotting analysis using the anti $(His)_6$-tag and S-tag antibodies revealed that the purified proteins both contained not only each OdhB, but also OdhA and OdhC (referred to as $BjOdhAB_1C$ and $BjOdhAB_2C$, respectively) (**Figure 4A**).

Their absorption spectra were similar to that of PpOpnDH, and FAD and FMN (the molar ratio of ~2) were extracted from (orange-brown) them. On the other hand, the significant dehydrogenase activities were detected only toward octopine, although the specific activity of $BjOdhAB_1C$ was ~200-fold lower than that of $BjOdhAB_2C$ (**Figure 3C**). The k_{cat}/K_m value for the octopine of $BjOdhAB_2C$ (using Cl2Ind; 224 min^{-1} mM^{-1}) was 178-fold lower than that

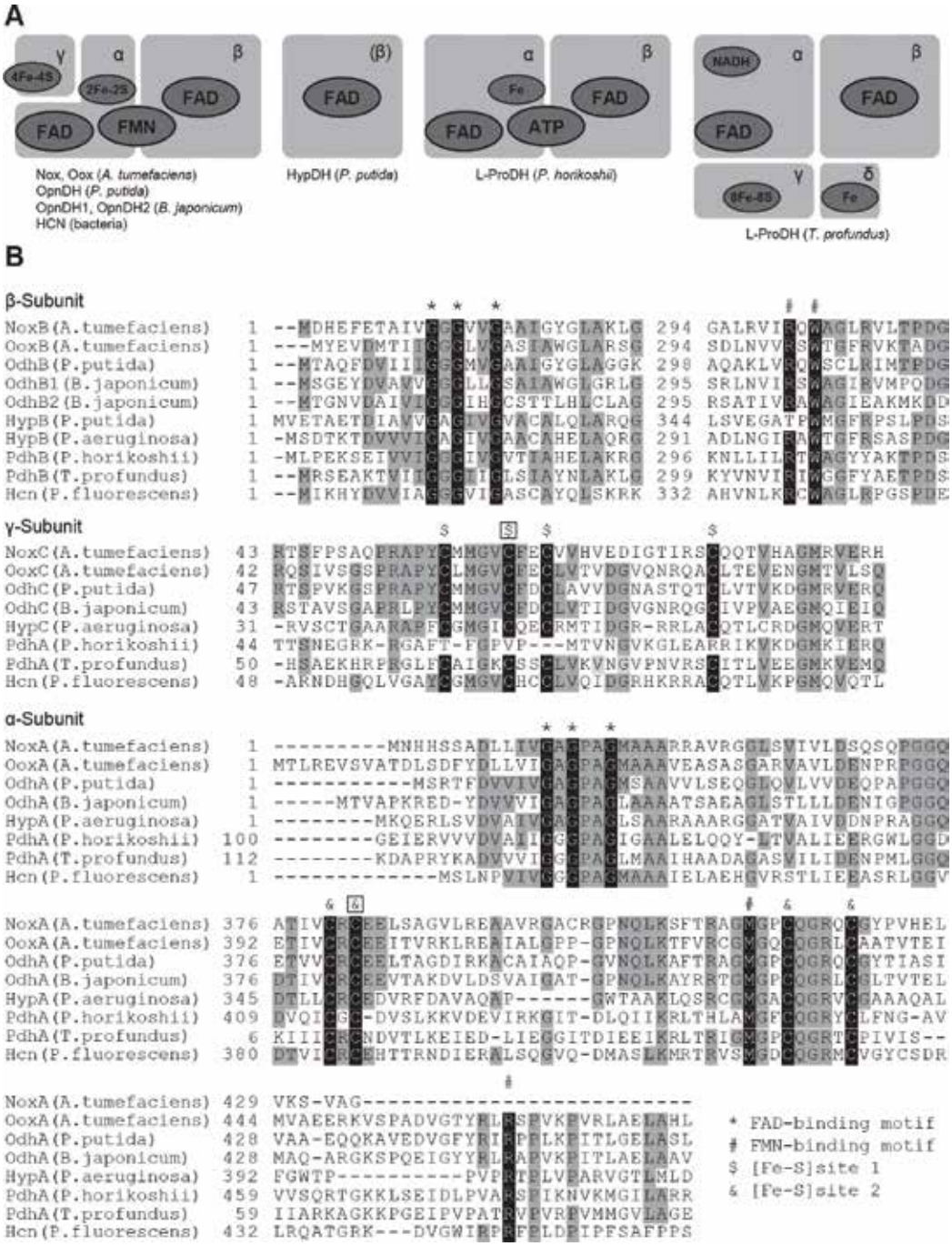


Figure 4. Schematic subunit assembly (A) and partial amino acid sequence alignment of dehydrogenases (B).

for the nopaline of PpOpnDH. These results suggested that BjOdhAB$_2$C and BjOdhAB$_1$C both functioned as octopine-specific OpnDH (referred to as BjOpnDH2 and BjOpnDH1, respectively).

2.6. Functional role(s) of iron-sulfur clusters

The γ- and α-subunits of OpnDH contain two different types of [Fe-S]-binding motifs consisting of four cysteine residues: $[Fe\text{-}S]_{site\ 1}$ and $[Fe\text{-}S]_{site\ 2}$, respectively (**Figure 4B**). An electron paramagnetic resonance (EPR) spectrum recorded for the frozen solution of the BjOpnDH2 wild-type enzyme (fully reduced by $Na_2S_2O_4$) at 40 K was composed of at least three paramagnetic species due to [Fe-S] clusters: species A, B, and C (**Figure 3E**). Among them, only species A was still detected at 60 K, indicating the assignment to be the fully reduced form of $[2Fe\text{-}2S]^{-}$ (therefore, species B and C were derived from the reduced form of $[4Fe\text{-}4S]^{3-}$). On the other hand, when one cysteine residue (Cys^{61}; boxed in **Figure 4B**) in $[Fe\text{-}S]_{site\ 1}$ was substituted to serine residue, the C61S mutant protein showed the significant activity, and the paramagnetic species was clearly similar to species A found in the wild-type enzyme (**Figure 3E**). No expression of C382S mutant in $[Fe\text{-}S]_{site\ 2}$ was found in *E. coli* (**Figure 4B**). Wholly, $[Fe\text{-}S]_{site\ 1}$ and $[Fe\text{-}S]_{site\ 2}$ of heteromeric OpnDH bind to the [4Fe-4S] and [2Fe-2S] clusters, respectively (**Figure 4A**), and the latter is important for structural folding and enzyme catalysis.

2.7. Phylogenetic relationship

Flavin-containing OpnDH (the β-subunit) belongs to the D-amino acid oxidase superfamily (pfam01266), and differed from the known $NAD(P)^{+}$-dependent enzyme (pfam02317) [8, 9]. Although this protein superfamily contains several oxidases for D-amino acid and sarcosine, a relatively high homology with OpnDH was found in hydrogen cyanide (HCN) synthase from bacteria [15], D-hydroxyproline dehydrogenase (HypDH) from bacteria (described in Section 3; [4]), and L-proline dehydrogenase (L-ProDH) from archaea with a heterooligomeric structure [16] (**Figure 5A**). The subunit assembly of OpnDH (αβγ) was the same as that of HypDH and HCN synthase (**Figure 4A**). PpOpnDH and BjOpnDH1 formed a single subfamily together with Nox and Oox from *A. tumefaciens* (referred to as the $OpnDH_{type\ 1}$ subfamily). Interestingly, BjOpnDH2 was not closely related to any of the subclasses including $OpnDH_{type\ 1}$

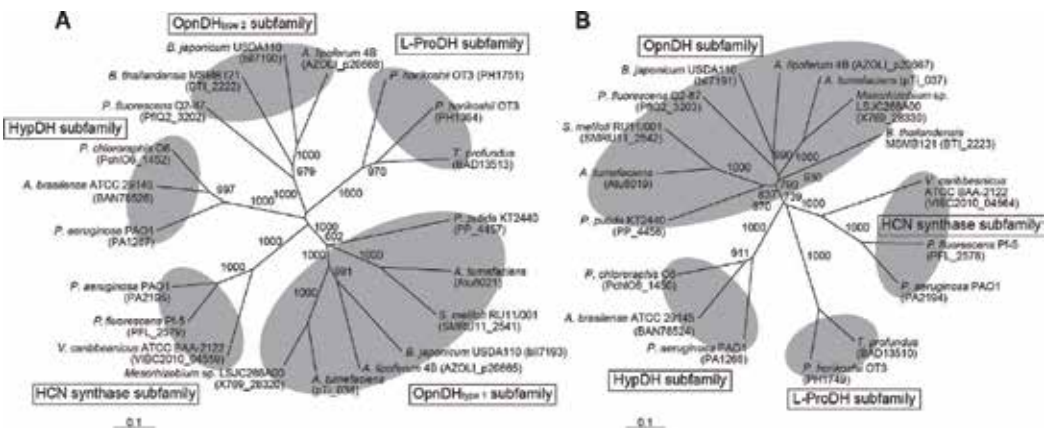


Figure 5. Phylogenetic trees using β- (A) and α-subunits (B) of flavin-containing dye-linked dehydrogenases.

(referred to as $OpnDH_{type\ 2}$ subfamily) (**Figure 5A**), whereas all OdhA proteins formed a single subfamily (**Figure 5B**). Several specific motifs (amino acid residues), previously proposed in L-ProDHs, are significantly conserved in the primary structure(s) of OpnDH: two typical ADP-binding motifs at the N-terminus of the β- and α-subunits as (putative) the binding sites of 2 FAD (Gly-*X*-Gly-X_2-Gly); a motif of Arg-*X*-Trp for (putative) the binding sites of FMN in the α-subunit (and β-subunit) (**Figure 4B**).

2.8. Physiological role

The degradation of toxic organic compounds by some *P. putida* strains has been extensively examined [17]. On the other hand, these bacteria, in addition to *B. japonicum*, also colonize the rhizosphere of agronomically relevant plants at high population densities; the origin of opines may be from rotting plants and/or plant exudates rather than biosynthesis. In case of endophytes, opines may be also provided without their being exuded. It is reported that a *P. putida* strain isolated from a commercial nursery catabolized nopaline [18], thereby conforming to the substrate specificity of PpOpnDH. In contrast to *Agrobacterium* species, PpOpnDH and BjOpnDH genes are located on the chromosome. Indeed, large numbers of *Bradyrhizobium* species possess the homologous genes, whereas all the other *P. putida* strains, except for KT2440, do not. These findings suggest that *P. putida* KT2440 very recently acquired this ability by horizontal gene transfer (not plasmid transfer).

3. D-Hydroxyproline dehydrogenase

3.1. L-Hydroxyproline in nature

trans-4-Hydroxy-L-proline (T4LHyp; generally called "L-hydroxyproline (L-Hyp)") is a non-standard amino acid, and detected in certain proteins including collagen, the cell wall of plants, and some peptide antibiotics. In the two former proteins, L-proline residue is post-translationally hydroxylated to L-Hyp by α-ketoglutarate and Fe(II)-dependent prolyl hydroxylase (① and ② in **Figure 6**). In case of so-called "hydroxyproline-rich glycoprotein (HRGP)" of plant cell walls including extensins and solanaceous lectins, Hyp-residues are subsequently glycosylated by hydroxyproline *O*-arabinosyltransferase (③) [19]. The β-L-arabinofuranoside (Ara)-containing HPRGs have repetitive Ser-Hyp_4 motifs and the majority of the Hyp-*O*-linked arabinofuranosides are Ara-Ara-Ara-Ara-Hyp and Ara-Ara-Ara-Hyp. In degradation system of the latter by bacteria such as *Bifidobacterium longum*, two β-L-arabinobiosidases HypBA2 and HypBA1 release Ara-Ara from Ara_3-Hyp (④), and subsequently liberate Ara from Ara-Ara, respectively (⑤), although no enzyme degrading Ara-Hyp to Ara and L-Hyp is known until now (⑥) and this bacterium may have no ability to metabolize L-Hyp by itself [20]. On the other hand, direct biosynthesis from free L-proline to L-Hyp by hydroxylase(s) from few bacteria (and fungi) such as *Streptomyces* sp. may be in the biosynthesis of peptide antibiotics containing L-Hyp such as etamycin (⑦) [21]. Therefore, free L-Hyp may also be produced by the degradation of these proteins and/or antibiotics through protease (peptidase), particularly in soil and water environments (⑧).

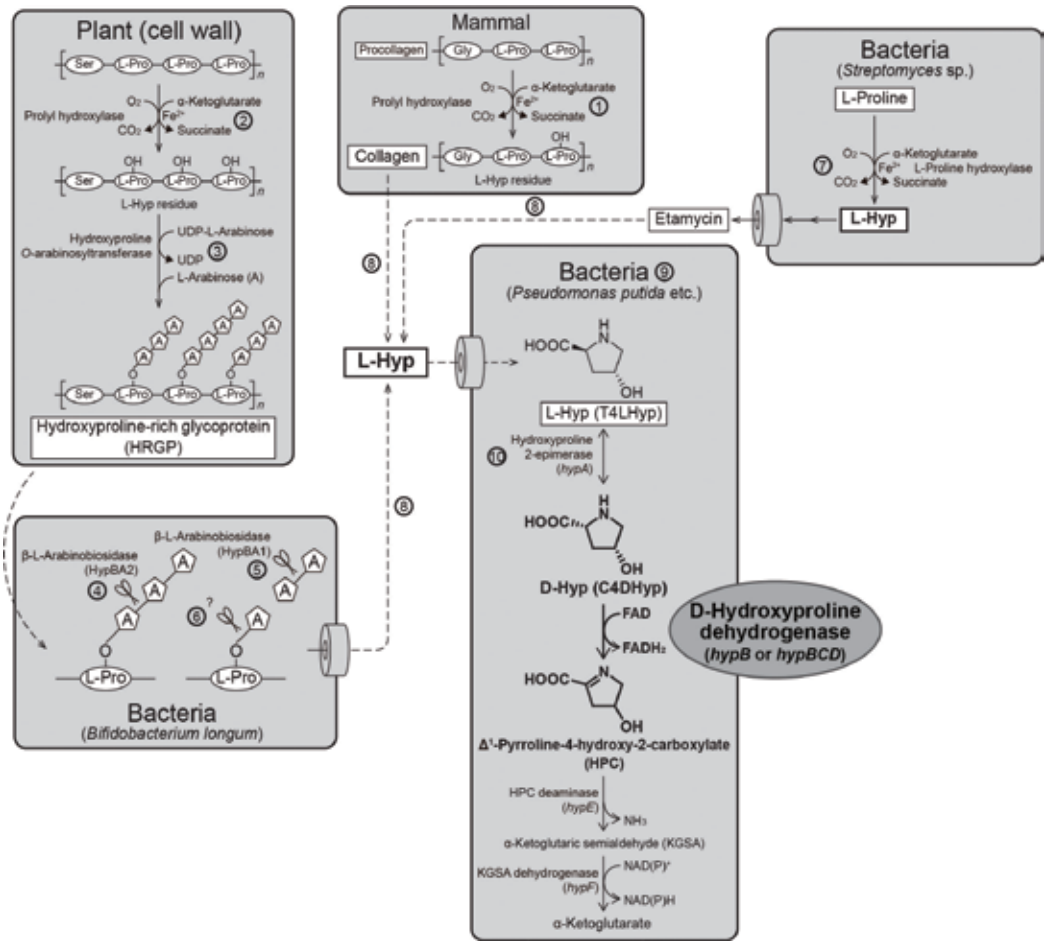


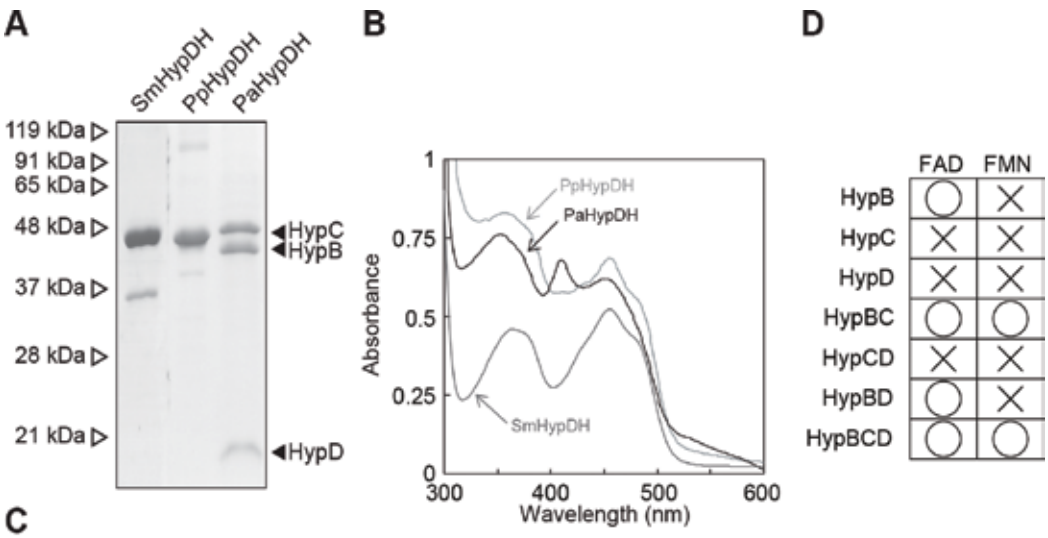
Figure 6. A hypothetical schematic between L-Hyp and organisms in nature.

3.2. Involvement in bacterial L-Hyp pathway

In contrast to mammals, bacteria metabolize free T4LHyp to α-ketoglutarate through four enzymatic steps via *cis*-4-hydroxy-D-proline (C4DHyp; generally called "D-hydroxyproline"), Δ^1-pyrroline-4-hydroxy-2-carboxylate, and α-ketoglutaric semialdehyde intermediates (⑨); therefore, bacteria with this pathway have the ability to grow using not only T4LHyp, but also C4DHyp as the sole carbon source. D-Hydroxyproline dehydrogenase (HypDH) catalyzes the dehydrogenation reaction of C4DHyp to Δ^1-pyrroline-4-hydroxy-2-carboxylate (second step of bacterial T4LHyp pathway): C4DHyp is produced from T4LHyp by hydroxyproline 2-epimerase (⑩). More than 40 years ago, it had been already known that the enzyme has the ability to utilize artificial electron acceptors including Cl2Ind as well as OpnDH, but there was no genetic report until now, probably because of the dye-linked dehydrogenase, as described in "Introduction" [22]. On the other hand, the genes (*hypA~hypF*) related to (putative) T4LHyp pathway are often clustered together with those encoding the transporter on bacterial genomes (referred to as T4LHyp gene cluster) (**Figure 2B**).

3.3. Homomeric HypDH

Among T4LHyp gene clusters of *P. putida* KT2440 and (nitrogen-fixing) *Sinorhizobium meliloti* 2011, PP_1255 and SM_b20267 genes, annotated as FAD-dependent oxidoreductase and D-amino acid dehydrogenase, respectively, were likely candidates for HypDH (referred to as *PphypB* and *SmhypB*, respectively) (**Figure 2B**). When *E. coli* (even *S. meliloti*) was used as a host, no expression of these recombinant proteins was found probably due to the membrane bound and dye-linked dehydrogenase as well as OpnDH: their biochemical characterizations were achieved by the same expression and purification systems as PpOpnDH (**Figure 7A**). Spectra of both the purified HypB proteins commonly showed the characteristics of a typical flavoprotein (**Figure 7B**), and only FAD was contained as a prosthetic group. Among several tested D-amino acids, their significant activities were observed only with C4DHyp and D-proline (referred to as PpHypDH and SmHypDH). The k_{cat}/K_m value with C4DHyp of PpHypDH



C

Enzymes	Substrates	Electron acceptors	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM)	C4DHyp / D-Proline
PpHypDH	C4DHyp	PMS/INT	0.276	280	1010	110
		PMS/NBT	0.0802	19.7	246	
	D-Proline	PMS/INT	6.58	59.9	9.15	
		PMS/NBT	3.49	9.95	2.86	
SmHypDH	C4DHyp	PMS/INT	0.188	125	665	5.5
		PMS/NBT	0.0602	61.0	1014	
	D-Proline	PMS/INT	0.942	115	122	
		PMS/NBT	0.401	67.4	169	
PaHypDH	C4DHyp	PMS/INT	0.109	2230	20500	190
		PMS/NBT	0.121	871	7200	
	D-Proline	PMS/INT	8.55	924	108	
		PMS/NBT	11.0	507	46.0	

Figure 7. Characterization of D-hydroxyproline dehydrogenase. An SDS-PAGE analysis (A) and absorption spectra (B) of purified recombinant HypDHs. (C) Kinetic parameters. (D) Functional characterization of α- (HypC), β- (HypB), and γ-subunits (HypC).

was 190-fold higher than that with D-proline, mainly caused by much lower K_m value. Only PMS/INT and PMS/NBT (but not Cl2Ind, ferricyanide, cytochrome *c* and NAD(P)$^+$) were available electron acceptor (**Figure 7C**). On the other hand, a ratio of C4DHyp to D-proline in k_{cat}/K_m of SmHypB was only 5.5.

3.4. Heteromeric HypDH

When compared with *P. putida* and *S. meliloti*, T4LHyp gene cluster of *Pseudomonas aeruginosa* PAO1 contained several additional genes, PA1267 (glycine/D-amino acid oxidase; *hypB*), PA1266 (heterotetrameric sarcosine oxidase α-subunit; *hypC*), and PA1266.5 (ferredoxin; *hypD*), in the order of *hypB-hypD-hypC* (**Figure 2B**). The *hypB-hypD-hypC* operon, in which the $(His)_6$-tag sequence was attached at the N-terminal of the *hypB* gene, was also expressed in *P. putida* cells (but not *E. coli*), and the recombinant protein was successfully purified to homogeneity. On SDS-PAGE analysis, three distinct bands with the estimated molecular mass of 48, 42 and 10 kDa were observed and corresponded to HypC (α-subunit), HypB (β), and HypD (γ) by LC-ESI-MS/MS analysis, respectively (**Figure 7A**). When compared with PpHypDH, this protein has similar specificity for C4DHyp and electron acceptor availability (referred to as PaHypDH). On the other hand, the k_{cat}/K_m value with C4DHyp was ~20-fold higher than that of PpHypDH probably due to the high stability (**Figure 7C**).

In contrast to homomeric HypDHs, both FAD and FMN (the molar ratio of ~2) were detected as prosthetic groups as well as OpnDH. On the other hand, when each subunit was functionally characterized, only proteins containing the HypB were active (**Figure 7D**). Only FAD was extracted from HypB (~0.8 mol/mol of protein), while FAD and FMN were extracted from HypBC (and HypBCD). These indicated that the dehydrogenation activity of heteromeric HypDH dependents only to β-subunit, and that the γ-subunit had no effect on the binding of FMN. Since two [Fe-S]-binding sites in the γ- and α-subunits were conserved, this enzyme might also contain [4Fe-4S] and [2Fe-2S] clusters as well as OpnDH (see Section 2.6) (**Figure 4A**).

3.5. Comparison with archaeal L-proline dehydrogenases

In spite of their same functions, there is only ~20% of sequence similarity of PpHypDH to PaHypDH (β-subunit) in D-amino acid oxidase superfamily. On the other hand, PaHypDH shows high sequential similarities to two dye-linked L-ProDHs from archaea (and OpnDH and HCN synthase) with heteromeric structure (**Figures 4** and **5**). First, L-ProDH of *Thermococcus profundus* is heterotetrameric structure of αβγδ containing 2 FAD (in α- and β-subunits) [23]. Second, L-ProDH (isozyme 1) of *Pyrococcus horikoshii* is heterooctameric structure of $\alpha_4\beta_4$ containing 1 FAD (β), 1 FMN (between α- and β-subunits), and 1 ATP (α) [24]. The γ-subunit of HypDH (and OpnDH and HCN synthase) corresponds to the N-terminal ~120 amino acid residues of the α-subunit of these L-ProDHs. Since the β-subunits show dehydrogenation activity by themselves, this subunit functionally corresponds to homomeric enzymes: so-called catalytic subunit [3, 5, 23]. On the other hand, the α-subunits may contribute to the regulation of catalysis, because of the binding of several prosthetic group(s) including FAD, ATP, Fe,

and/or NADH (**Figure 4B**). In fact, the heteromeric subunit assembly of HypDH significantly increases catalysis: 39-fold higher k_{cat}/K_m value for C4DHyp than that of the β-subunit alone due to 21-fold lower K_m value [3, 5].

3.6. Physiological role

It was believed that HypDH is involved only in T4LHyp but not in proline metabolism. In case of *S. meliloti*, the *hypB* mutant continues to grow on T4LHyp, but at half the wild-type rate, which may be due to alternative D-amino acid oxidases with broad specificity [25]. In fact, Rmar_0499 protein from *Rhodothermus marinus* JCM9785, whose gene is located within T4LHyp metabolic gene cluster, shows dehydrogenation activity not only toward C4DHyp but also several D-amino acids including (the most preferable) D-phenylalanine [1, 2]. Furthermore, the disruption of *hypB* also leads to a significant decrease of growth on D-proline. Since a ratio of C4DHyp to D-proline in k_{cat}/K_m of SmHypB is significantly low compared with those of PpHypDH and PaHypDH [3], this enzyme must physiologically function as a D-proline dehydrogenase in D-proline metabolism.

In addition to T4LHyp, other relatively rare L-Hyp compounds such as *cis*-4-hydroxy-L-proline (C4LHyp), *trans*-3-hydroxy-L-proline (T3LHyp), and *cis*-3-hydroxy-L-proline (C3LHyp) are found in nature. Among them, genetic and molecular information of T3LHyp and C3LHyp degradation has only recently been elucidated [26–28]. Interestingly, these metabolic genes are often contained within T4LHyp gene cluster on genomes of *S. meliloti* and *P. aeruginosa*, but not *P. putida*. Therefore, it is possible that each L-Hyp gene cluster may be responsible for the metabolism of L-Hyp(s) produced from different natural sources; collagen (containing only T4LHyp and T3LHyp), HRGP (only T4LHyp), and peptide antibiotics (T4LHyp, C4DHyp, C4LHyp, T3LHyp, and/or C3LHyp) (**Figure 6**). In fact, collagenase, peptidase, and/or the peptidase gene are located (closely) within the L-Hyp gene cluster of several bacteria. In addition to (homomeric and heteromeric) HypDH, among metabolic enzymes involved in these L-Hyp pathways, there are several examples of convergent evolution. In different bacterial species, these enzymes are involved in L-Hyp pathways with several combinations, suggesting that even the same L-Hyp pathway(s) evolved independently, at least partially.

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Over the last few decades, the prevalence of studies about symbiosis has dramatically grown in most regions of the world. Many aspects have been investigated related to this phenomenon. If we can gain understanding about symbiosis, then we may be able to manipulate it to reduce both chemical fertilizer use and environmental impact without decreasing the yield.

This book provides information about the symbiotic relationship between a fern and *Azolla*, plant control over thread development during legume-rhizobia symbiosis, bacterial leaf nodule symbiosis in flowering plants and the potential of rhizobium strains in improving nitrogen fixation and legume yields. We hope this information will be useful to all people working on a hot topic.

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