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Fusarium

Plant Diseases, Pathogen Diversity, Genetic Diversity, Resistance and Molecular Markers

Edited by Tulin Askun

FUSARIUM - PLANT DISEASES, PATHOGEN DIVERSITY, GENETIC DIVERSITY, RESISTANCE AND MOLECULAR MARKERS

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Meet the editor

Tulin Askun is a professor of Biology and Molecular Biology at the Department of Biology, Balikesir University, and the head of the Department of Molecular Biology in Balikesir, Turkey. She obtained her undergraduate degree in 1981 from the Ege Universitesi, Izmir, Turkey; master's degree in Scientific Research in 1997, with the thesis entitled "Macrofungi of Balya (Balıkesir) County"; and PhD degree in 2002 from the Balikesir University, with the thesis entitled "Isolation and Identification of Potential Ochratoxigenic Moulds from Raisins and Investigation of Time-Dependent Change of Oratratoxin-A Formation in Some Ochratoxigenic Species."

Before she was appointed as a full-time professor at the Balikesir University in 2014, she spent 5 years as an associated professor in the same university. Her research interests are fungal diversity, fungal identification, mycotoxins and fungal infections. She also has an interest in secondary metabolites, antifungal activity and fungal enzymes.

She is a member of several professional organisations and working groups acting as the project leader in more than 20 funded research projects (funds from the Scientific and Technological Research Council of Turkey [TÜBİTAK]; Ministry of Science, Industry and Technology; and Balikesir Universitesi scientific research projects). She has published many scientific papers, book chapters, and conference papers (http://www.balikesir.edu.tr/bau_yonetim/yukleme/birim/5/CV-Tulin%20Askun-ING.pdf).

Contents

Preface

This book introduces the properties of the genus *Fusarium* with several aspects. The primary purposes of the book are as follows:

- To provide an overview of historical importance and taxonomy of *Fusarium* species
- To help understand the mechanism of *Fusarium* infections and the factors that caused the infections
- To discuss diseases caused by *Fusarium* species and the pathogen diversity and host range of *Fusarium* species in plants
- To discuss mycotoxin contaminations in cereals, plant secondary metabolites, and antifungal and anti-mycotoxigenic compounds
- To discuss plant-*Fusarium* interactions, particularly the antagonistic activity of *Trichoderma* against *Fusarium* species
- To study genetic diversity, genetic resistance, and molecular markers with an aim to investigate the population diversity of *Fusarium* and the environmental conditions that enable its opportunistic growth
- To study the distribution and evolution of the genes responsible for mycotoxin biosynthesis and the steps to prevent toxin production
- To study suitable approaches for the management of diseases caused by *Fusarium* species and the development of *Fusarium*-resistant cultivars to reduce the diseases caused by *Fusarium* species on a wide scale

The book comprises 10 chapters from different countries including the USA, Mexico, Indonesia, China, India, Croatia, and Turkey. I am grateful to all the contributors, leading experts, and my colleagues who have collaborated with me on this book project and submitted their chapters for this book on *Fusarium*. They shared their valuable experiences, scientific works, original photographs, figures, and graphics related to the topics discussed in the book. I believe that this book will be beneficial to biologists, mycologists, chemists, molecular biologists, genetics, agriculturists, and scientists working in related fields.

I thank my family who supported me with their love and understanding. I offer my special thanks and appreciation to Ms. Kristina Kardum, the IntechOpen Author Service Manager, for her encouragement and help with recommendations for bringing out this book in the present form as well as for her concern, efforts and support in the task of publishing this volume. I also wish to express my sincere gratitude to other workers of IntechOpen for their efforts for publishing the book. I would like to thank IntechOpen for the opportunity given me to participate in this project.

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Chapter 1

Introductory Chapter: *Fusarium* - Pathogenicity, Infections, Diseases, Mycotoxins and Management

Tulin Askun

Additional information is available at the end of the chapter

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1. Introduction

The genus *Fusarium* contains pathogens that can cause significant harm to humans, animals and plants by infecting vegetables, grains and seeds and causing diseases in humans and animals. *Fusarium oxysporum*, *F. solani* and *F. fujikuroi* complexes are of great importance worldwide especially as a plant, human and animal pathogen.

Identifying Fusarium species is not easy. Currently, scientists are focused on identifying *Fusarium* species using molecular techniques, such as genetic markers and polymerase chain reaction-restriction fragment length polymorphism, for analyzing the rDNA internal transcribed spacer region.

The aim of this book is to highlight the new information reported by numerous studies on *Fusarium* species. The primary aims of this book are the following:

(a) To provide an overview of historical importance and taxonomy of *Fusarium* species

(b) To understand the mechanism of *Fusarium* infections and the factors that cause the infections

(c) To discuss *Fusarium* species-caused diseases, pathogen diversity and host range in plants

(d) To discuss mycotoxin contaminations in cereals

(e) To discuss plant secondary metabolites as well as anti-fungal and anti-mycotoxigenic compounds

(f) To discuss plant-*Fusarium* interactions

(g) To discuss the antagonistic activity of *Trichoderma* and *Fusarium* species

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h) To discuss genetic diversity, genetic resistance and molecular markers to investigate population diversity

(i) To discuss the environmental conditions that enable the opportunistic growth of *Fusarium*

(j) To discuss the distribution and evolution of the genes responsible for mycotoxin biosynthesis

(k) To discuss the steps that can be taken to prevent toxin production

(l) To discuss suitable approaches for *Fusarium* species disease management

(m) To discuss development of *Fusarium*-resistant cultivars to reduce the diseases caused by *Fusarium* species on a wide scale

2. Plant pathogens and cereals

Fusarium attacks numerous plants and cereals that are important for human and animal nutrition. It specifically infects certain parts of them, such as grains, seedlings, heads, roots or stem, and causes various diseases, reduced commercial yield, and decrease in product quality [1]. *Fusarium* head blight (FHB) [2, 3], foot (FR) and root rot (RR) [4] and crown rot (CR) are among the major diseases caused by them. FHB produced by *F. graminearum* (teleomorph Gibberella zeae, Schwabe) causes starch and protein losses in cereals [5]. *Fusarium* species are saprophytic and are found commonly growing on the plants as a pathogen. *F. proliferatum* is a plant pathogen that is capable of infecting many important crops. *F. oxysporum* f.sp. *cubense* (FOC) causes *Fusarium* wilt, which is the most destructive disease of banana [6]. Many *Fusarium* species from the *F. solani* species complex (FSSC) are pathogenic and virulent. FSSC causes diseases in many agriculturally important crops, such as FR and/or RR of the infected host plant and causes necrosis. Symptoms, such as wilting, stunting and chlorosis, vary widely according to FSSC pathogenesis and the host plant species. Necrosis depends on the severity of fungal development [4]. Two of the most serious diseases of wheat known globally are *Fusarium* CR and *Fusarium* FHB.

Stephens et al. [7] investigated the CR disease in wheat infected by *F. graminearum* and reported that CR developed in three stages. In the first stage, the *F. graminearum* biomass significantly increased within 2 days after inoculation. At this stage, there was germination of spores and superficial hyphal growth on the leaf sheath. In the second stage, the fungal biomass significantly decreased over 2 weeks. At this stage, the fungus penetrated from the outer parts of the leaf sheath to the leaf sheath base. In the third stage, biomass of *F. graminearum* increased significantly, and this increase correlated with fungal colonization on wheat and showed that the fungal biomass was being formed as fungal colonization on wheat crown parenchyma.

3. Fusarium infections in humans

Fusarium species cause superficial, locally invasive and diffuse infections in humans. Although *Fusarium verticillioides*, including *F. moniliforme* and *F. fujikuroi* species complex [8],

are opportunistic pathogens, the species in the *F. solani* complex include pathogenic species [9]. *F. solani*, *F. oxysporum*, *F. verticillioides* and *F. proliferatum* infect the immune-compromised patients. Sidhu et al. [10] reported that prevalent meningospondylodiscitis in an elderly diabetic patient caused by *F. oxysporum*. *F. sacchari*, *F. anthophilum*, *F. chlamydosporum* and *F. dimerum* was also thought as related to human disease. Guendouze-Bouchefa et al. [11] reported a rare case of perinephric abscess in a child caused by *F. chlamydosporum*.

The members of *F. solani* and *F. oxysporum* species complexes are known to include the agents that cause human infections worldwide. *F. solani* can adhere to and damage the corneal membrane [12]. Some *Fusarium* species, such as *F. dimerum*, are associated with keratomycosis, particularly in the bad hygiene conditions.

4. Fusarium diseases in animals

Fusarium mycotoxins affect the growth, reproduction and hormonal condition of the animal. The effect of these mycotoxins on animals depends on the quantity of mycotoxin intake. After intake, these mycotoxins arrive at the gastrointestinal epithelial cell layer which is covered by the mucous secreted from goblet cells [13, 14].

Although deoxynivalenol (DON) and fumonisin-B1 (FB1) increase the permeability of intestinal epithelial cell layer in humans, animals and birds, they worsen the viability and proliferation of intestinal epithelial cells. High doses of mycotoxins may cause abdominal distress, diarrhea, cardiac insufficiency, emesis and even death in pigs and equine leukoencephalomalacia (ELEM) in horses [15]. Through *in vivo* and *in vitro* experimental studies, Cortinovis et al. [16] demonstrated that ZEN and its metabolites markedly up-regulated estrogen secretion in the reproductive organs.

ZEN is closely associated with infertility, decreased milk production and hyperestrogenism [17]. Cortinovis et al. [16] reported that ZEN directly affect ovarian cells and alter oocyte maturation under in vitro conditions; conversely, under in vivo conditions, this mycotoxin affected ovulation and puberty onset and caused morphological and functional disorders. T-2 toxin (T-2) causes cutaneous lesions in the mount and intestinal membrane and reduces egg production in poultry [18].

5. Mycotoxins and mycotoxin-producing conditions

Fusarium mycotoxins are very common worldwide. They exist in many plants and in various compositions. The major *Fusarium* mycotoxins are FB1, trichothecenes [e.g. DON, nivalenol (NIV), T-2 and ZEN] [19–21]. The most important species that is common in Europe is *F. graminearum*. In the past, *Fusarium* genus members were mostly not considered as pathogens in the field. However, *F. proliferatum* and *F. verticillioides* are of great importance as the main producers of the most dangerous *Fusarium* mycotoxins [22, 23]. Worldwide mycotoxin occurrence in maize and wheat/bran samples with their median and maximum levels were given in **Figure 1** [24, 25].

Shi et al. [5] evaluated the mycotoxins from 20 of the most common *Fusarium* species and sorted them into the following three groups based on their molecular characterization (**Figure 2**). Group-1 comprised fusaric acid producers and was further divided into two subgroups. Subgroup-I comprised *F. fujikuroi*, *F. solani*, *F. verticillioides* and *F. proliferatum* that produce fusaric acid and fumonisins; subgroup-II comprised *F. musae*, *F. equiseti*, *F. temperatum*, *F. subglutinans*, *F. tricinctum*, *F. oxysporum*, *F. concentricum*, *F. sacchari* and *F. andiyazi* that produce only fusaric acid. According to the classification of *Fusarium* mycotoxins, type-A trichothecene producers comprising *F. polyphialidicum*, *F. sporotrichioides* and *F. langsethiae* formed the Group-II, and type-B trichothecene producers comprising *F. meridionale*, *F. culmorum*, *F. graminearum* and *F. poae* formed Group-III.

In the presence of *Fusarium* species in plants, the contamination with fumonisins was shown in wheat [26], garlic [27], and asparagus [28]. The most affected plants, that is, maize, beans, soybean [29], rice [30], and sorghum [31] were specifically infected by *Gibberella fujikuroi* species complex (*F. proliferatum, F. verticillioides* and *F. andiyazi*) [29, 32].

Guidance values for *Fusarium* mycotoxins were set in Commission Recommendation 2006/576/EC [33]. Recommended values for the *Fusarium* mycotoxins DON, ZEA and fumonisins were set in "Commission Recommendation 2006/576/EC" [33]. For T-2 and HT-2 toxin, indicative

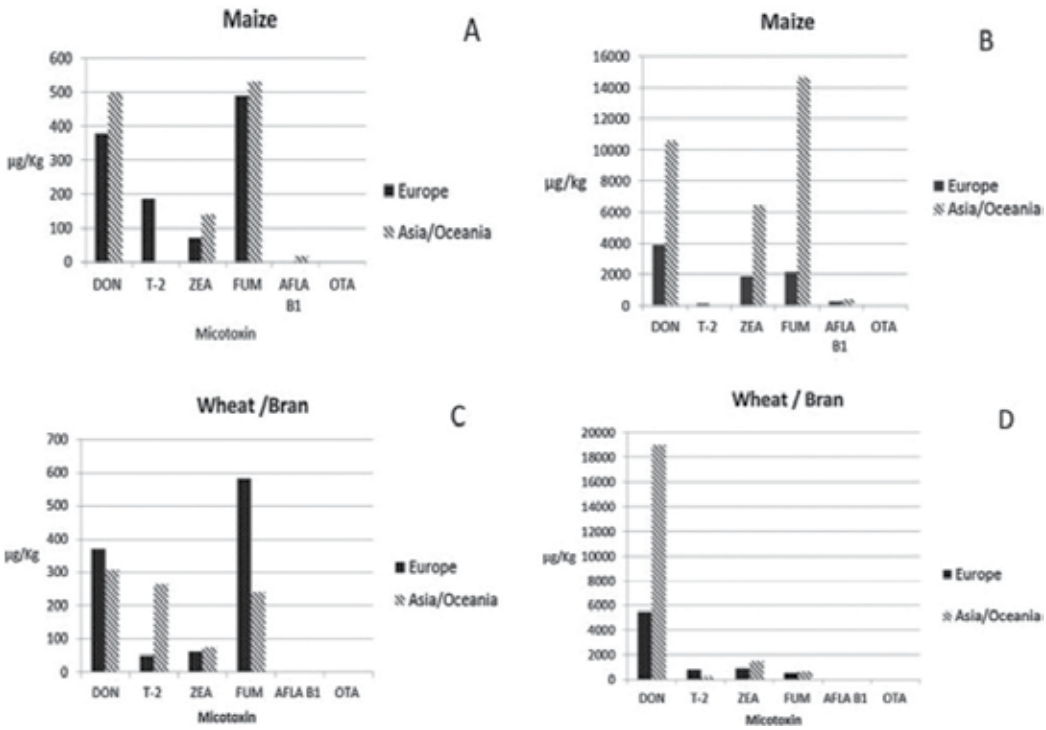

Figure 1. Worldwide mycotoxin occurrence (µg/kg) in maize and wheat/bran samples (A, C: Median of positive samples; B, D: Maximum levels) [24, 25].

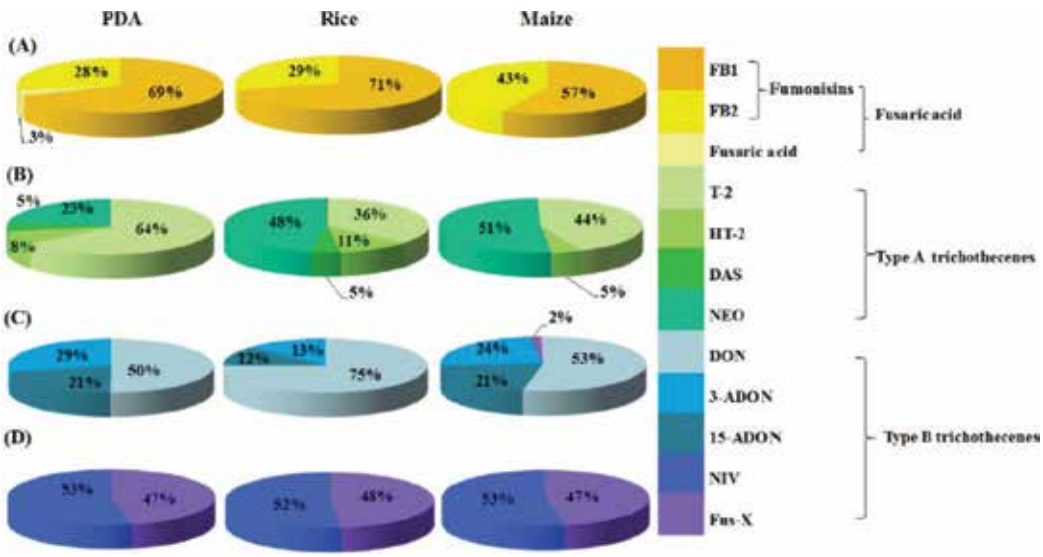

Figure 2. Mycotoxin classification according main four *Fusarium* species (A) *F. proliferatum* (B) *F. langsethiae* (C) *F. graminearum* and (D) in PDA, rice and maize medium [5].

levels for cereals and cereals products were set in "Commission Recommendation 2013/165/EU" [34]. Maximum limits for DON, ZEA, fumonisins, T-2 and HT-2 toxins have been set for cereals and by-products according to the production technology used [35].

6. Fungal resistance

Resistance of *Fusarium* to antifungal drugs has been defined by many researchers. It is known that many FSSC members cause fusarial onychomycosis [36]. *F. solani* showed more resistance to antifungal agents than others [37]. The effect of azole antifungals used clinically is depend on a particular site, lanosterol-14α-demethylase. While imidazole or triazole rings are important for conferring the therapeutic effect in animals, epoxiconazole, propiconazole, difenoconazole, bromuconazole and tebuconazole are used for plants. *Fusarium* spp. are resistant to azoles [38].

Tupaki-Sreepurna et al. [39] reported that FSSC members, mainly *F. falciforme* and *F. keratoplasticum*, showed multi-drug resistance against caspofungin and azoles. Only a few antifungal agents (voriconazole, posaconazole and amphotericin B) showed *in-vitro* activity against *F. falciforme* and *F. keratoplasticum* [40].

Conversely, the echinocandins are lipopeptide molecules which effectively work by inhibiting 1,3-β-D-glucan synthase of the fungal membrane. If a change occurs in the amino acid residues of β-1,3-glucan synthase enzyme subunits (FKS subunits) in the treatment process, it may lead to increased drug resistance [41, 42].

Polyenes, which are fungicidal, are known as amphipathic drugs, such as nystatin and amphotericin-B. The complexes show efficacy via destroying the proton gradient, allowing for the leakage of ions and removal of ergosterol from phospholipids in the membrane, thus causing fungal cell death in the process [43, 44].

7. Plant disease resistance mechanism

Plants, humans and animals give instant response to the pathogen. In animals, this effect is seen as antibody production, while in plants, it is seen in the form of secretion of various proteins, such as defense-related enzymes and pathogenesis-related proteins [45]. Defense-related enzymes are of great importance in the plant disease resistance mechanism. Immunized plants have rich defense-related enzymes that prevent them from suffering large losses.

If a plant is stimulated by a pathogen, early local defense reactions (a local programmed cell death) are followed by systemic responses (signal is transmitted from infected tissue to the whole plant). At the end, overall defense gene expression gets induced. Consequently, signal perception is essential for plants to combat pathogens [46, 47].

Numerous studies have been done on the transporter genes of plants for improved resistance to *Fusarium* spp. A sucrose transporter gene (IbSWEET10) of the SWEET gene family obtained from the sweet potato line ND98 was tested for this purpose. This overexpression of the gene has been shown to reduce sugar levels and has a potential use to lower carbohydrate levels and increase the resistance of the plant [48].

8. Fungal transporters

Transporters are of great importance in protecting fungi against plant defense compounds. Transporters enable efflux of the plant-originated defense compounds. Although resveratrol (from grape) and camalexin (from Arabidopsis) transport via the transporter BcatrB of *Botrytis cinerea*, pisatin (from pea) transports by the NhABC1 transporter of *F. solani* f. sp. *pisi*, and rishitin (from potato) transports via the GpABC1 transporter of *F. sambucinum* [4, 49–51].

Transporters are divided into two major classes: the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters. ABC transporters are known to be important for resistance against fungal pathogens, particularly for pleiotropic drug resistance or multidrug resistance domains [52]. Although some transporters produce specific or non-specific toxins, some of them show very specific responses to fungicide sensitivity or resistance [53].

9. Identification, control and management

It is possible to identify the genus *Fusarium* by several methods. On culturing, hyaline, banana-shaped and multicellular macroconidia are very common; however, to identify them at the

species level is not easy. Therefore, molecular methods are needed. Some of the most commonly used molecular methods are the genus-specific PCR, 28 s rRNA gene sequencing, sequence-based PCR, multiplex tandem PCR and automated repetitive sequence-based PCR [54].

As a biological control, Ben Amira et al. [55] showed that when *Trichoderma harzianum* was co-cultured with *F. solani*, the former happened to have an antagonistic effect *in-vitro*. Then, they repeated this experiment by inoculating olive tree roots with the same *T. harzianum* and *F.* solani combination. They reported that the former showed a mycoparasitic reaction and antagonistic effects on *F. solani*. Therefore, mycoparasitic fungi, such as *T. harzianum* may be used as a biocontrol agent against *Fusarium*.

Notably, agricultural and chemical precautions cannot be completely successful in preventing *Fusarium*-related diseases in plants [56]. Therefore, synthetic fungicides are not a true approach for preventing the *Fusarium*-related diseases due to their harmful effects on the ecosystem and environment, and growing disease-resistant species to combat *Fusarium*-related diseases seems a more sustainable approach. Resolving the concern of plant diseases caused by *Fusarium* using biological control methods seems to be a more efficient and eco-friendly approach for agricultural products.

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Chapter 2

Fusarium: Historical and Continued Importance

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Additional information is available at the end of the chapter

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Abstract

Historically, *Fusarium* has been important because: (i) taxonomy of *Fusarium* species has been a controversial issue, (ii) *Fusarium* species are among the most important plant pathogens in the world, and (iii) many *Fusarium* species produce mycotoxins that cause animal and human diseases. The genus *Fusarium* was introduced by Link in 1809. "Die Fusarien" was published by Wollenweber and Reinking in 1935, described 65 species, 55 varieties, and 22 forms of *Fusarium*. In 1945, Snyder and Hansen reduced number of species of *Fusarium* to nine. In 1990s, the application of phylogenic species concept based on the DNA sequencing resulted in introducing new species of *Fusarium* that cannot be distinguished morphologically. In 2006, Leslie and Summerell integrated the morphological, biological, and phylogenic species concepts and published "The *Fusarium* Laboratory Manual," which provides details of identification of 70 *Fusarium* species. Although considerable research studies on *Fusarium* have been accomplished in the past 200 years, yet *Fusarium* diseases continue to be among the most important plant diseases. *Fusarium* fungi are the most widespread in cereal-growing areas of the world and produce a diversity of mycotoxins, including zearalenone, fumonisin, moniliformin, and trichothecenes, which cause various disorders, including cancer, in animals and humans.

Keywords: *Fusarium*, taxonomy, fungi, plant diseases, mycotoxins

1. Introduction

The genus *Fusarium* was introduced by Link in 1809 [1]. However, *Fusarium* received more attention when "Die Fusarien" was published by Wollenweber and Reinking in 1935 [2]. In the past 80 years, tremendous investigations have been carried out on the taxonomy, biology, and mycotoxins of *Fusarium* species [3–5]. Although Wollenweber and Reinking described 65 species, 55 varieties, and 22 forms of *Fusarium* in 1935 [2], Snyder and Hansen reduced number of species of *Fusarium* to nine [6]. During 1940–1980, several mycologists developed different

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taxonomy of *Fusarium*, but none of them received a global agreement. During 1980s, *Fusarium* taxonomists in the world collaborated to offer a unique agreement on *Fusarium* taxonomy. In 1990s, however, the application of phylogenic species concept based on the DNA sequencing resulted in introducing new species of *Fusarium* that often cannot be distinguished morphologically. In 2006, Leslie and Summerell published "The *Fusarium* Laboratory Manual" and described 70 *Fusarium* species [3].

Fusarium species are among the most common and widespread plant pathogens in the world and are of great economic importance [4]. Every plant pathologist, mycologist, agronomist, and horticulturist encounters them in the course of work. They are serious pathogens on a wide range of crops. In spite of worldwide investigations on *Fusarium* in the past 200 years, *Fusarium* diseases continue to be among most important plant diseases and cause widespread crop losses throughout the world [4, 7].

Several *Fusarium* species produce mycotoxins, which cause various disorders, including cancer in animals and humans [5]. Zearalenone, fumonisin, moniliformin, and trichothecenes are among most important *Fusarium* mycotoxins, especially in grains [5]. The objective of this chapter is to provide details of *Fusarium* taxonomy, pathology, and mycotoxins.

2. Taxonomy

The genus *Fusarium* was introduced in 1809 [1]. During 1809–1935, much of the works on *Fusarium* were focused on identification of *Fusarium* species and diagnosis of *Fusarium* diseases. In the past 100 years, the taxonomy of *Fusarium* has undergone a number of changes, which is adapted and "The *Fusarium* Laboratory Manual" [3].

2.1. Wollenweber and Reinking

The basis for all modern taxonomic systems of *Fusarium* species is the work of Wollenweber and Reinking, which was published in "Die Fusarien" in Germany. In this publication, 16 sections, 65 species, 55 varieties, and 22 forms of *Fusarium* were introduced, which were separated based on the morphological differences [2]. Prior to this publication, about a thousand *Fusarium* species had been described, often a different species for every host. Wollenweber and Reinking offered an order to a chaotic situation of *Fusarium* taxonomy. In their taxonomic system, each section contained species that were united by critical morphological characteristics (e.g., macroconidia morphology and pigment). Each section contained only a few species. Other taxonomists used the sections created by Wollenweber and Reinking to develop their taxonomic systems.

2.2. Snyder and Hansen

During 1940s and 1950s, Snyder and Hansen in the United Sates (US) developed a new taxonomy system and reduced number of species of *Fusarium* to nine [6, 8, 9]. Their identification was based on using cultures derived from single spores. The Snyder and Hansen species taxonomy was easy to use and identify any *Fusarium* isolate to species level.

2.3. Gordon

Gordon published a number of papers on *Fusarium* species collected from Canada [10–12]. He used *Fusarium* taxonomy system developed by Wollenweber and Reinking with some concepts of Snyder and Hansen system.

2.4. Messiaen and Cassini

These French scientists developed a *Fusarium* taxonomy system based on Snyder and Hansen system [13]. They used varieties for the subspecific level instead of cultivars, which was used by Snyder and Hanson.

2.5. Matuo

Matuo was a Japanese scientist who used the system developed by Snyder and Hanson and introduced a new *Fusarium* taxonomy system with 10 species [14].

2.6. Raillo

Raillo, a Russian scientist, published a taxonomic system based on the shape of macroconidia, and the presence of microconidia and chlamydospores [15].

2.7. Bilai

Bilai, a Ukrainian scientist, studied variability in characteristics related to temperature, moisture, and culture media composition and offered her own revision of the taxonomy of the genus *Fusarium* and recommended combining some sections suggested by Wollenweber and Reinking [16, 17].

2.8. Booth

A significant development in the taxonomy of *Fusarium* was made by Booth from England during 1960s and 1970s. He published a monograph "The Genus *Fusarium*" [18], which was a revision of the Wollenweber and Reinking's system. Booth introduced the use of the morphology of the conidiogenous cells, especially those producing the macroconidia.

2.9. Gerlach and Nirenberg

Based on the taxonomy published in "Die Fusarien," Gerlach and Nirenberg published their own *Fusarium* taxonomy system in Germany in 1982 [19]. In spite of the criticism of their taxonomic system, their work was a significant step forward in understanding of *Fusarium* taxonomy and many of the suggested species in their system are now accepted.

2.10. Joffe

Joffe, originally a Russian scientist and then in Israel, began his studies on *Fusarium* in Russia in 1940s. He included *Fusarium* isolates collected from Russia, Israel, and some other

countries in his studies and evaluated their taxonomic and mycotoxicological issues. His work was published as a monograph "*Fusarium* Species: Their Biology and Toxicology" [20]. His taxonomic approach was based on the taxonomic systems of Wollenweber and Reinking [2] and Gerlach and Nirenberg [19].

2.11. Nelson, Toussoun and Marasas

Toussoun and Nelson from the United States published a pictorial guide for identification of *Fusarium* species, in which 9 species and 10 cultivars were described [21]. In 1983, Nelson and Toussoun together with W. F. O. Marasas from South Africa published an illustrated manual of *Fusarium* species and described 46 species [22]. Their taxonomic approach began a definitive shift toward a more complicated taxonomy and a larger set of recognized species and away from the nine species of Snyder and Hansen system. This manual has been widely used by scientists.

2.12. 1980s and 1990s

During 1980s, *Fusarium* taxonomists, including Burgess and Summerell from Australia, Gerlach and Nirenberg from Germany, Marasas from South Africa, and Nelson and Toussoun from the US collaborated to offer a unique agreement on *Fusarium* taxonomy based on fungal morphological characteristics. In 1990s, however, the application of phylogenic species concept to DNA sequencing resulted in introducing new species of *Fusarium* that often cannot be distinguished morphologically. Thus, the relatively unique uniformity of 1980s shifted toward another chaos on *Fusarium* taxonomy.

2.13. Leslie and Summerell

In 2006, Leslie from the United States and Summerell from Australia integrated the morphological, biological, and phylogenic species concepts and published "The *Fusarium* Laboratory Manual" with 70 species [3]. This manual, which is based on the outcomes of workshops conducted at the Kansas State University, is widely used by mycologists and plant pathologists to identify *Fusarium* isolates.

Although taxonomy of *Fusarium* species has been historically a complex issue, and no unanimous agreement available among the *Fusarium* taxonomists, using morphological characteristics combined by the molecular data minimizes differences in identification of *Fusarium* isolates. As more information is generated, more accurate taxonomic systems are expected to be developed for the identification of species of *Fusarium*.

3. Pathology

The members of genus *Fusarium* can incite diseases in plants, animals, and humans [23]. The mortality rate for human patients with systemic *Fusarium* infection is reported to be greater than 70% [24]. In addition, *Fusarium* species produce secondary metabolites that are associated with plant diseases, as well as with diseases of animals and humans [25, 26]. In this chapter, only *Fusarium* diseases in plants will be discussed.

Fusarium has been known for over 200 years. Despite universal effort on developing effective management of *Fusarium* in plants, *Fusarium* diseases continue to be among the most important plant diseases. *Fusarium* species are among the most widespread fungi in the world and are of great economic importance. Many plant species are affected with at least one *Fusarium* disease [3, 4]. The American Phytopathological Society reported that 81 of 101 economically important plants have at least one *Fusarium* disease (www.apsnet.or/online/common/search.asp). To understand importance of *Fusarium* diseases in plants, **Table 1** was prepared that shows *Fusarium* species, their host plants, and geographical distributions.

***Fusarium* species**	**Host plants**	**Geographic distribution**
F. acuminatum	Legumes	Temperate regions
F. andiyazi	Sorghum	Africa, Australia, US
F. anthophilum	Many plant species	Temperate regions
F. avenaceum	Carnations, cereals, legumes	Temperate regions
F. aywerte	Grasses	Australia
F. babinda	Soil	Australia
F. begoniae	*Begonia* species	Germany
F. brevicatenulatum	Millet, *Striga asiatica*	Africa
F. bulbicola	Bulb plant species	Europe
F. camptoceras	Banana, cacao	Tropical and subtropical regions
F. circinatum	Conifers	Chile, Japan, Mexico, South Africa, US
F. concentricum	*Musa* species	Central America
F. crookwellense	Potato, cereals	Temperate regions
F. culmorum	Cereals	Temperate regions
F. decemcellulare	Trees	Tropical regions
F. denticulatum	Sweet potato	Brazil, Cuba, Indonesia, US, Zambia
F. foetens	*Begonia* species	Germany, Netherlands
F. fujikuroi	Rice	Rice-growing areas
F. globosum	Corn, wheat	Africa, Japan
F. graminearum	Barley, corn, wheat	Worldwide
F. guttiforme	Pineapple	Cuba, South America
F. heterosporum	Millet, other grasses	Africa
F. hostae	*Hosta* species	South Africa, US
F. konzum	Grasses	US
F. lactis	Fig	US
F. lateritium	Woody plants	Worldwide
F. mangiferae	Mango	Africa, Asia, US
F. musarum	Banana	Panama
F. napiforme	Millet, sorghum	Africa, Argentina, Australia
F. nelsonii	Alfalfa, sorghum	South Africa
F. nisikadoi	Bamboo, wheat	Japan

Fusarium species	Host plants	Geographic distribution
F. nygamai	Sorghum	Arid regions
F. oxysporum	Many plant species	Worldwide
F. phyllophilum	*Dracaena* and *Sansevieria* species	Europe, Japan
F. poae	Cereal	Worldwide
F. polyphialidicum	Sorghum grain	Australia, Italy, South Africa
F. proliferatum	Asparagus, corn, mango, sorghum	Worldwide
F. pseudoanthophilum	Corn	Southern Africa
F. pseudograminearum	Barley, triticale, wheat	Drier areas worldwide
F. pseudonygamai	Pearl millet	Africa, US
F. ramigenum	*Ficus carica*	US
F. redolens	Many hosts	Temperate regions
F. sacchari	Corn, sugarcane	Mexico, Philippines
F. semitectum	Banana	Subtropical regions
F. solani	Many plant species	Worldwide
F. sterilihyphosum	Mango	South Africa
F. subglutinans	Corn	Cooler corn-growing areas
F. succisae	*Succisa pratensis*	Europe
F. thapsinum	Sorghum	All sorghum-growing areas
F. torulosum	Several plant species	Temperate regions
F. udum	Pigeon pea	Southern Asia, sub-Saharan of Africa
F. venenatum	Several plant species	Europe
F. verticillioides	Corn	Worldwide

Source: The *Fusarium* Laboratory Manual [3].

Table 1. Plant pathogen *Fusarium species*, their host, and geographic distribution.

4. *Fusarium* toxins

Mycotoxins are toxic secondary metabolites produced by fungi and are capable of causing diseases in both animals and humans. Mycotoxins may produce birth defects, abortion, tremors, and cancers [27–30]. Among the major mycotoxin-producing fungi are *Aspergillus*, *Claviceps*, *Fusarium*, and *Penicillium* species [27, 28]. *Fusarium* fungi are the most widespread in cereal-growing areas of the world and produce a diversity of mycotoxin types. The most prevalent *Fusarium* toxins are zearalenone, fumonisin, moniliformin, and trichothecenes (T-2/HT-2 toxin, deoxynivalenol, diacetoxyscirpenol, nivalenol) [5, 28, 30, 31].

Zearalenone is a group of estrogenic metabolites produced by several species of *Fusarium*, the most known of which is *F. graminearum* [5, 28]. Zearalenone is the generic name for a complex macrocyclic molecule and is derived from the perfect stage of the fungus *F. graminearum* (*Gibberella zeae*) [28]. *Fusarium* infection and zearalenone production are most notable on

barley, corn, oat, sorghum, and wheat. *Fusarium* causes crown rot of corn, and scab of barley, oat, and wheat. Zearalenone is produced in infected plants in the field and in stored food and feed stuffs including cereal grains [5, 28].

Zearalenone is frequently implicated in reproductive disorders of farm animals and occasionally in hyperoestrogenic syndromes in humans [28, 32]. It has been reported that zearalenone possess estrogenic activity in cattle, pigs, and sheep. The biotransformation for zearalenone in animals involves the formation of two metabolites α-zearalenol and β-zearalenol, which are subsequently conjugated with glucuronic acid [32]. Moreover, zearalenone has also been shown to be hepatotoxic, hematotoxic, immunotoxic, and genotoxic.

Fumonisins are hydroxylated long-chain alkylamines esterified with propanetricarboxylic acid moieties produced by *Fusarium moniliforme* worldwide [33, 34]. The fumonisins have been reported carcinogenic in laboratory rats. It has also been reported that consumption of corn contaminated with *Fusarium moniliforme* is associated with higher than average incidence of esophageal cancer, and fumonisins may be responsible. Fumonisins are structurally similar to sphingosine and may exert their biological activity through their ability to block key enzymes (sphinganine- and sphingosine-N-acyltransferases) involved in sphingolipid biosynthesis.

Moniliformin is produced by several *Fusarium* species on cereals worldwide [31, 35]. Moniliformin is a small and ionic molecule that forms only a single sensitive fragment ion in the collision cell of a tandem mass spectrometer. There is great variability in the moniliformin synthesized by *Fusarium* spp. [35]. It has been reported that moniliformin in large amounts acts at the level of sugar metabolism and is cytotoxic at high concentrations in mammalian cells [35]. In addition, this toxin causes intoxication, and the lesions include intestinal hemorrhage, muscle weakness, breathing difficulty, cyanosis, coma, and death.

Trichothecenes are a very large group of mycotoxins produced by various species of *Fusarium*, *Cephalosporium*, *Myrothecium*, *Stachybotrys, Trichoderma*, *Trichothecium*, and *Verticimonosporium*. The generic name "trichothecene" has been derived from a *Trichothecium* species from which the first of these related compounds was isolated [28]. Trichothecenes belong to sesquiterpene compounds. They are produced on many different grains, e.g., corn, oats, and wheat by various *Fusarium* species such as *F. graminearum*, *F. poae*, and *F. sporotrichioides* [28, 36, 37].

There are several types of trichothecene mycotoxins, including deoxynivalenol, diacetoxyscirpenol, HT-2 mycotoxins, neosolaniol, nivalenol, satratoxin-H, T-2 mycotoxins, verrucarin A, and vomitoxin. Exposure to trichothecene mycotoxins can cause different symptoms in people such as dry eyes, tiredness, fatigue, vomiting, diarrhea, abdominal pain, mental impairment, rash, and bleeding [28]. In addition, T-2 mycotoxins are also substances for biological warfare that can be absorbed through a person's skin [37].

Trichothecenes are typically found in plants when the autumn is cool and wet that delays harvest of grains such as corn. The toxins are also found in animal feeds that contain contaminated grain with *Fusarium*. Joffe [39] reported that trichothecenes are among the most toxic mycotoxins. He found that the LD50 rate for laboratory mice given trichothecene mycotoxins is between 1 and 7 mg/kg, depending on the specific type of trichothecene and the method of exposure [38, 39]. Toxicity of trichothecene in human is documented since 1913 when people in Russia consumed cereals that overwintered in the field [38, 39].

5. Conclusions

After about 200 years from the first introduction of *Fusarium*, there is not a universal agreement on the taxonomy of *Fusarium* species yet. However, considerable efforts are underway to use the available information to develop a uniform taxonomy system for *Fusarium*. *Fusarium* species infect most of plant species and cause substantial crop and yield losses. Effective management of *Fusarium* diseases in crops is not only essential for preventing crop losses but also needed to minimize mycotoxin production in food and feed products. Major strategies for preventing/minimizing mycotoxin production should be based on preventing growth of *Fusarium* in plants and therefore mycotoxin formation, reducing or eliminating mycotoxins from contaminated food and feed stuffs, or diverting contaminated products to low risk uses.

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Chapter 3

Fusarium Mycotoxins and Metabolites that Modulate Their Production

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Abstract

The genus *Fusarium* is a group of fungi producing several types of toxins with toxicological effect in both humans and animals. Such fungi are commonly found in soils so it can contaminate various types of crops, preferably cereals, leading to significant economic losses. Relative humidity, storage temperature and various handling in cereales increase the possibility of contamination by *Fusarium* toxins. Cereals naturally have secondary metabolites that may help attenuate contamination by these toxins, but it is necessary to know strategies and mechanisms that generate inactivation mycotoxins. This chapter reviews relevant information about cereal mycotoxin contamination, as well as the production of cereal secondary metabolites as a strategy to reduce the possibility of mycotoxin contamination.

Keywords: *Fusarium*, mycotoxin, secondary metabolites, cereals, detoxification

1. Introduction

Mycotoxins produced by fungi of the genus *Fusarium* have the universal distribution, and economic importance given their toxicity for animals, humans and plant pathogens, which infect and colonize various cereal crops such as maize, rice, wheat and oats in temperate and semi-tropical areas. Among the mycotoxin-producing species are *F. sporotrichioides*, *F. graminearum* and *F. verticillioides*, which produce toxins such as zearalenone, zearalene, deoxynivalenol or nivalenol, T-2 toxin and diacetoxyscirpenol [1]. These toxins generate diverse diseases to crops

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and contamination to diverse types of cereals mainly to maize being of toxicological concern the ear rot [2]. Therefore, the contamination prevention could be generated by the biosynthesis the *Fusarium* during the crop. Then, the development of *Fusarium* can be triggered by the environmental conditions, agricultural practices and range of susceptibility [3]. Biochemical resistance is directly associated with specific proteins and metabolites that focus on the biosynthetic analysis of mycotoxins explaining the sporadic occurrence of the mycotoxins as fungal metabolites. Several studies indicate that secondary metabolites present in cereals can modulate the production of mycotoxins, and these are important in plant response to fungal contaminations, such as, the phenolic compounds that control or prevent the response to mycotoxins [4]. Phenolic acids, including ferulic acid, tannins and proanthocyanidins, are the most abundant in cereal showing the highest potential to function as fungal growth inhibitor [1, 3]. In this sense, the objective of this chapter is the review of the main mycotoxins of the genus *Fusarium* that affects cereals, as well as the production of secondary metabolites that can modulate their production. The above will gather relevant information on possible inhibition options in cereal contamination by mycotoxins of the genus *Fusarium*, including major mycotoxin-producing species, cereal contamination by mycotoxins (economic losses, implications to food safety and health), cereal secondary metabolites with antifungal activity and possible mechanisms that modulate inhibition of mycotoxin production of *Fusarium* species.

2. Overview of major mycotoxin-producing species

The genus *Fusarium* comprises an outsize cluster that includes animal and plant pathogenic species with great biological properties [5]. Some species are used as biocontrol agents, as industrially applicable enzymes, and some cause diseases in many agronomical crops and are probably the most prevalent toxin-producing fungi [6]. The genera *Aspergillus*, *Penicillium* and *Fusarium* are filamentous fungi and produce mycotoxins that are toxic and/or carcinogenic secondary metabolites produced under appropriate environmental conditions [7]. *Fusarium* produces three of the most important of mycotoxins, such as *fumonisins*, *trichothecenes* or *zearalenone*, and these furthermore produce emerging mycotoxins as well as *fusaproliferin*, *beauvericin*, *enniatins* and *moniliformin* [8].

Mycotoxins possess biological activities that represent a problem for both human and animal health (**Figure 1**). The ingestion of these compounds can cause chronic disease, morbidity and death and reduce the resistance to pathogens [9]. Most mycotoxin are stable during food processing, and these are commonly resistant to chemical and thermal changes. Mycotoxins can also come to the human by animal products [10, 11].

2.1. Aflatoxins

Aflatoxins (B1, B2, G1, G2) are difuranocoumarin synthesized by *Aspergillus flavus* and *Aspergillus parasiticus* present in soil and various organic materials. Aflatoxin-producing species has been reported in a wide variety of food commodities (maize, peanuts, barley oats, rice, cottonseed, spices and figs [12]. Optimal conditions for their propagation are high temperature

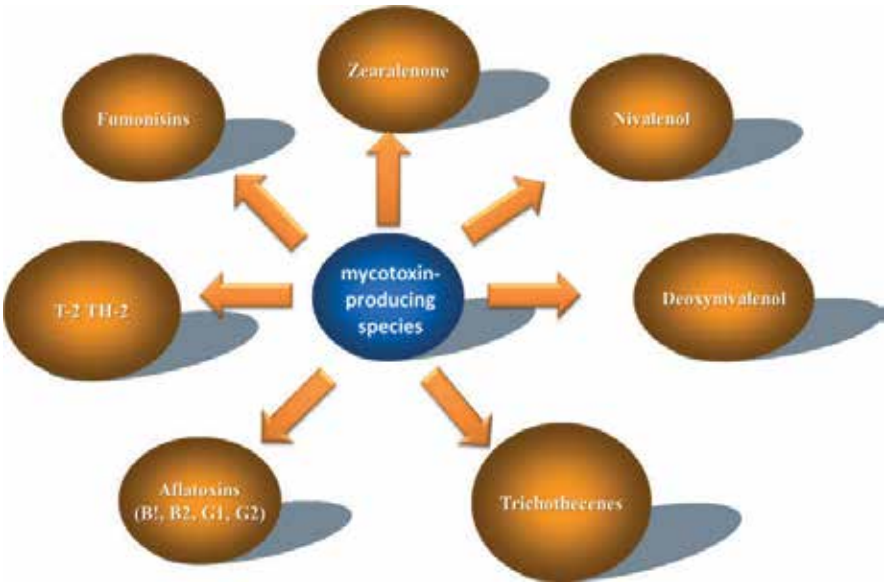

Figure 1. Group of mycotoxin-producing species.

and humidity (30–33°C, 0.99 water activity) [13]. Aflatoxins showed carcinogenic, teratogenic, hepatotoxic, mutagenic and immunosuppressive effects; specific limits have been set on average to 50 mg/kg for total aflatoxins.

2.2. Trichothecenes

Trichothecenes can be divided into four types: A (T-2 and HT-2 toxins, diacetoxyscirpenol), B (deoxynivalenol, nivalenol), C and D, and these are the main and most diverse chemical groups of the three major classes of *Fusarium* mycotoxins [14, 15]. These are shaped by a set sesquiterpenoids with or without a tricyclic nucleus. Trichothecenes are small, amphipathic molecules that can move passively across cell membranes [16, 17]. The most prevalent contaminants in wheat, barley, oats and maize are trichothecenes of types A and B. Exposure to these toxins can cause immunological problems, vomiting, skin dermatitis, hemorrhagic lesions, acute diseases and gastroenteritis. Trichothecenes in wheat behaves as phytotoxic were causing chlorosis, inhibition of root elongation, and dwarfism [4, 9]. Trichothecenes show several inhibitory effects such as inhibition of proteins, DNA and RNA synthesis on the primary metabolism of eukaryotic cells [18].

2.3. Deoxynivalenol

Trichothecene of type B (deoxynivalenol) is produced by *Fusarium graminearum* and *Fusarium culmorum*; these mycotoxin-producing species are found in wheat, rye, barley and oats [18]. These are a group of toxins with a keto group at carbon 8 of the parent epoxytrichothecene nucleus [19]. Deoxynivalenol is divided into five types (deoxynivalenol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X and nivalenol). "ribotoxic stress response" is produced

by deoxynivalenol added the ribosome in eukaryotic cells [20]. The impact of deoxynivalenol on the immune system ranges from immunosuppression to immunostimulation, according to its concentration, duration and time of exposure [19].

2.4. Nivalenol

Nivalenol are the main mycotoxins produced by *F. cerealis*, *F. poae*, *F. nivale*, *F. culmorum* and *F. graminearum*. Maize red ear rot throughout is caused by nivalenol [21]. As expected, they reportedly also share many toxicological properties, such as the inhibition of cell proliferation, induction of interleukin-8 secretion and the involvement of stress-activated MAPKs and nuclear factor-κB in the signal transduction pathways of toxicities [15].

2.5. Zearalenone

Zearalenone is a mycotoxin, which have a structure of estrogenic lactone; they have sufficient structural similarity and these synthesized by various *Fusarium* species—*F. graminearum*, *F. culmorum* and *F. crookwellense*. Zearalenone is found in cereals, mainly maize, and processed foods and these are a non-highly toxic mycotoxin [10, 22]. These mycotoxins have been producing of estrogenic effects in animals and the stimulation of human breast cancer cells growth. Zearalenone is a mycotoxin producing of host-contaminated corn [9]. Also, inhibiting the gene expression caused by zearalenone produced severe hepatic illness. Zearalenone has been shown to be immunotoxin and hepatotoxic and nephrotoxic and an enhancer of lipid peroxidation [19, 23].

2.6. Fumonisins

Fusarium verticillioides and *F. moniliforme* produced by Fumonisins (A, B, C, P) are toxic secondary metabolites, mycotoxins non-fluorescent, common fungal contaminants in grains and agricultural commodities [24]. These are analogous to sphingolipids, and intake of contaminated foods with fumonisins B1 has been associated with equine leukoencephalomalacia, porcine pulmonary edema and liver cancer in rats and decreased body weights in chickens [8, 23]. The exposure levels ranging from 0.02 to 0.2 mg/kg in body weight have been found of fumonisin concentration; these are within the limit of intake. Although fumonisins are relatively thermal stability, these may undergo reactions in food systems that alter their chemical structure and toxicity and is potentially hazardous to the health of both humans and animals [25].

3. Overview of mycotoxin-contaminating cereals

Mycotoxin contamination can occur pre-harvest when the crop plant is growing or post-harvest during processing. Storage of cereals at temperatures over 37°C increases humidity during prolonged storage times is a factor for crops and cereals to be susceptible to mold growth and mycotoxin contamination [16]. The susceptibility of the grain is another factor to consider, presenting greater susceptibility maize and lower rice. Animal pests, weeds and pathogens impact yield and quality of cereals. *F. graminearum* mostly affects cereals, including maize, wheat and barley. The predominant *Fusarium* species associated with ear and stalk

rots are *F. graminearum* followed by *F. verticillioides*, *F. proliferatum* and *F. culmorum* [21]. These *Fusarium* species are also capable of producing mycotoxins, which contribute to pre-harvest contamination of human food and animal feed impacting health [7]. Among *Fusarium* spp., *F. graminearum* is the most common agent causing *Fusarium* head blight [26]. The major mycotoxin type of *F. graminearum*, *F. sporotrichioides* and *Fusarium avenaceum* is the trichothecene type-B mycotoxin class of fungi capable of producing deoxynivalenol and its derivatives (3Ac-deoxynivalenol, 15Ac-deoxynivalenol) or nivalenol. The nivalenol-producing isolates of *F. graminearum* have been found to be more aggressive in maize than the deoxynivalenol-producing isolates [15]. On the other hand, maize production is mainly affected by diseases caused by the species *Fusarium proliferatum*, *F. verticillioides* and *F. subglutinans* and mycotoxin generators including fusaric acid, fusarins and fumonisins. Among fumonisins, fumonisin B1 (FB1), FB2 and FB3 are most frequently encountered in maize kernels [1, 27]. *Fusarium sporotrichioides* is a common soil-borne plant pathogen causing dry rot of potato [28].

4. Cereal secondary metabolites with antifungal activity

A component of the plant resistance to *Fusarium* and their toxins is related to the capacity of plant tissues to reduce the fungal infestation and mycotoxin accumulation (e.g. zearalenone, type B trichothecenes, fumonisins) throughout the presence of secondary metabolites. Secondary metabolites are compounds produced by plants for which no role has yet been found in growth, photosynthesis, reproduction or other "primary" functions; however, it has been found that they are implicated in plant defense. The presence of secondary metabolites along with temperature, water activity, pH and nutrients have been identified as key features regulating *Fusarium* and their mycotoxins [29].

Plant endogenous compounds can be both constitutively synthesized and induced in response to pathogen infection. Recent metabolomic studies have pointed an important amount of cereal metabolites produced by cereals such as fatty acids, amino acids and their derivatives, carbohydrates, amines and polyamines, terpenoids, benzoxazinoid derivatives and phenylpropanoids that contribute to the resistance of *Fusarium* and low mycotoxin accumulation (**Figure 2**). These metabolites are derived from primary and secondary metabolism [30]. Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups: phenylpropanoids, terpenoids and nitrogen-containing alkaloids. The secondary metabolites that play a role in the plant resistance to *Fusarium* and mycotoxin accumulation are listed below.

4.1. Phenylpropanoids

Phenolic compounds are secondary metabolites that are produced by descend from the phenylpropanoid pathway and are synthesized by plants from the amino acid phenylalanine. Plant biosynthesis produces various phenols that can be grouped commonly as flavonoids and phenolics. Flavones, flavonols, flavanones, flavan-3-ols, anthocyanidins, isoflavones, coumarins, stilbenes and lignans are the main flavonoids. These are structurally distinct because of their specific hydroxylation, methylation and conjugation patterns, with various monosaccharides and disaccharides. Phenolic acids found in cereals exist in both soluble (free) and insoluble

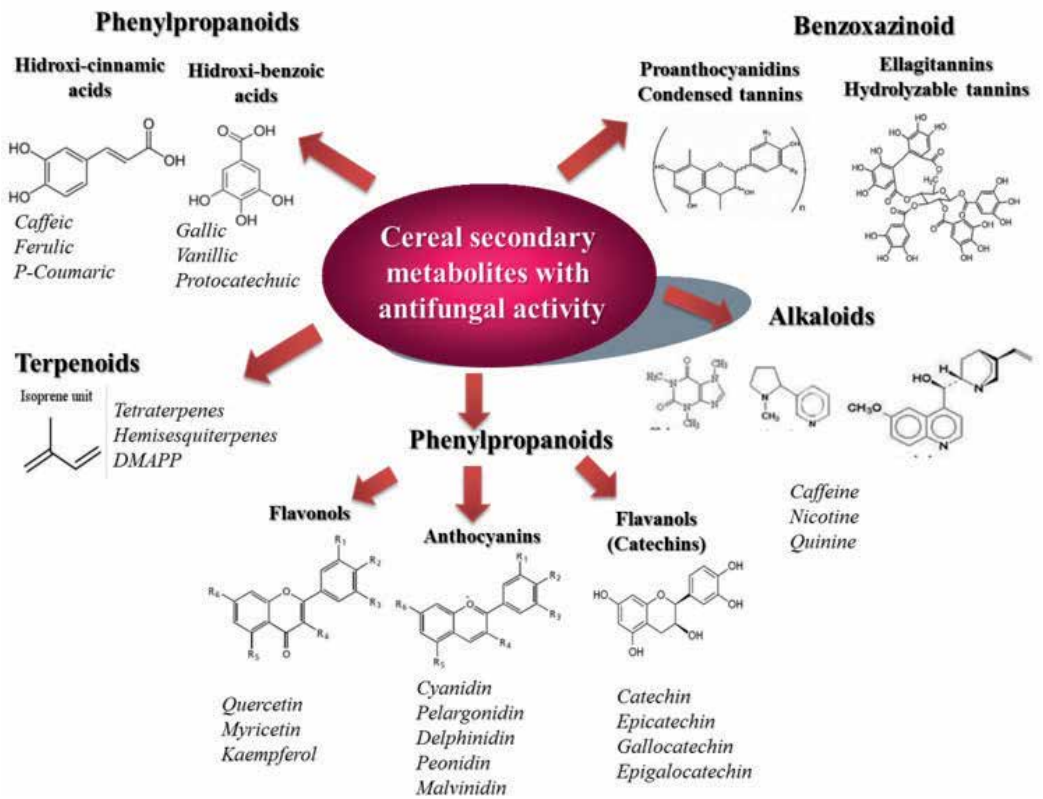

Figure 2. Cereal secondary metabolites with antifungal activity.

(cell wall-bound) forms [31]. The major portion of phenolic compounds is in the outer part of grains. Moreover, phenolic acids, predominantly ferulic and coumaric acid, play an important role in limiting polysaccharide degradation by exogenous enzymes, where they act as a cross-link between polysaccharides and between polysaccharides and lignin [32].

Phenolic compounds in plants are involved in the interaction between the pathogen and the plant. For example, the phenolic acids accumulated throughout the development of wheat-kernel development impact positively the resistance to *Fusarium* [33]. It has been reported the fungicidal efficiency of phenolic compound considering IC_{50} values. These values rank between 0.7 and >10 mM [30].

It has been stated that the most maize-resistant genotypes exhibited high levels of phenylpropanoids, which were related to low levels of disease severity and grain fumonisin (FUMO) concentration [34]. In a study using wheat cultivars (winter and spring), significantly higher amounts of free phenolic compounds were found in the glumes, lemmas and paleas of the spring cultivar prior to and at all sampling times after inoculation, in comparison to the winter wheat cultivar. The spring cultivar exhibited resistance against initial infection by the fungus. It was found that the amount of *p*-coumaric acid increased significantly in the glumes,

lemmas and paleas of the spring cultivar concluding that phenolic compounds appear to play a role in the resistance of the cultivars to *F. culmorum* [35]. In the same way, a study with date palm roots showed that date palm roots contain four cell wall-bound phenolics identified as *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid and sinapic acid. The contents of *p*-coumaric acid and ferulic acid, *p*-hydroxybenzoic acid, sinapic acid and lignin in the resistant cultivars to *F. oxysporum* were about 2, 8.4, 4.5 and 1.8 times higher than those in the susceptible cultivars [36].

Regarding mycotoxin production, cinnamic acid derivatives such as sinapic, caffeic, *p*-coumaric, chlorogenic and ferulic acids are efficient inhibitors of TCTB (type B trichothecenes) production by *F. graminearum* and *F. culmorum.* It is important to mention that the effect of phenolic compounds is strain and molecule dependent [37].

An amount of studies support that phenolic compounds have a role in enhanced plant resistance to *Fusarium* [38–43]. Besides, number of studies related to phenolic acids supports that in cereals, cell wall-bound ferulic acid along with its dehydrodimers and free chlorogenic acid could be pivotal components of the resistance to toxigenic *Fusarium* species [34].

4.2. Terpenoids

Terpenes are the most numerous and structurally diverse plant natural products. The plethora of terpenoid compounds is biosynthetically assembled from only two simple precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Plant terpenoids include compounds ranging from C5 hemisesquiterpenes to C40 tetraterpenes, with diverse physical and chemical properties leading to lipophilic or hydrophilic, volatile or non-volatile metabolites [44].

Several terpenoids have their roles in plant defense against biotic and abiotic stresses, or they are treated as signal molecules to attract the insects of pollination. In a study using cyclic terpenes (limonene, menthol, menthone and thymol) against *F. verticillioides*, limonene and thymol showed the highest inhibitory effects on *F. verticillioides* development. Thymol was the most active inhibitor of fumonisin B1 biosynthesis [45].

In the last year, essential oils, which composition mainly include terpenes and terpenoids, from different plants were used in the prevention of fungi and mycotoxins accumulation in cereals. A study using *Melissa officinalis*, *Salvia officinalis*, *Coriandrum sativum*, *Thymus vulgaris*, *Mentha piperita* and *Cinnamomum zeylanicum* showed that all these essential oils have an inhibitory effect on fungal contamination of wheat seeds. This ability was dose-dependent. Regarding mycotoxin development, the best control on fumonisins production was recorded for *Cinnamomum zeylanicum* [46]. Similar findings regarding essential oils were done by Daferera et al. [47]; *Fusarium* sp. was completely inhibited by oregano, thyme, dictamnus and marjoram essential oils at moderately low concentrations (85–300 μg/mL). Also, oils from *Cymbopogon citratus*, *Ocimum basilicum* and *Ocimum gratissimum* were the most effective *in vitro*, completely inhibiting the growth of *F. verticillioides*. The application of these oils at concentrations of 8, 6.4 and 4.8 μL/g inhibit the growth of *F. verticillioides* in maize for a period of 21 days. It was also observed that the production of fumonisin was not affected by the lower concentration (4.8 μL/g) [48].

On the other hand, in a cromatografy study, volatile organic compounds (VOCs) were identified using GC-MS in oats, barley and wheat infected by three species of *Fusarium*, including species that caused cortical rot disease in wheat, and two terpenes were identified (linalool and β-caryophyllene), which found higher concentrations with respect to the controls [49].

The metabolomics as a tool helped in to identify the metabolites in barley that are related to resistance against *Fusarium* head blight FHB exposed that metabolites conferring resistance mainly belonged to phenylpropanoid, flavonoid, fatty acid and terpenoid metabolic pathways [50]. A research by Wang et al. [51] exposed a number of genes involved in secondary metabolites biosynthesis are specifically responsive to *F. verticillioides* inoculation in BT-1 kernels. Terpenoid biosynthesis and diterpenoid biosynthesis were particularly increased by *F. verticillioides* inoculation. See Ref. [29] to review a list of terpenoids conferring resistance to *Fusarium*.

4.3. Alkaloids

Alkaloids are a group of chemical compounds that mostly contain basic nitrogen atoms. Saponins are a class of glycosylated triterpenes; steroids and steroidal alkaloids synthesized from the mevalonate or non-mevalonate pathway in plants. These compounds are absent in most monocotyledon plants and all cereals except in oat. The glycosylated form confers activity to avenacins, contrary to other compounds such as avenacosides, benzoxazanoids and other compounds with antifungal activity, then only with active in its form of aglycone [52]. Vacuoles are the reservoir of inactive avenosides, which allow them to be available when there is tissue damage caused by pathogenic fungi causing their activation; this results in alteration of the membranes and consequently the formation the biologically active aglycone. In a research performed by the homozygous mutant, *A. strigose* lines and the wild-type line were inoculated with fungal pathogens to assess the effects of the saponin-deficient mutations on plant disease resistance. The results exhibited that mutant plants showed increased susceptibility to *Fusarium culmorum* and *Fusarium avenaceum* revealing an implication of saponins in the plant resistance [53].

The best-known alkaloids of grasses are hordenine and gramine. Hordenine is found in many plant species and in cereals; it has been reported in barley, millet and sorghum. The reports of their allelopathic effects may imply a resistance to *Fusarium* and their mycotoxins; however, no specific reports have been found.

Several compounds within the monoterpene indole alkaloid class are known to exhibit antifungal properties. Secologanin production is induced by the application of methyl jasmonate in *C. roseus*, perhaps suggesting a link between defense-related signaling pathways and monoterpene indole alkaloid production. A study using double haploid barley lines differing in *Fusarium* head blight sensitivity observed metabolite accumulation and found secologanin was constitutively produced in resistant lines [54]. Few alkaloid compounds have been identified within wheat. A more detailed understanding of how cereal crops and related grass species respond to *Fusarium* pathogens will reveal novel mechanisms of resistance.

4.4. Benzoxazinoid

Benzoxazinoids (Bxs) are widely distributed in cereals discovered in the 1950s. A range of biological roles such as allelopathy, resistance to insects and defense against pathogens has

been attached to them [55]. Benzoxazinoids are synthesized in the shikimate pathway from the amino acid tryptophan. They are present in maize; wheat, rye and certain wild barley species, however, have not been found in cultivated barley varieties, oat or rice. Bxs are stored in an inactive glucoside form in plant vacuoles or plastids to avoid toxicity to the plant itself; through the enzymatic activation and chemical degradation, the tissue disrupted form the active benzoxazinoid [56]. In a research using wheat, principal component analyses demonstrated a correlation between the susceptibility to FHB and the concentrations of range of Bxs [57]. The benzoxazinoid 2-β-glucopyranoside-2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-glc), α-tocopherol and the flavonoids homo-orientin and orientin were identified as potential inhibitors of (deoxynivalenol) DON accumulation in a study with wheat that correlates accumulation in *Fusarium*-infected winter and spring wheat cultivars [58].

A plethora of secondary metabolites have been reported to inhibit *Fusarium* and their mycotoxins; however, the molecular mechanisms of plant resistance to both are needed to provide a deeper understanding of the mode of actions of the metabolites as well as the mechanisms of detoxification.

5. Possible mechanisms and management that modulate inhibition of mycotoxin production of *Fusarium* species

Mycotoxins produced by *Fusarium* spp. include different compounds with trichothecenes, fumonisins, zearalenone and emerging toxins such as fusaproliferin, enniatins, beauvericin and moniliformin [10]. This mycotoxins genus can infect cereals directly during ripening, harvesting or storage, the crop soil affecting plant growth and development, which makes its eradication complex and difficult, but various strategies are used to reduce this contamination, butte the best strategies cannot completely eradicate mycotoxin contamination. Prevention strategies during cultivation and storage aim to eliminate mycotoxins; some of the strategies used are crop rotation; in this sense, Schaafsma et al. [59] observed in a 4-year study that planting a crop other than wheat 2 years previous to planting a wheat crop significantly decreased the level of DON in wheat grain in 1 year out of four. This type of studies support the theory that crop residues are the source of *Fusarium* toxin inoculum, so alternating crops would reduce the possibility of contamination. However, studies such as that reported by Fernández-Blanco et al. [25] indicate that wheat grown consecutively (each year) has less contamination by *Fusarium* toxins than alternately grown wheat. Urea fertilization is another strategy to reduce contamination by *Fusarium* sp. as mentioned by Teich [60] and Martin et al. [61], where they applied urea instead of ammonium nitrate, with fewer pollution symptoms observed. Among the aspects to consider in order reducing *Fusarium* contamination is the cultivation season, since it has been documented that winter varieties develop and mature before spring varieties, which reduce the risk of *Fusarium* infection, for avoid that flowering coincides with spore release.

Alternatives to chemical fungicides, such as biocontrol agents, have been tested extensively in both the greenhouse and field environment, but the toxins of *Fusarium* control efficacy under field conditions have not been consistent [62]. However, some of the strategies to reduce contamination at the crop level are not always effective, so at the storage level it is sought to address other types of strategies.

5.1. Storage

The mycotoxins generated by *Fusarium* sp. usually present with greater incidence during the storage. The conditions for the mycotoxins biosynthesis are the grain with temperature 25–32°C, moisture between 16 and 30% and air RH of 80 and 100% [63]. This is why the strategies to mitigate and inhibit mycotoxins are postharvest management and storage strategies. Postharvest management has a significant role in mitigation of mycotoxins through good management in grain food chains during harvesting, cleaning, drying, storage and processing. The control of moisture, temperature and humidity to safe storage levels laid a key to mitigate mycotoxins in grains. Ouzounidou et al. [64] indicate that reduction in oxygen and increase in carbon dioxide concentrations generate effects on the growth of fungi. Decreasing O_2 to minor of 0.14% and increasing CO_2 to more of 50% are required for inhibition of mycelial growth and will prevent mycotoxin [65]. The degree of inhibition achieved by elevated CO_2 concentrations is dependent on other environmental factors, such as relative humidity (RH) and temperature [66]. Irradiation is usually used as a mitigation of mycotoxins; 4–6 kGy gamma-irradiation reduces *Fusarium* toxins and was eliminated at 8 kGy [67]. Both inhibition and elimination of *Fusarium* mycotoxins can be attributed to providing energy, which results in reactions and changes molecular structures.

5.2. Chemical and biological control

Another strategy is the application of chemical control as fungicides; however, this application can sometimes be ineffective and even increase the production of mycotoxins [68, 69]. That is why another alternative is the use of natural products in specific essential oils and antioxidant compounds. In stored cereals, the application of natural preservatives and essential oils generate inhibition on *Fusarium* mycotoxins production is found [46]. On the other hand, the agreement of chemical compounds and natural products can generate a reduction of 90% in deoxynivalenol (DON) (*Fusarium* toxin) as reported by Magan [70] in agreeing BHA (butyl hydroxyl anisole), PP (propyl paraben), resveratrol and cinnamon oil. In relation to the use of natural compounds, a study of phenolic extract of *Spirulina* sp. reported by Pagnussatt et al. [71] indicates that the *Spirulina* LEB-18 extract led to mycelial growth inhibitions that ranged between 50% and 90% in addition, the extract inhibits production of nivalenol (NIV) and deoxynivalenol (DON) in 73%. This may be attributed to the extract composition (main constituents were gallic and caffeic acid). Apparently, these compounds act as fungal stressors when they hamper the energy abstention due to the lower glucose availability [72]. This may trigger the production of secondary metabolites to compensate and limit the apparent competition by the substrate of the medium [73].

Biological control is another strategy in the reduction and incidence of *Fusarium* toxin using living microorganism's whit *Bacillus* spp. [74], *Pseudomonas* spp. [75] and *Streptomyces* spp. [74]. The lactic acid bacteria (LAB) strains have been examined for their potential to detoxify zearalenone (ZEA) that is an estrogenic mycotoxin produced by *Fusarium* [76]. Sangsila et al. [77] showed that these strains of LAB are capable of ZEA detoxification in a range of 29.74–83%, where the strain with the best binding capacity was JM0812 with 83% at an initial concentration of ZEA of 74.7 μg/ml, followed by UM054 and UM055 with 82.78 and 81.69%, respectively.

Mycoparasitism is the mechanism by which a fungus parasitized another fungus and is used with biocontrol strategy. Many studies suggested that mycoparasitism was associated with competition for nutrients and space, generation of antibiotic and induction of systemic resistance on *Fusarium* spp. [78–80]. Competition for nutrients and space in the soil is considered to be responsible for the phenomenon of fungistasis via the inhibition of the germination of fungal spores in soil [81]. The deprivation of the resource in the soils is partly responsible for the suppressive nature of soils. When the antagonists present in sufficient quantity at the right time and place and can use nutrients more efficiently than the pathogen, this competition can be used as an effective biological control.

On the other hand, the production of metabolites toxic is another strategy used for the control of diverse strains of *Fusarium*. Dunlap et al. [82] in *B. amyloliquefaciens* AS 43.3 identified nine gene clusters encoding for the biosynthesis of secondary metabolites associated with the biological control of *Fusarium*. The application of gases like ozone is another strategy for the detoxification of mycotoxins; Li et al. [83] obtained a reduction of 57.3% in DON by ozonation, with the moisture content of 17% in wheat. The ozone is a gas, has a favorable penetration and can decompose the double bonds in organisms and further produces simple products with less double bond and low molecular weight; in addition, it can decompose to oxygen voluntarily with non-toxic residual. Other strategie is the application of photocatalytic activity of graphene/Zno hybrids can be useful to degrade DON up to 99% according to Bai et al. [84]. The information on possible mechanism and strategies that can help detoxification of mycotoxins has increased, however, the road is still long.

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Chapter 4

The Incidence of T-2 and HT-2 Toxins in Cereals and Methods of their Reduction Practice by the Food Industry

Jelka Pleadin, Ana Vulić, Jurislav Babić and Drago Šubarić

Additional information is available at the end of the chapter

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Abstract

T-2 toxin and its major metabolite HT-2 toxin are type A trichothecene mycotoxins produced by the *Fusarium* moulds, present mainly in cereals and cereal-based products. The studies quoted under this chapter bring data on the incidence of T-2 and HT-2 toxin in unprocessed cereals (n = 285) harvested in Croatia during a two-year period. They also demonstrate the influence of certain thermal food processing methods on the reduction of T-2 and HT-2 toxin in naturally contaminated cereals. In analysed cereals, the highest percentage of T-2- and HT-2-positive samples was determined in oat samples (56.9%), followed by triticale (34.5%), maize (32.1%) and barley samples (22.7%), whereas the highest mean (94.8 ± 63.7 μg/kg) and maximal concentration (420 μg/kg) of the toxins in reference were determined in maize. The summary T-2/HT-2 concentrations found in one maize and one triticale sample were higher than the indicative levels, necessitating further sampling and investigations of conditions under which the production of these toxins takes place. Thermal food processing in terms of roasting (which enabled 28.8–54.4%-toxin reduction) and especially extrusion (which enabled 73.0–92.5%-toxin reduction) efficiently reduces T-2/HT-2 levels, whereas cooking does not significantly aid in their reduction (<10%-toxin reduction achieved).

Keywords: T-2 toxin, HT-2 toxin, cereals, occurrence, thermal reduction, food industry

1. Introduction

Worldwide, cereals are at risk from contamination with mycotoxins, i.e. secondary mould metabolites, both while still in field and during storage. *Fusarium* fungi produce several

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trichothecene mycotoxins having a common chemical structure and a similar mode of action [1]. Among them, T-2 toxin (3α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutoxy)-12,13-epoxytrichothec-9-ene), and its major metabolite HT-2 toxin (3α,4β-dihydroxy-15-acetoxy-8α-(3-methylbutoxy)-12,13-epoxy-trichothecene), represent not only type A trichothecene mycotoxins produced mainly by the *Fusarium langsethiae*, but also the *Fusarium poae* and the *Fusarium sporotrichioides* [2, 3]. These toxins are produced at temperatures ranging from −2 to 35°C and with water activities above 0.88 [4, 5], and are frequently responsible for the contamination of different grains such as maize, oat, barley, wheat, rice and soya beans. Weather conditions, varieties and the sowing time are the most important factors influencing the T-2 and HT-2 toxin production [6].

Type A trichothecenes, T-2 and HT-2 toxins included, are generally more toxic than type B trichothecenes (e.g. deoxynivalenol and nivalenol). Structure/activity-relationship studies revealed that 12,13-epoxide group and C9-C10-double bond are essential for their toxicity [7]. Toxicological studies show that T-2 toxin is a very potent cytotoxic and immunosuppressive agent, which can cause acute intoxication and chronic diseases in both humans and animals [6]. Given that T-2 toxin is metabolised into HT-2 toxin after ingestion, they are considered to be equally toxic [8]. The symptoms of acute T-2 intoxication of different mammalian species include skin necrosis, asthenia, lack of appetite, panting, vomiting, diarrhoea, anorexia, myocardial damage, lethargy, as well as haemorrhages and necrosis of the epithelium of the stomach and intestines, bone marrow, spleen, testis and ovary [1, 8–10]. The International Agency for Research on Cancer (IARC) classified T-2 toxin into Group 3 carcinogens because of the lack of data on its carcinogenicity in humans and only limited evidence on its carcinogenicity in experimental animals [11].

Data collected in a number of European countries have shown substantial variations in *Fusarium* mycotoxin levels across various cereal types, various countries and various investigated periods [12, 13]. Croatia, as a Central European country, falls into the group of countries in which contamination with *Fusarium* mycotoxins is a frequent occurrence [14, 15]. Since data on the occurrence of T-2 and HT-2 toxins in cereals and cereal by-products are still very limited, the European Commission recommended the member states to gather reliable data on year-to-year variations of these mycotoxins in order to be able to establish their maximal levels (MLs) in different food and feed in the near future [6, 16, 17]. In 2013, the European Commission gave recommendations (Commission Recommendation 2013/165/EU) regarding indicative levels of T-2 and HT-2 toxin in cereals and cereal-based products intended for food and feed. In this document, the Commission also recommended further investigations into the effects of food processing and agronomic factors on the presence of T-2 and HT-2 toxin and different factors favouring high level-contaminations with these toxins, so as to be able to identify measures to be taken to avoid or reduce the above [18]. In view of the evidenced toxicity of T-2 and HT-2 toxin, there exists the need for further collection of data on their presence in different cereals intended for food and feed production. In their recent study, Pleadin et al. [17] stated that further studies shall also be performed in Croatia in order to investigate into the conditions favouring T-2 and HT-2 production and to identify measures that are to

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be implemented in order to prevent contamination during cultivation and storage of cereals and their final products.

Literature data on the impact of cereal processing on the levels of T-2 and HT-2 contamination are also very scarce. Data gathered insofar have suggested that toxins in reference, when milled, get to be relocated into various milling fractions, but are not eliminated [19]; in addition, they have been proven resistant to processing. Even more so, due to their hull binding, subsequent processing of cereals leads to significant rise in levels of these toxins in finished products [16]. Scudamore [20] concluded that final processing, such as boiling, fermentation, baking, frying, and extrusion, has no impact on the level of contamination with these mycotoxins. Some studies evidenced that processing of raw cereals greatly reduces T-2 and HT-2 levels in food products, but makes these toxins concentrate in high levels in by-products [6]. In industrial food processing settings, the processing time and temperature combination has been shown to be crucial for the reduction of mycotoxin content in a finished food product. While conventional food preparation at temperatures up to 100°C has a negligible effect on most mycotoxins, higher temperatures used with frying, roasting, toasting and extrusion have been shown to be capable of decreasing mycotoxin contamination [21].

The studies quoted under this chapter bring data on the incidence of T-2 and HT-2 toxin in different unprocessed cereals during a two-year period. They also demonstrate the influence of certain food processing methods, such as cooking, roasting and extrusion, performed under predefined conditions, on the levels of T-2 and HT-2 toxin in contaminated cereals. For this purpose, after application of the quantitative screening method termed the enzyme-linked immunosorbent assay (ELISA), which enabled the determination of summary T-2 and HT-2 toxin concentrations, the concentrations of each mycotoxin in positive samples were determined using a confirmatory method in terms of liquid chromatography tandem-mass spectrometry (LC-MS/MS), also used in the investigations devoted to the possibilities of reduction of concentrations of these mycotoxins.

2. Materials and methods

2.1. Sampling and sample preparation

During the period spanning from May 2015 to April 2017, a total of 285 samples of unprocessed cereals, in specific maize (n = 84), wheat (n = 56), oat (n = 72), barley (n = 44) and triticale (n = 29), were sampled from households situated in the Northern, Central and Eastern part of Croatia. All cereals were grown in the crop season 2015 and 2016 and had not undergone any physical or thermal treatment other than drying, cleaning and sorting prior to sampling. Sampling and sample preparation of unprocessed cereals were performed in full line with the Commission Regulation 401/2006 [22], laying down the methods of sampling to be exercised within the frame of the official control of mycotoxin levels in foodstuffs. The aggregate

cereal samples were combined of three, five or ten incremental samples, depending on the lot weight, each lot thereby weighing at least 1 kg.

The samples were stored in a cool and dry place and transported to the laboratory within 48 h. The prepared test portions (500 g per sample) were ground into a fine powder having a particle size of 1.0 mm using an analytical mill (Cylotec 1093, Tecator, Sweden), and then stored at 4°C pending analyses.

2.2. Chemicals, standards and reference materials

T-2 toxin (Art. No. 34071, 100 μg/mL in acetonitrile) and HT-2 toxin (Art. No. 34136, 100 μg/mL in acetonitrile) standards were provided by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). A RIDASCREEN® T-2/HT-2 toxin kit (Art. No. R3805) was provided by R-Biopharm (Darmstadt, Germany). PuriTox Total Myco-MS solid phase clean-up columns (Art. No. TC-MT3000) were produced by R-BiopharmRône LTD (Glasgow, Scotland). The Certified Reference oat flour Material (CRM) (Art. No. TET039RM) having the reference values of 85.3 ± 13.7 μg/kg for T-2 toxin and 86.9 ± 11.9 μg/kg for HT-2 toxin, was purchased from Fapas, Fera Science Ltd. (York, UK).

All chemicals used for ELISA and LC-MS/MS analyses were of an analytical grade (acetic acid, Art. No. 33209, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) or a HPLC grade (acetonitrile, Art. No. 34851, and methanol, Art. No. 34885 Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Ultrapure water was supplied by the Merck system Direct-Q 3 UV (New Jersey, USA).

2.3. Analytical methods

All samples were first analysed using the validated ELISA method. After grinding, to 5 g of a homogenised sample, 25 mL of methanol/distilled water solution (70/30; v/v) were added, except for oat samples, to which 25 mL of the appropriate extraction buffer provided with the ELISA kit were added. The extraction of T-2/HT-2 toxin was performed using a head-over-head shaker for 10 min (100 rpm, room temperature) followed by an additional 10-min centrifugation (4000 rpm, room temperature). When it comes to oat samples, the obtained supernatant was diluted in methanol/distilled water (70/30; v/v) in 1:2 ratio, while the supernatant obtained with all other biological materials under study was diluted in distilled water in 1:2 ratio. The obtained solutions were then transferred into the wells of the ELISA microtitration plate. The ELISA tests were performed using a ChemWell autoanalyser (Awareness Technology Inc. 2910, Palm City, USA), observing thereby the instructions given by the kit provider. Once the stop solution had been injected, the absorbances were determined at 450 nm. In order to calculate the summary T-2/HT-2 concentration in an individual sample, the results provided by the calibration curve were multiplied by the corresponding sample dilution factor. The calculation of the summary toxin concentrations was guided by the average recovery values ascertained by method validation.

Samples, in which T-2/HT-2 concentrations higher than the ELISA's Limit of Detection were established, were further analysed using the LC-MS/MS method. To that effect, to 2.5 g of a test sample, 10 mL of 80%-acetonitrile were added and vortexed for 30 s; afterwards, the samples

were put on a head-over-head shaker for 10 min (100 rpm, room temperature). The samples were then centrifuged (10 min, 4000 rpm, room temperature). Two millilitres of the obtained supernatant were acidified with 20 μL of (glacial) acetic acid; after that, 1.4 mL of the obtained solution was passed through the PuriTox Total Myco-MS columns (R-Biopharm, Glasgow, Scotland). Five hundred microliters were then evaporated under a nitrogen stream at 40°C and reconstituted in 250 μL of 1%-acetic acid in 20% acetonitrile. Fifty microliters were injected onto the LC-MS/MS system. The LC-MS/MS system consisted of a degasser, a binary pump, an auto-sampler and a column compartment (Infinity 1260, Agilent Technologies, Santa Clara, USA) coupled with a triple quadrupole mass detector (6410 QQQ, Agilent Technologies, Santa Clara, USA). The chromatography separation was performed on an XBridge BEH C18 column (particle size 2.5 μm, dimensions 4.6 × 150 mm) (Waters, Milford, Massachusetts, USA). The mobile phase consisted of 0.1%-acetic acid (constituent A) and acetonitrile (constituent B). The flow rate was 0.8 mL/min and the temperature was set at 40°C. A gradient elution program was employed: 0–3 min 90% A, 18 min 10% A, 18.1 min 90% A with a 3-minute post-run time. Mass spectrometry conditions were as follows: electrospray ionisation (ESI), positive polarity, source temperature 350°C, gas flow 9 L/min, nebulizer 45 psi, and capillary voltage 6 kV. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. **Table 1** shows the ions monitored within the frame of LC-MS/MS analyses targeted at T-2 and HT-2 toxin. The final concentrations of T-2 and HT-2 toxin were calculated based on the dilution factor and the average recovery values obtained during the validation process.

2.4. Validation of the analytical methods used

For the ELISA method, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the mean value of 10 control samples of a given cereal (maize, wheat, oat, barley or triticale) plus 3- and 10-fold standard deviation. For each cereal, the recoveries were determined in three replicates per concentration level per day. To that goal, the control samples were supplemented with 50% T-2 and 50% HT-2 toxin standard working solutions (either 500 μg/L or 1000 μg/L).

As for the LC-MS/MS method, the LOD and LOQ values were estimated according to the Guidance document on the estimation of LOD and LOQ for measurements in the field of contaminants in feed and food [23] via paired observations. In brief, 10 pseudo-blank samples of

Mycotoxin	Precursor ion	Fragmentor voltage (V)	Product ions	Collision energy (eV)	Cell accelerator voltage (V)
T-2 toxin	489.2	200	387.1[b]	20	1
			245.1[a]	27	
HT-2 toxin	447.2	100	345.1[a]	18	1
			285.1[b]	20	

[a]A more intense ion—used as a quantifier.
[b]A less intense ion—used as a qualifier.

Table 1. Ions monitored within the frame of LC-MS/MS analyses targeted at T-2 and HT-2 toxin.

different cereals, i.e. maize, oat, wheat, barley and triticale, were chosen based on the results obtained using the ELISA technique (<LOD) and spiked with the T-2 and HT-2 standard at the level of 25 μg/kg. Then, both spiked and pseudo-blank samples were analysed, and the difference in signal abundances and the standard deviation was calculated. The obtained data were used for the calculation of the LOD and the LOQ. Linearity was tested in the range of 5–100 ng/mL, using the standard solution of each mycotoxin. The recovery was determined by virtue of analysing six blank maize samples spiked with T-2 and HT-2 toxin at 50 μg/kg, while the trueness was tested using six replicates, making use of oat flour as the CRM, and later compared with the values assigned for each mycotoxin by the manufacturer.

2.5. Thermal processing

Three samples, which were found to be the most contaminated with T-2 and HT-2 toxins, underwent thermal processing in terms of cooking, roasting and extrusion. These samples and the concentrations of T-2/HT-2 toxins in them were as follows: a maize sample (summary toxin concentration 384 μg/kg that of T-2128 μg/kg and that of HT-2256 μg/kg), an oat sample (summary toxin concentration 267 μg/kg that of T-2107 μg/kg and that of HT-2160 μg/kg) and a triticale sample (summary toxin concentration 151 μg/kg, that of T-2 47.0 μg/kg, and that of HT-2104 μg/kg). From each sample, three parallels were used during processing, and after that three replicates of each were used for the analyses.

The above described contaminated maize, oat and triticale samples were cooked in boiling water (96°C) for 10, 20 and 30 min. After that, cereals were filtered and the samples were left to dry overnight. As for roasting, the contaminated cereals were roasted in an oven (LV9/11/P 320, Nabertherm, Germany) at three different temperatures (180°C, 200°C and 220°C) for 30 min (at each temperature). Once cooked and roasted, the cereals were milled into a fine powder having a particle size of 1.0 mm using an analytical mill (Cyclotec 1093, Tecator, Sweden), intended to be analysed for the levels of T-2 and HT-2 toxin using the LC-MS/MS method.

Before the extrusion cooking, all samples were milled using an IKA MF10 laboratory mill (IKA Werke GmbH, Staufen, Germany) having a 2-mm sieve. The blend preparation was performed based on 1 kg d. m. The samples were conditioned at 25% moisture by spraying an adequate amount of distilled water, while continuously mixed using a laboratory mixer (Kenwood KMM020, JVC Kenwood, Uithoorn, The Netherlands). The prepared mixtures were then put into plastic bags (one bag per sample) and stored overnight in the refrigerator at 4°C in order to equilibrate the moisture. Before the extrusion, the samples were brought to room temperature. The prepared samples were extruded in a single-screw laboratory extruder (Brabender GmbH, Model 19/20DN, Duisburg, Germany) at three different temperature profiles: 135/150/150°C; 135/170/170°C and 135/190/190°C (extruder's dosing/compression/ejection zone). Other constant extrusion parameters were as follows: screw: 4:1; die: 4 mm; screw speed: 100 rpm; and dosing speed: 40 rpm. After the extrusion, the samples were air-dried overnight.

In all cereal samples, the moisture content was measured before and after thermal processing by taking a 5-g sample and heating it in an oven at 105 ± 2°C. Moisture was determined according to the 712:2009 ISO standard [24].

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2.6. Data analysis

Concentrations (μg/kg) obtained by the ELISA assay are expressed as mean summary values (T-2/HT-2) ± standard deviation (SD), while in toxin-positive samples, the concentrations are given as individual concentrations of each mycotoxin (T-2 or HT-2) obtained by the LC-MS/MS. Statistical analysis was performed using the Statistica ver. 10.0 software (StatSoft Inc. 1984-2011, Tulsa, OK, USA) and made use of the analysis of variance (ANOVA), the statistical significance thereby being set at 95% ($p = 0.05$).

3. Results and discussion

3.1. Validation of analytical methods used

The ELISA assay, used as a quantitative screening method for the determination of summary concentrations of T-2/HT-2 toxins, was first validated and then applied for the analyses of the sampled cereals. Its cross-reactivity declared by the kit manufacturer is approximately 85% for T-2 toxin and 100% for the HT-2 toxin. Validation of the ELISA method resulted in LODs ranging from 20.6 to 30.1 μg/kg and LOQs ranging from 26.7 to 37.4 μg/kg, depending on the type of cereal under consideration. The mean recovery value equalled to 90.1%, with the mean coefficient of variation (CV) of 7.8% (**Table 2**).

Validation of the LC-MS/MS method resulted in LODs spanning from 5.5 to 8.3 μg/kg, and LOQs ranging from 18.2 to 27.5 μg/kg for T-2 and HT-2 toxin, respectively. The mean recovery values were 89.6 and 77.0%, with the mean CV of 5.1% and 8.9%, respectively. The analyses of oat flour sample (CRM) used for the determination of trueness, resulted in concentrations of 77.9 μg/kg for T-2 (91.3% of the mean certified value) and 75.6 μg/kg for HT-2 (86.9% of the mean certified value). **Figure 1** presents a typical LC-MS/MS-MRM chromatogram of the analysed CRM. The determination of linearity resulted in correlation coefficients higher than 0.99 for both analytes (**Table 3**).

Given the obtained validation results, both analytical methods were recognised as suitable for analyses of different cereals, the ELISA assay thereby being employed as a screening method used for the determination of summary concentrations of T-2/HT-2 toxins, and the LC-MS/MS thereby being used as a confirmatory method exploited to the effect of determination of individual concentrations of mycotoxins under study.

3.2. The occurrence of T-2/HT-2 toxin in cereals

Data published insofar have revealed that the exposure to T-2 and HT-2 toxins primarily comes as a result of the consumption of cereal grains and cereal by-products, wherein the levels of these toxins found in forages and oilseed meals are generally low. It has been established that T-2 and HT-2 toxins occur together, HT-2 thereby representing approximately two-thirds of the summary T-2/HT-2 concentration. The highest mean T-2/HT-2 concentrations were determined in grains and milled grain products, in particular in oat and oat-based

Cereals	LOD (μg/kg)	LOQ (μg/kg)	Level of fortification (μg/kg)	Mean recovery[a] (%)	CV (%)
Maize	20.6	26.7	50	94.3	6.3
			100	97.8	5.8
			200	100.5	9.2
Wheat	27.3	33.6	50	90.7	7.2
			100	89.3	8.3
			200	95.8	6.4
Oat	25.8	31.9	50	72.2	6.1
			100	75.3	7.6
			200	78.5	7.9
Barley	30.1	37.4	50	88.3	7.4
			100	92.6	8.9
			200	94.8	10.3
Triticale	26.2	34.7	50	90.7	8.6
			100	94.1	7.7
			200	96.2	9.3

[a]Three replicates per concentration level per day; analyses were performed by spiking the negative material (before validation, confirmed by the LC-MS/MS).

LOD: limit of detection; LOQ: limit of quantification; CV: coefficient of variation.

Table 2. Validation of the ELISA method used for the determination of summary concentrations of T-2/HT-2 toxins in cereals.

products, the aforementioned applying equally for food, feed and raw cereals. Higher toxin concentrations established in unprocessed vs. processed grains indicate that grain processing succeeds in lowering, at least to some point, both T-2 and HT-2 concentrations [16].

In this study, the levels of T-2 and HT-2 were analysed in different unprocessed cereals (maize, wheat, oat, barley and triticale) sampled from Croatian households during a two-year period. In the first study step, summary concentrations of these toxins were established using the ELISA method. The results obtained in unprocessed cereal samples are shown in **Table 4**.

The study results show a significantly higher ($p < 0.05$) incidence of T-2/HT-2 toxins in oats (56.9%) in comparison to other unprocessed cereals. However, the maximal summary concentration was determined in maize sample (420 μg/kg), but the maximal mean value was observed in oat (136 ± 55.6 μg/kg). The results also show that in two samples, one maize (mentioned above) and one triticale (169 μg/kg), summary concentrations of T-2/HT-2 were higher than stipulated indicative levels from which onwards/above investigations should be performed in case of repetitive findings according to the Commission Recommendation [18]. Such a finding imposes the need for further sampling and investigations of the causes of such a high level of mycotoxin contamination. The lowest percentage of positive samples was

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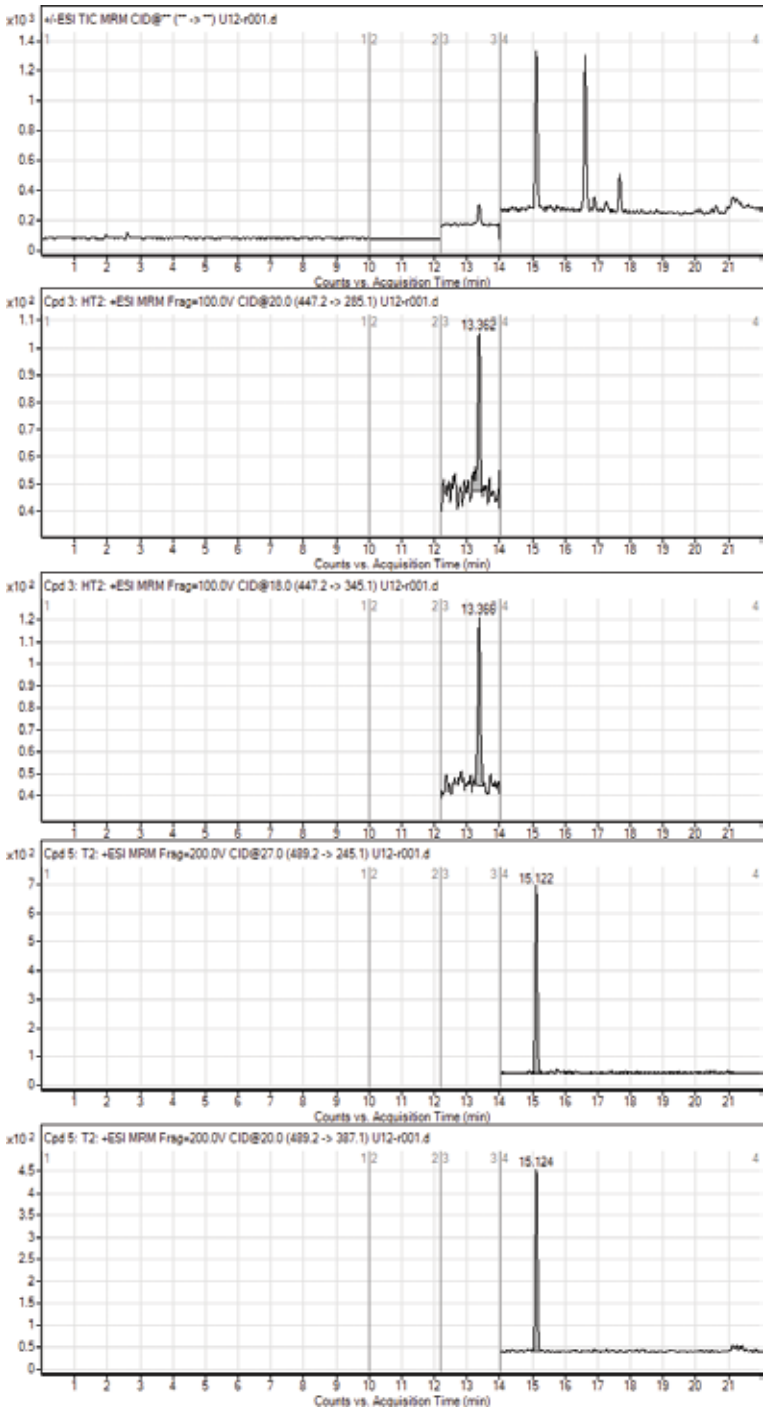

Figure 1. A typical LC-MS/MS-MRM chromatogram of the certified reference material (CRM, oat flour) with values of 77.9 μg/kg determined for T-2 and 75.6 μg/kg determined for HT-2; TIC; HT-2 MRM 447.2 → 285.1; HT-2 MRM 447.2 → 345.1; T-2 MRM 489.2 → 245.1; and T-2 MRM 489.2 → 387.1.

Analyte	LOD (μg/kg)	LOQ (μg/kg)	Correlation coefficient	Mean recovery[b] (%)	CV (%)	Trueness[c] (%)
T-2 toxin	5.5	18.2	0.998	89.6	5.1	91.3
HT-2 toxin	8.3	27.5	0.995	77.0	8.9	86.9

[a]Samples in which T-2/HT-2 summary concentrations were higher than the LOD of the ELISA method.

[b]Six blank maize samples spiked at 50 μg/kg per day.

[c]Six oat flour CRM replicates with the assigned reference values of 85.3 ± 13.7 μg/kg for T-2 and 86.9 ± 11.9 μg/kg for HT-2 toxin.

LOD: limit of detection; LOQ: limit of quantification; CV: coefficient of variation.

Table 3. Validation of the LC-MS/MS method used for the determination of T-2 and HT-2 toxin in positive cereal samples[a].

established in the wheat (21.4%) and barley sample pool (22.7%), in which the lowest mean T-2/HT-2 concentrations were determined, as well.

Literature data mainly address individual T-2 toxin concentrations rather than the summary T-2/HT-2 concentrations. Of note, the level of contamination greatly varies depending on the geographical region (country), the period of investigation and the type of cereal and/or final product investigated [25–27]. It is known that T-2/HT-2 toxins are produced by moulds of the *Fusarium* genus in high-temperature environments, with the maximum productivity at temperatures lower than 15°C accompanied by high relative humidity (of 60–95%) [3, 5]. Higher T-2/HT-2 concentrations ascertained in certain samples (although below the indicative levels) come, first and foremost, as a consequence of the plenitude of rain that facilitated the production of *Fusarium* moulds and therefore also the production of T-2/HT-2 toxins. The occurrence of these toxins in higher concentrations is also linked to other factors that could cause their formation independent of climate conditions.

T-2 toxin levels in different food components retrieved from different parts of the world were found to range from 6 to 2406 μg/kg [28–33]. Investigations performed in eight European countries on over 3000 samples showed 20% of T-2-positive and 14% of HT-2-positive samples. In the United Kingdom, T-2 was found in 16% of wheat and 12% of barley samples, the LOD thereby being 10 μg/kg. When it comes to oat, T-2 was identified in 84% of samples, with the mean and

Cereals	Indicative level[a] (μg/kg)	Positive (%)	Mean[b] (μg/kg)	SD (μg/kg)	Min (μg/kg)	Max (μg/kg)
Maize (n = 84)	200	32.1	94.8	63.7	26.9	420
Wheat (n = 56)	100	21.4	65.6	25.2	29.4	50.6
Oat (n = 72)	1000	56.9	136	55.6	32.4	273
Barley (n = 44)	200	22.7	61.3	20.6	31.6	70.2
Triticale (n = 29)	100	34.5	76.6	30.4	36.0	169

[a]Indicative levels of the summary T-2/HT-2 concentrations from which onwards/above investigations should be performed in case of repetitive findings according to the Commission Recommendation 2013/165/EU [18].

[b]Mean concentration found in positive samples (>LOD).

Table 4. Summary concentrations of T-2/HT-2 toxins determined in unprocessed cereal samples using the ELISA method.

the maximal concentration of 84 and 2406 μg/kg, respectively [31–33]. In a study performed in Germany, toxin concentrations found in oats varied from 14 to 214 μg/kg [34]. Within the 2005–2008 timeframe, T-2 toxin was detected in 50% of different barley cultivars coming from the Czech Republic, in the mean concentration of 30 μg/kg [35]. In Serbia, the toxin concentrations determined in 54 analysed samples were lower than the LOD of 0.3 μg/kg [36]. The most frequently *Fusarium*-contaminated T-2-positive food items are maize (28%), wheat (21%) and oats (21%); when it comes to the HT-2 toxin, the most frequently contaminated foodstuffs are oat (41%), corn (24%) and rye (17%) [37]. In a study by the JECFA [8], the T-2 contamination rate was found to be 11% on the overall, with annual variations dependent on the cereal type. In the large-scale study by Pettersson et al. [38] on 243 raw oat samples, 529 oat flake samples and 105 oat meal samples, T-2 contamination was corroborated in 73%, 24% and 17% of samples, respectively, the mean concentrations of the toxin thereby being 32 μg/kg, 5 μg/kg and 4 μg/kg, respectively.

Data published earlier in Croatia showed the T-2 toxin presence in 57% of maize, 25% of wheat, 32% of barley and 18% of oat samples [15]. The highest detected concentration of T-2 toxin in maize was 210 μg/kg, with the pertaining mean value of 110 μg/kg pointing towards a *Fusarium*-induced maize contamination that occurred after heavy rainy periods [14]. In a study performed on different cereals harvested in 2011, the maximal level of T-2 toxin was 42 μg/kg, with the mean value of 24 ± 27 μg/kg found in maize the maximal and the mean T-2 level found in oat on the same occasion was 10 μg/kg and 7 ± 2 μg/kg, respectively [15]. In the study by Pleadin et al. [17], the highest mean summary concentration of T-2/HT-2 toxins was, as also in this study, found in oats (102.2 ± 73.6 μg/kg), followed by maize (63.1 ± 36.7 μg/kg) and barley (51.7 ± 18.6 μg/kg), while the lowest concentration was found in wheat (34.3 ± 11.2 μg/kg). The maximal toxin level was determined in oats (304.1 μg/kg), but all of the obtained summary T-2/HT-2 concentrations were below the indicative levels necessitating further investigation as advised under the Commission Recommendation 2013/165/EU [18].

In this study, after the implementation of the ELISA method, which was used for the determination of summary values of T-2/HT-2 toxins in all samples under investigation, the LC-MS/MS method was implemented for the determination and confirmation of each mycotoxin in positive samples (>LOD). **Table 5** shows individual levels of T-2 and HT-2 toxin determined using the LC-MS/MS method.

Cereals	T-2 toxin (μg/kg)				HT-2 toxin (μg/kg)				Share T-2:HT-2[a]
	Mean	SD	Min	Max	Mean	SD	Min	Max	
Maize (n = 25)	27.6	10.7	23.1	128	66.3	21.9	30.9	256	1:2.4
Wheat (n = 12)	23.1	6.8	18.2	42.4	39.0	16.1	28.4	89.4	1:1.7
Oat (n = 44)	45.2	21.1	32.4	107	89.1	38.3	31.6	160	1:2.0
Barley (n = 10)	22.4	8.7	18.5	50.3	37.6	15.9	28.0	80.4	1:1.7
Triticale (n = 11)	26.4	6.8	21.0	47.0	47.6	24.9	27.7	104	1:1.8

[a]the share of mean values of T-2 and HT-2.

Table 5. Levels of T-2 and HT-2 toxin determined in positive unprocessed cereal samples using the LC-MS/MS method.

When the individual concentrations of T-2 and HT-2 toxin obtained by the LC-MS/MS method were summed up, it was established that the latter sum slightly differs from, and is mainly lower than, the summary concentrations of these mycotoxins determined using the ELISA method. This can be explained by the cross-reactivity and a lower specificity of the ELISA method. It is known that the ELISA represents an easy-to-use purification technique with a lesser need for an extensive clean-up, but it may suffer from undesired cross-reactivity with other trichothecenes that give rise to metric uncertainty [16].

Figure 2 presents the LC-MS/MS-MRM chromatogram of the most contaminated sample (maize), in which the concentration of T-2 toxin of 128 μg/kg and the concentration of HT-2 toxin of 256 μg/kg was determined (summary T-2/HT-2 toxins concentration, 384 μg/kg). Together with the most contaminated oat and the most contaminated triticale sample, this sample was further subjected to thermal processing.

Based on the comparison of the mean T-2 and HT-2 toxin concentrations, the shares of T-2:HT-2 were established to be in the range from 1:1.7 in wheat and barley to 1:2.4 in maize, with the mean share value of 1:1.9 in all investigated cereal samples. The determined share values are comparable to those stated by other literature sources, which show that HT-2 is present in cereals and their products in levels higher than those of T-2 toxin, representing approximately two-thirds (1:2) of the summary T-2/HT-2 concentrations [16].

3.3. The reduction of T-2 and HT-2 toxin

In general, different cereal treatments implemented by the food industry are known to decrease mycotoxin concentrations, but mostly do not eliminate these toxins completely. These food processing operations include sorting, trimming, cleaning, cooking, baking, frying, roasting, flaking and extrusion, and have variable effects on the level of contamination. In their recently published study, Schmidt et al. [39] stated that in comparison to other mycotoxins, thermal degradation of T-2 and HT-2 has not been the subject of many studies. In the last decades, some research on the effects of thermal degradation has mainly been performed on oats, known to be the cereal most contaminated with these mycotoxins [34, 39–41]. Scudamore [20] concluded that final processing, such as boiling, fermentation, baking, frying, and extrusion, has no impact on T-2 and HT-2 contamination. A greater extent of thermal degradation of T-2 as compared to HT-2 has been established, as well [34, 39, 41].

Nevertheless, the efficiency of T-2 and HT-2 toxin reduction using thermal processing techniques is still under-established, mostly because of the fairly small data pool on the subject-matter provided insofar, obtained under various, mutually different thermal degradation conditions, which, in turn, yields inconsistent study outcomes and study conclusions. In light of the foregoing, in order to establish the degree of thermal degradation and reduction of T-2 and HT-2 toxin in naturally contaminated cereals, this study made use of three thermal processing methods, that is to say, cooking, roasting and extrusion, each of them running at three different temperatures for different lengths of time.

Cooking is the preparation of a meal using heat [42]. Several studies have reported about the effect of cooking on the reduction of *Fusarium* mycotoxins in contaminated cereals [34, 43, 44].

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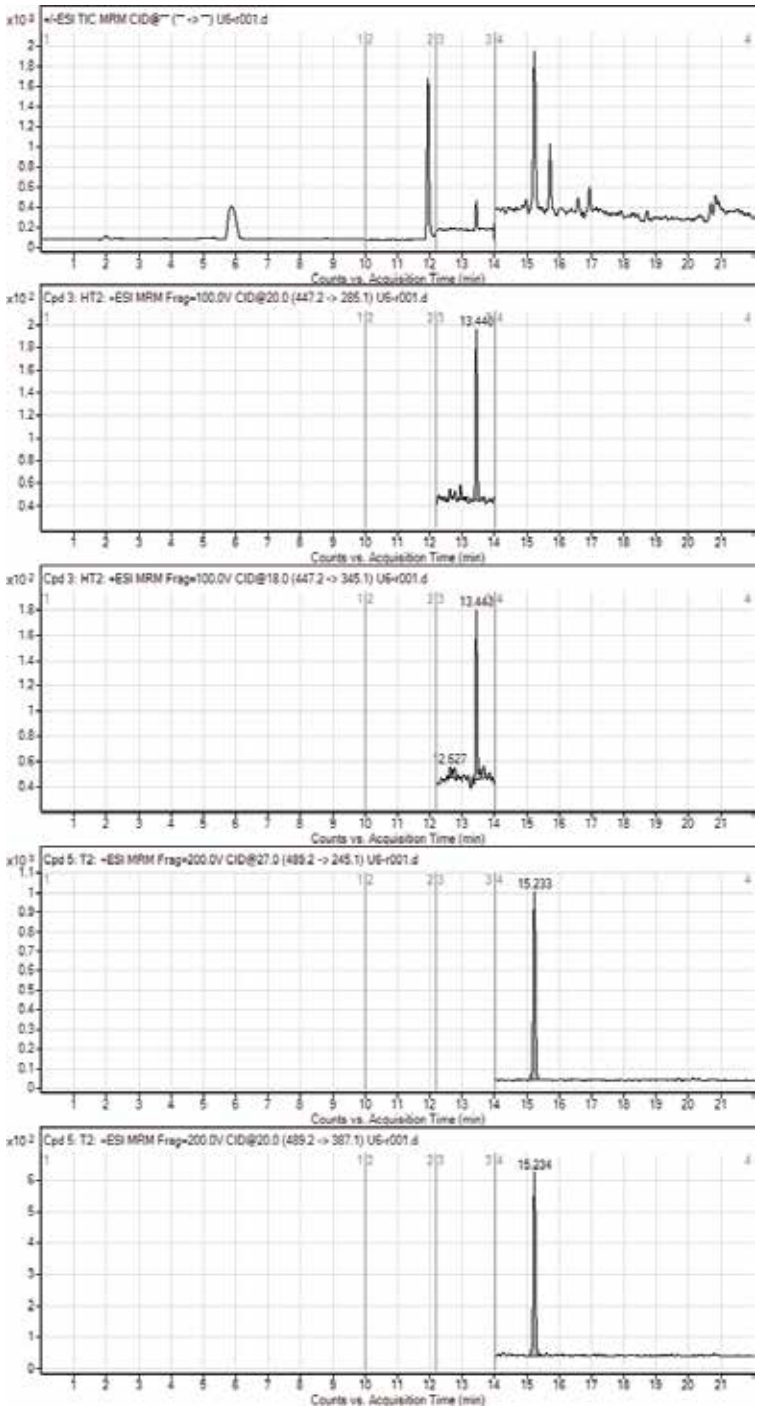

Figure 2. A typical LC-MS/MS-MRM chromatogram of the contaminated maize sample subsequently subjected to thermal reduction processing (128 μg/kg of T-2 and 256 μg/kg of HT-2); TIC; HT-2 MRM 447.2 → 285.1; HT-2 MRM 447.2 → 345.1; T-2 MRM 489.2 → 245.1; and T-2 MRM 489.2 → 387.1.

The results descriptive of T-2 and HT-2 reduction achieved by various cooking times (10:30 min) are presented in **Table 6**.

The maximal reduction of T-2 toxin was observed in oat (14.7%) cooked for 30 min, whereas the greatest HT-2 (7.5%) and summary T-2/HT-2 concentration reduction (9.3%) were obtained in oat cooked for 20 min. A slightly greater reduction was observed for T-2 toxin (9.3%) in comparison to the HT-2 toxin, which was mostly unreduced in all three types of cereals despite cooking. A prolonged cooking time (30 min) achieved no significantly greater reduction of summary T-2/HT-2 concentrations or individual toxin levels. The results show that the reduction of T-2/HT-2 summary concentrations achieved by cooking can be considered negligible (R < 10%), suggesting that cooking as a thermal processing method does not represent a valuable tool when it comes to the decontamination of cereals contaminated with T-2 and HT-2. After cooking of noodles, Kamimura et al. [43] determined the residual rate

Cereals[a]	T-2/HT-2 after cooking	Cooking time (96°C)		
		10 min	20 min	30 min
Maize	T-2/HT-2 (μg/kg)	375	366	372
	T-2/HT-2 R (%)	2.3	4.7	3.1
	T-2 (μg/kg)	117	123	110
	T-2 R (%)	8.6	3.9	14.1
	HT-2 (μg/kg)	258	243	270
	HT-2 R (%)	NR	5.1	NR
Triticale	T-2/HT-2 (μg/kg)	252	271	262
	T-2/HT-2 R (%)	5.6	NR	1.9
	T-2 (μg/kg)	104	99.5	94.8
	T-2 R (%)	2.8	7.0	11.4
	HT-2 (μg/kg)	172	151	167
	HT-2 R (%)	NR	5.6	NR
Oat	T-2/HT-2 (μg/kg)	152	137	144
	T-2/HT-2 R (%)	NR	9.3	4.6
	T-2 (μg/kg)	43.2	40.8	40.1
	T-2 R (%)	8.1	13.2	14.7
	HT-2 (μg/kg)	108	96.2	104
	HT-2 R (%)	NR	7.5	NR

[a]Concentrations in cereals before cooking: 384 μg/kg (128 μg/kg of T-2 and 256 μg/kg of HT-2) in maize, 267 μg/kg (107 μg/kg of T-2 and 160 μg/kg of HT-2) in oat and 151 μg/kg (47.0 μg/kg of T-2 and 104 μg/kg of HT-2) in triticale.
T-2 and HT-2 toxin concentrations are presented as the mean value of three replicates; R: reduction; NR: not reduced.

Table 6. Reduction of T-2/HT-2 toxins achieved by various cooking times.

of T-2 toxin in fortified samples of up to 76%. Schwake-Anduschus et al. [34] stated that T-2/HT-2 toxin levels are relatively stable during short-time cooking. This was also confirmed by this study, as it resulted in a very small reduction of both mycotoxins despite the prolonged cooking time (30 min).

Roasting is a cooking method that uses dry heat in form of an open flame, oven, or other heat sources. Roasting can enhance flavour through caramelisation and Maillard browning of the food surface [42]. The results of T-2 and HT-2 reduction via roasting, obtained in this study at three different temperatures (180–220°C), are presented in **Table 7**. **Figure 3** presents a typical LC-MS/MS-MRM chromatogram of a contaminated maize sample obtained after roasting at the temperature of 220°C during 30 min, in which a significant reduction of both T-2 toxin (60.7%) and HT-2 toxin (46.1%) can be seen.

Cereals[a]	**T-2/HT-2 after roasting**	**Temperature of roasting**[b]		
		180°C	**200°C**	**220°C**
Maize	T-2/HT-2 (μg/kg)	270	225	188
	T-2/HT-2 R (%)	29.7	41.4	51.0
	T-2 (μg/kg)	80.1	65.3	50.3
	T-2 R (%)	37.4	49.0	60.7
	HT-2 (μg/kg)	190	160	138
	HT-2 R (%)	25.8	37.5	46.1
Triticale	T-2/HT-2 (μg/kg)	190	173	151
	T-2/HT-2 R (%)	28.8	35.2	43.4
	T-2 (μg/kg)	56.2	43.3	38.2
	T-2 R (%)	47.5	59.5	64.3
	HT-2 (μg/kg)	138	130	113
	HT-2 R (%)	13.8	18.8	29.4
Oat	T-2/HT-2 (μg/kg)	101	84.3	68.9
	T-2/HT-2 R (%)	33.1	44.2	54.4
	T-2 (μg/kg)	28.1	22.7	ND
	T-2 R (%)	40.2	51.7	CR
	HT-2 (μg/kg)	72.9	61.6	59.3
	HT-2 R (%)	29.9	40.8	43.0

[a]Concentrations in cereals before reduction: 384 μg/kg (128 μg/kg of T-2 and 256 μg/kg of HT-2) in maize, 267 μg/kg (107 μg/kg of T-2 and 160 μg/kg of HT-2) in oat and 151 μg/kg (47.0 μg/kg of T-2 and 104 μg/kg of HT-2) in triticale.

[b]Roasting was carried out for 30 minutes at the default temperatures.

T-2 and HT-2 toxin concentrations are presented as the mean value of three replicates; R: reduction; ND: not detected; CR: completely reduced.

Table 7. Reduction of T-2/HT-2 toxins achieved by roasting of contaminated cereals at different temperatures.

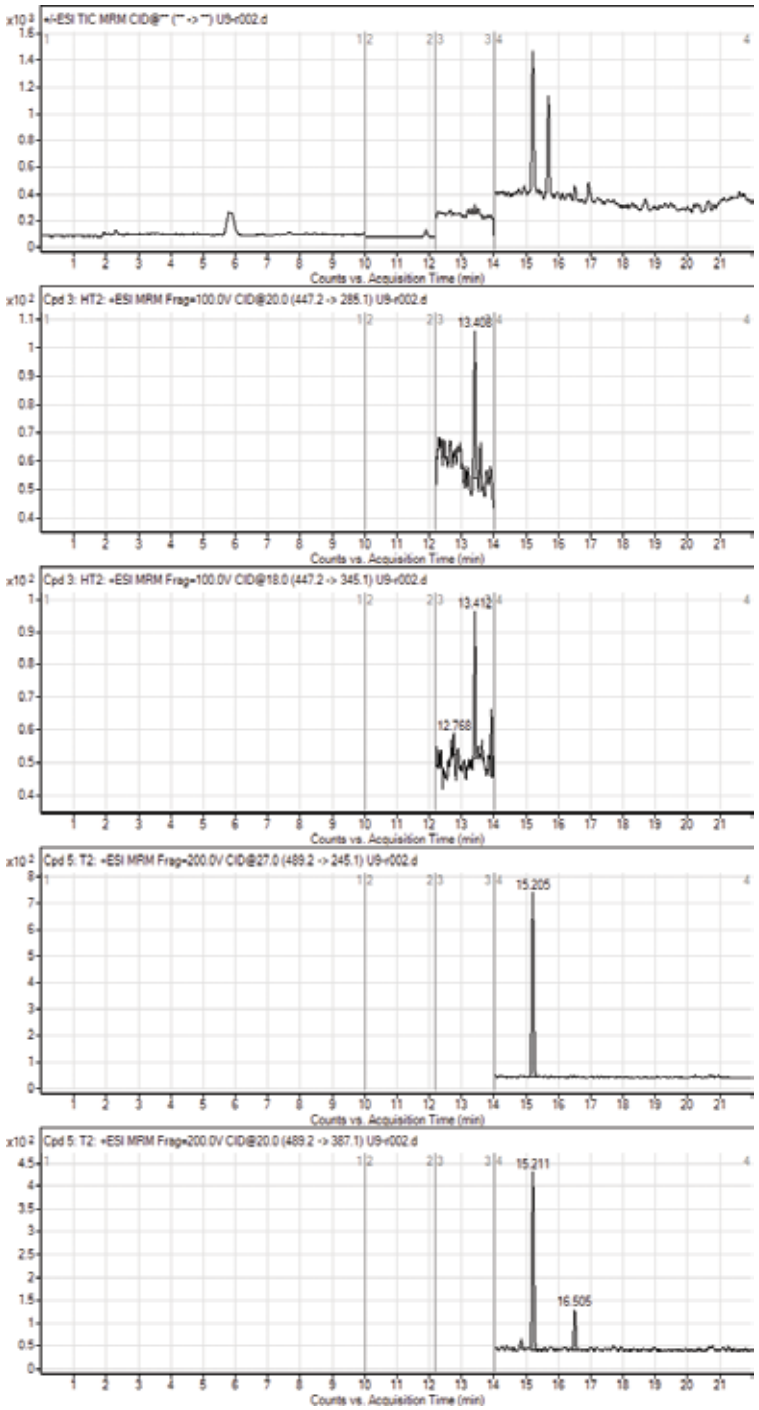

Figure 3. A typical LC-MS/MS-MRM chromatogram of a contaminated maize sample after roasting at the temperature of 220°C for 30 min (50.3 μg/kg of T-2 and 138 μg/kg of HT-2); TIC; HT-2 MRM 447.2 → 285.1; HT-2 MRM 447.2 → 345.1; T-2 MRM 489.2 → 245.1; and T-2 MRM 489.2 → 387.1.

Published data on the influence of roasting on the reduction of mycotoxins are mostly linked to aflatoxins, ochratoxins or some *Fusarium* mycotoxins other than T-2/HT-2 [45–48] and refer to baking [34, 49]. In this study, the reduction of summary T-2/HT-2 toxin levels, achieved with roasting at 180°C, ranged from 28.8 to 33.1%; at 200°C, the achieved reduction ranged from 35.2 to 44.2%, while at 220°C, a 43.4 to 54.4% reduction was witnessed. A complete T-2 toxin reduction was achieved when roasting oats; after roasting at 220°C, no detectable concentration of this mycotoxin was determined. The results show that in comparison to the HT-2 toxin, a significantly higher (nearly 2-fold) reduction of T-2 was achieved, which is in line with an earlier observation that thermal processing reduces T-2 concentrations to a greater extent as compared to those of HT-2 [39]. Roasting can be considered as an effective thermal processing method suitable for cereal decontamination, as it resulted in a roughly 40% T-2/HT-2 toxins reduction in contaminated samples.

Extrusion is used in the production of cereal products such as breakfast foods, snacks and animal feedstuffs, and represents one of the fastest-growing food-processing operations in the recent years due to its many advantages. Extrusion cooking of cereals combines pumping, kneading, mixing, shearing, cooking and forming, all in one processing session. As several operations are carried out simultaneously, they interact with each other [39]. Cereals are passed through an extruder under pressure, undergo mechanical shearing stresses at elevated temperature, and rapidly expand when forced through the outlet die [50]. Apart from its main goal in terms of improving the product quality, extrusion may also significantly improve the product safety because of its potential to reduce mycotoxin levels in cereals [51]. The effectiveness of mycotoxin reduction also depends on the presence of minor ingredients or additives. Scudamore et al. [20] explained that during extrusion, contaminants are subjected to both high temperatures and chemical reactions mediated by free radical mechanisms so that they might be susceptible to some degree of breakdown, the effects on mycotoxins thereby generally being variable.

The reduction of T-2/HT-2 toxins achieved by extrusion cooking of contaminated cereals in this study using three different regimes of extrusion is shown in **Table 8**. **Figure 4** presents a typical LC-MS/MS-MRM chromatogram obtained after extrusion cooking of a contaminated maize sample at the defined temperatures of 135-190-190°C.

By virtue of extrusion cooking under three temperature regimes and with the same moisture content (25%), an almost complete reduction of T-2 and HT-2 toxin was achieved. The results show a similar effect of extrusion independent of the type of cereal and the applied temperature regime, based on which this method can be considered as the most effective and most valuable when it comes to the reduction of mycotoxins. As the LC-MS/MS method failed to determine any of the two mycotoxins in any of the extruded samples, the summary T-2/HT-2 concentrations were analysed using the ELISA method. The results showed T-2/HT-2 concentrations slightly higher than the method's LOQs, except for the oat subjected to the extrusion temperature regime of 135-190-190°C, in which the presence of the above toxins was not detected at all. The reduction of T-2/HT-2 achieved under the 135-150-150°C extrusion regime ranged from 73.0% in oats to 92.5% in maize. However, it should be taken into account that the presence of individual mycotoxins was not confirmed by the LC-MS/MS method and that the ELISA method showed higher T-2/HT-2 summary concentrations.

Cereals[a]	T-2/HT-2 after extrusion	Regime of extrusion[b]		
		135-150-150°C	135-170-170°C	135-190-190°C
Maize	T-2/HT-2 (μg/kg)	28.9	49.7	32.2
	T-2/HT-2 R (%)	92.5	87.1	91.6
	T-2 (μg/kg)	ND	ND	ND
	T-2 R (%)	CR	CR	CR
	HT-2 (μg/kg)	ND	ND	ND
	HT-2 R (%)	CR	CR	CR
Triticale	T-2/HT-2 (μg/kg)	37.7	45.9	41.3
	T-2/HT-2 R (%)	85.9	82.8	84.5
	T-2 (μg/kg)	ND	ND	ND
	T-2 R (%)	CR	CR	CR
	HT-2 (μg/kg)	ND	ND	ND
	HT-2 R (%)	CR	CR	CR
Oat	T-2/HT-2 (μg/kg)	40.7	38.1	ND
	T-2/HT-2 R (%)	73.0	74.8	CR
	T-2 (μg/kg)	ND	ND	ND
	T-2 R (%)	CR	CR	CR
	HT-2 (μg/kg)	ND	ND	ND
	HT-2 R (%)	CR	CR	CR

[a]Concentrations in cereals before reduction: 384 μg/kg (128 μg/kg of T-2 and 256 μg/kg of HT-2) in maize, 267 μg/kg (107 μg/kg of T-2 and 160 μg/kg of HT-2) in oat and 151 μg/kg (47 μg/kg of T-2 and 104 μg/kg of HT-2) in triticale.

[b]Moisture in extruded samples ranges from 11.0 to 12.0%.

T-2 and HT-2 toxin concentrations are presented as the mean value of three replicates; R: reduction; ND: not detected; CR: completely reduced.

Table 8. Reduction of T-2/HT-2 toxins achieved by extrusion cooking of contaminated cereals at different temperatures.

When comparing the degradation rates of T-2 against those of HT-2 toxin, it was revealed that T-2 shows a higher mitigation in the extrusion cooking process [39, 41]. Some investigations showed that T-2 and HT-2 degradation during extrusion are not influenced by the heating temperature to the same extent and that other variables present during processing are responsible for a more complex degradation pattern. This observation can be linked to the results of this study, in which a significant influence of the extrusion temperature regime was not determined. Schmidt et al. [39] stated that due to the strong interference of various parameters during extrusion, it is not possible to attribute toxin degradation to just one of them. Among the factors of influence, the water content plays an important role in

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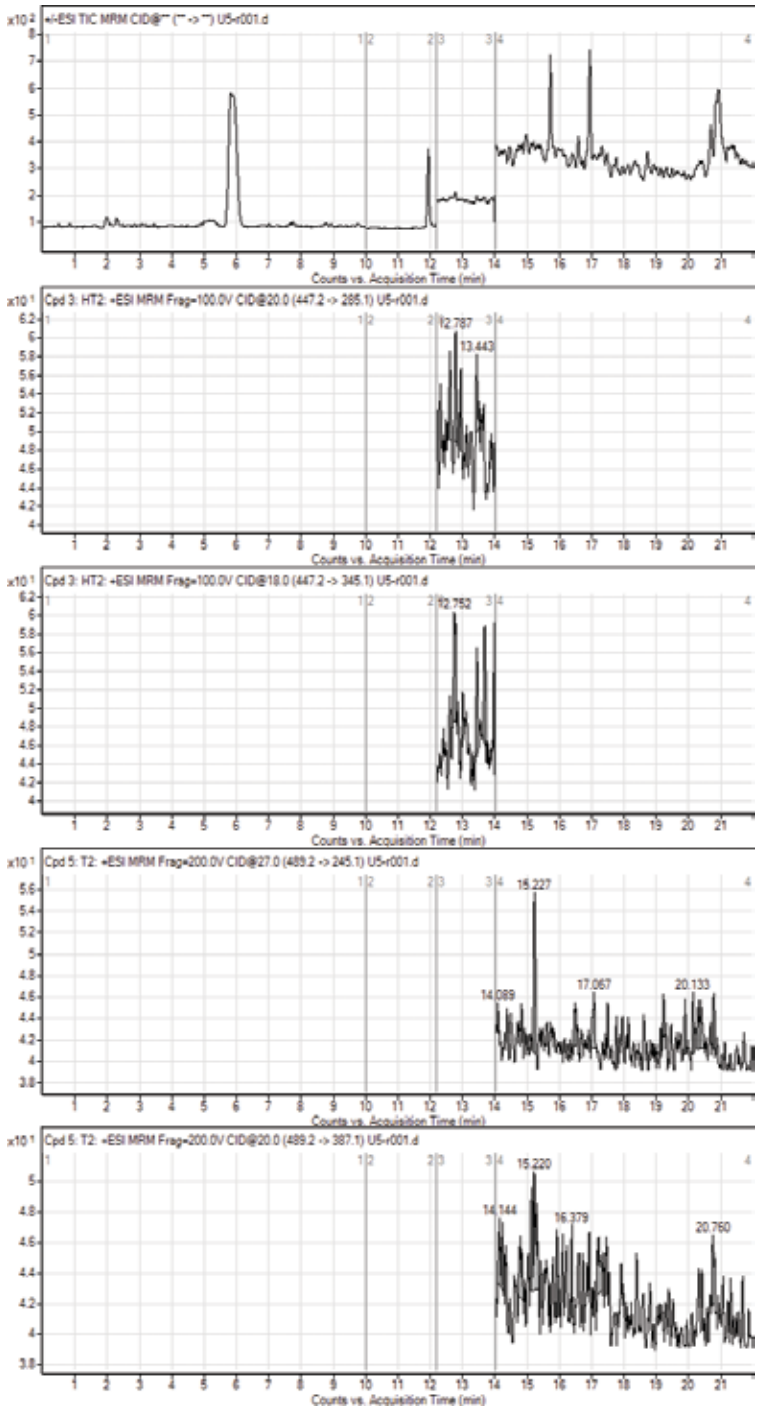

Figure 4. A typical LC-MS/MS-MRM chromatogram of a contaminated maize sample after extrusion at 135-190-190°C (T-2 and HT-2 toxin not detected); TIC; HT-2 MRM 447.2 → 285.1; HT-2 MRM 447.2 → 345.1; T-2 MRM 489.2 → 245.1; and T-2 MRM 489.2 → 387.1.

extrusion cooking, because it is essential for starch gelatinization and strongly affects fluid viscosity and the expansion ratio. Extrusion cooking was shown to decrease the mycotoxin content at rates depending on the moisture level, screw centrifugation, extruder geometry, die temperature, die size, screw speed and additives [51], while the extrusion temperature was found to be a minor factor of influence. As opposed to that, high moisture levels and high shear rates substantially contribute to the toxin degradation [39, 52]. The authors elaborated that since the fate of T-2 and HT-2 and the formation of so far unknown degradation products or bound forms remains unclear, it cannot be concluded that extrusion cooking of contaminated oats is accompanied by a detoxification process. Scudamore et al. [52] pointed out that the inconsistency of the results presented in the literature may be a consequence of failure to control or report all conditions under which the extrusion process was taking place. For example, chemical breakdown taking place during an extrusion process is related to the duration of the process, so that the loss of mycotoxin will depend on the residence time of the material in the extruder. Differences in parameters implemented during extrusion cooking carried out in this study may also explain an almost complete reduction of T-2 and HT-2 toxin achieved, as opposed to other studies quoted above, in which only partial or smaller toxin reduction has been witnessed when using this thermal processing method.

4. Conclusions

Among the analysed cereals, the highest percentage of T-2- and HT-2-positive samples were determined in oats, followed by maize, triticale and barley, whereas the highest mean and maximal concentration of the toxins was determined in maize. The summary T-2/HT-2 concentrations found in one maize and one triticale sample were higher than the indicative levels stipulated by the European Commission, suggesting that further sampling should be performed and that the production conditions should be investigated more thoroughly. As for the thermal methods of toxin reduction, roasting appears to efficiently reduce T-2 and HT-2 concentrations, whereas cooking does not significantly reduces these mycotoxins. Extrusion cooking seems to be far more efficient since it resulted in an almost complete T-2/HT-2 elimination in all cereals, independent of the temperature regime applied during the extrusion process.

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Chapter 5

Plant Secondary Metabolites for Antifusarium and Antiphytophthora

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Additional information is available at the end of the chapter

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Abstract

Plants produce secondary metabolites that are essential for survival of the producing plants such as to attract insect for pollination and defend against pest and environmental stress. Plant secondary metabolites are widely exploited by the mankind especially for medicine, one of which is to protect against infection by microorganism including fungi. Many medicinal plants have been traditionally used and/or studied for the fungicidal activity. Most of the plants studied or traditionally used as antifungi show antiphytophthora activity and some of them also active as antifusarium. Higher concentration plant extract is needed to inhibit the growth of *Fusarium* than *Phytophthora*. Considering the concentration in plant and activity as antifungi, eugenol is considered to be the most effective to be used as antiphytophthora and antifusarium. The presence of aromatic moiety, orthodioxy substitution, and double bond in the terminal of site chain is considered to be essential for the antifungal activity of the eugenol derivative.

Keywords: secondary metabolites, plant, antifungal, antifusarium, antiphytophthora

1. Introduction

Plants conduct primary metabolism to support the growth and development. In addition to primary metabolism, plant also produce and collect secondary metabolites. Compared to primary metabolites that are found in every organism, secondary metabolite has limited distribution, has been produced and collected in specific organ, and has no physiological role in the producing plants. Secondary metabolites may function in protecting the producing plant from pests and facilitate plant breeding by spreading seeds through organisms consuming

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fruits produced by the plant. The mankind, however, has used secondary metabolite produced by plants since the ancient time. People use plant secondary metabolites as medicine, spices, perfumery, poison, pest control, etc.

Plants have been traditionally used as fungicide for various purposes such as food preservatives and treatment of skin diseases. Tofu that is made from soya curd is rinsed with yellow pigment of turmeric to make the color of tofu into yellow and to extend the shelf life of tofu. Garlic is traditionally used together with cassava starch to catch spore of yeast needed for fermentation of soya bean to make *tempeh* (fermented soya bean) and *tape* (fermented cassava). The addition of garlic is intended to inhibit the growth of microorganism, but the spore can still survive and can grow under suitable environment, when it is mixed with the appropriate substrates such as cassava, rice, soya bean, and other carbohydrate-containing materials to produce fermented products. *Alpinia galanga* rhizome is sliced, and the rough surface is rubbed on the skin infected by *Trichophyton*. *Cassia alata* leaves are boiled and used for bathing to treat itchy and ringworm caused by fungal infection. *Piper betel* leaves are boiled and used for washing vaginal area to treat and to prevent candidiasis. *Ageratum conyzoides* leaves is rolled up and patched on a new cut to protect the tissue from infection by microorganisms and accelerate wound healing.

Many secondary metabolites from plants have been extracted, fractionated, isolated, and studied for the antifungal activity. Volatile oil is the secondary metabolites that play many important roles in human daily life, such as perfumery, spices, essence, medicine, aromatherapy, insect repellent, and also as fungicide [1]. Many medicinal plants contain volatile oil; many of them have been traditionally used in cut healing or as natural preservatives due to their capacity to control the growth of bacteria and or fungi [2]. Coumarin is reported to be one of the antifungal compounds present in the leaves of *Ageratum conyzoides* in addition to the volatile oil components. So far not much information on the traditional use of plants as antifungi for plants infected by fungi including soil-borne pathogenic fungi such as *Fusarium* and *Phytophthora*. Plants infected by soil-borne fungi are extremely hard to eradicate. In some cases, burning of the remaining plant is the only way to eradicate the pest accompanied by replacement with different crops. The possibility of plant secondary metabolites to be developed as source for natural fungicide especially for antifusarium and antiphytophthora is explored and discussed and supported with published reports and experimental data.

2. Plants with antifungal activity

The mankind has been using plants as medicine to treat different kinds of diseases, including fungal infection. *Acorus calamus* (sweet flag), one of the medicinal plants, has antifungal activity, and the compound responsible for this activity is α-asarone that was tested on *Fusarium oxysporum* [3, 4]. *Ageratum conyzoides* that belongs to the family Asteraceae is traditionally used to treat fresh cut. It accelerates the recovery of the tissue and prevents infection. The leaves are hairy consisting ordinary trichome and glandular hair containing volatile oil. The

extract of *Ageratum* leaf contains antifungal compound that is active toward *Aspergillus niger*, *Pestalotiopsis theae*, *Rhizoctonia solani*, and *Candida albicans* [5, 6]. The responsible compounds for the antifungal activity are volatile oil components and chromene compound that was further identified as coumarin [6, 7]. Garlic (*Allium sativum*) is commonly used as spices and herbal medicine and used as antibacteria, antifungi, antivirus, antihyperlipidemia, antiplatelet aggregation, and blood fibrinolytic agent [8]. The extract of garlic is active toward *Fusarium oxysporum*, *Phytophthora capsici*, *Aspergillus niger*, *Aspergillus flavus*, *Trichophyton rubrum*, and *Trichoderma harzianum* [9–12]. The compound that is responsible for the antifungal activity is allicin and ajoene.

Alpinia galanga is one of the medicinal plants that belongs to family Zingiberaceae and also used as seasoning. Zingiberaceae is a plant family by which the member of the family is widely used as spices and herbal medicines. The organ used is mostly the subterranean part of the plant known as rhizome. When the rhizome of *A. galanga* is sliced transversally, it produces a rough surface and traditionally used by rubbing on the skin infected by fungi, such as ringworm. The extract of the rhizome is active against *Fusarium oxysporum*, and one of the active compounds is acetoxychavicol [13]. *Curcuma domestica* known as turmeric is popular as the main spice in making curry, a popular cuisine in India and South East Asia. Turmeric extract is active toward *Phytophthora infestans*, *Exserohilum turicicum*, *Fusarium oxysporum*, and *Colletotrichum cassiicola* [14–16]. The active compounds are the component of volatile oil, i.e., eucalyptol, β-pinene, and camphor. Differed from *Curcuma domestica*, *Curcuma xanthorrhiza*, known as Java turmeric due to its bitter taste is mainly for herbal medicine. Java turmeric extract is active against *Candida albicans*, *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosisi*, and *Candida tropicalis*, and the active compound is xanthorrhizol [17].

Curcuma zedoaria is also widely used as herbal medicine and even claimed as anticancer. The extract obtained from the rhizome of *C. zedoaria* has antifungal activity toward *Trichophyton rubrum*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *Epidermophyton floccosum*, *Aspergillus fumigates*, *Penicillium purpurogenum*, *Trigonopsis variabilis*, *Microsporum gypseum*, *Sclerotium rolfsii*, *Geotricullar candiade*, *Fusarium oxysporum*, *Helminthosporium oryzae*, *Candida krusei*, and *Trichophyton mentagrophytes*, and the active compound is ethyl-p-hydroxycinnamate [18]. Ginger (*Zingiber officinale*) is mainly used as spices and also as herbal medicine. The rhizome contains volatile oil and pungent compounds gingerol and shogaol that are well recognized as antiemetic agent. Ginger extract is active toward *Aspergillus flavus*, *Aspergillus niger*, *Penicillium griseofulvum*, *Fusarium oxysporum*, and *Pyricularia oryzae* [5, 19] with zingerone as the active compound. Zingerone is one of the ginger oil components that belongs to the group of phenylpropanoid compounds.

Cassia alata is a shrub that belongs to the Caesalpiniaceae family. Traditionally, *C. alata* leaves are boiled and used by bathing to treat fungal infection causing skin diseases. The extract of the leaves is active toward *Trichophyton rubrum*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, *Aspergillus niger*, *Phytophthora notatum*, and *Fusarium solani* [3, 20–22], and the active compound was identified to be anthraquinones. Cinnamomum leaves contain volatile oil that contains cinnamaldehyde and eugenol and are reported to

be active toward *Candida albicans* [23–25]. *Cymbopogon nardus* leaves contain high quantity of volatile oil that is frequently used as insect repellent. The oil is also active as antifungal agent toward *Erysiphe cichoracearum*, *Aspergillus, Penicillium*, and *Erollium*, and the active antifungal compounds are citronellal and linalool [26]. *Eclipta alba* is commonly used as an ingredient in making hair tonic. The extract was reported to be active toward *Candida tropicalis* and *Candida albicans* [27]. *Garcinia mangostana* fruit is one of the most delicious tropical fruit. Garcinia fruit cortex that is rich in tannin and mangosteen is now commercially used as raw material for herbal medicine. Garcinia extract is active as antifungi toward *Candida albicans*, *Epidermophyton floccosum*, *Alternaria solani*, *Mucor* sp., *Rhizopus* sp., and *Cunninghamella echinulata* [28].

Piper betel leaf is traditionally chewed together with limestone and gambier by Melanesian to stain teeth and protect from infection. The leaf is also used to treat and to prevent vagina and mouth cavity from candidiasis. Betel leaf extract is reported to be active as antifungal agent for *Colletotrichum gloeosporioides*, *Botryodiplodia theobromae*, *Rhizoctonia solani*, *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. Hydroxychavicol and eugenol were reported to be the responsible antifungal compounds [29]. *Piper crocatum* is locally named as red betel; it has more bitter taste than the ordinary betel. It is considered to be more potent as herbal medicine compared to the ordinary betel; however, the volatile oil content and the antimicrobial activity were lower. The extract of red betel is active toward *Candida albicans*, *Colletotrichum gloeosporioides*, and *Botryodiplodia theobromae* [30, 31]. *Syzygium aromaticum* flower bud and leaves contain volatile oil with eugenol as the major component. The oil content of the flower bud is much higher compared to the leaves and so the eugenol content [32]. The flower bud is usually used as seasoning in cigarette-making. It is also used as local anesthesia for dental illness. The extract of clove is an active antifungi and is also active toward *Fusarium oxysporum* [4, 33, 34]. Eugenol is one of the clove oil components that has antifungal activity [35, 36].

The antifungal plants described above were extracted using methanol, and the extract obtained were tested toward *Fusarium oxysporum* and/or *Phytophthora palmivora*. The relative activities were compared, and the results are described in Section 3.

3. Plant extract antifungal activity on *Phytophthora palmivora* and *Fusarium oxysporum*

Most plants reported or traditionally used as antifungi are indeed all active toward *Phytophthora palmivora*. **Table 1** shows the antiphytophthora activity of 11 Indonesian plants that are traditionally used or experimentally reported as antifungi [36]. The capacity to inhibit the growth of *P. palmivora* can be detected by testing methanol extract that was prepared by soaking dried powder plant material at concentration 0.5%. Their activity however is different to one another. The strongest capacity is demonstrated by extract obtained from clove bud followed by *C. xanthorrhiza*, *C. zedoaria*, and *C. domestica*. These differences may be due to the concentration and the capacity of the active substances present in the individual plant

No.	Plant	Plant organ	Concentration	
			0.5%	1.0%
1	*Ageratum conyzoides* Linn.	Aerial part	+	++
2	*Cassia alata* L.	Leaves	+	+++
3	*Piper betel* L.	Leaves	++	++
4	*Allium sativum* L.	Bulb	++	++
5	*Alpinia galanga* L.	Rhizome	++	++
6	*Curcuma domestica* Val.	Rhizome	++	+++
7	*Curcuma xanthorrhiza* Roxb.	Rhizome	+++	+++
8	*Curcuma zedoaria* (Berg.) Roscoe.	Rhizome	+++	+++
9	*Zingiber officinale* Rosc.	Rhizome	++	++
10	*Cymbopogon nardus* L.	Leaves	++	++
11	*Syzygium aromaticum* L.	Flower bud	++++	++++

Note: Extract was made by maceration in methanol: +: 1–25% inhibition; ++: 26–50% inhibition; +++: 51–75% inhibition; ++++: 76–100% inhibition.

Table 1. Activity of 11 antifungal plants on ***Phytophthora palmivora***.

material. The individual active compound may be very active but present only at low concentration; consequently, the activity becomes low when the sample concentration is calculated based on the plant material. The flower bud of *Syzygium polyanthum* contains approximately 15% with eugenol content that can reach 80%. Coumarin in *Ageratum conyzoides* on the other hand only presents at very low concentration. However, for the application purpose, clove is considered potential to be used as source of antiphytophthora from plants. The rhizome of *C. xanthorrhiza*, *C. zedoaria*, *C. domestica*, and *Cassia alata* leaves can be used as alternatives. The price of the plant materials are much cheaper compared to clove bud.

Compared to *P. palmivora*, *F. oxysporum* is less susceptible toward the extract of antifungal plant. Higher concentration of plant extract is needed to observe the growth inhibition of the *F. oxysporum* culture by the extract. On *P. palmivora*, culture growth inhibition can be observed at extract concentration lower than 0.5%, whereas on culture of *F. oxysporum*, the inhibition can be observed at higher than 1%. Seven of 17 plants reported or traditionally used as antifungal agents inhibit the growth of *F. oxysporum* (**Table 2**). High inhibition activity is demonstrated by clove bud extract, and relatively high activity is shown by clove leaf extract. Inhibition by the extract of *Piper betel*, *Curcuma domestica*, *Curcuma xanthorrhiza*, *Zingiber officinale*, and *Acorus calamus* can be considered to be low [31].

Tables 1 and **2** show that clove bud and clove leaves are potential source of secondary metabolite for antifusarium and antiphytophthora. Clove bud contains 15–20% volatile oil with major components consisting eugenol (80–90%), eugenol acetate (10–15%), and caryophyllene (3%). Clove leaves also contain volatile oil, but the composition is different, and the content is much lower

No.	Plant	Plant organ	Concentration		
			2.5%	5%	10%
1	*Ageratum conyzoides* Linn.	Aerial part	—	—	—
2	*Eclipta alba*	Aerial part	—	—	—
3	*Cassia alata* L.	Leaves	—	—	—
4	*Piper betle* L.	Leaves	—	++	+++
5	*Piper crocatum*	Leaves	—	—	—
6	*Cymbopogon nardus* L.	Leaves	—	—	—
7	*Cinnamomum burmannii*	Leaves	—	—	—
8	*Syzygium aromaticum* L.	Leaves	—	+	++++
9	*Syzygium aromaticum* L.	Flower bud	+++	++++	++++
10	*Garcinia mangostana*	Fruit cortex	—	—	—
11	*Allium sativum* L.	Bulb	—	—	—
12	*Alpinia galanga* L.	Rhizome	—	—	—
13	*Curcuma domestica* Val.	Rhizome	++	++	++
14	*Curcuma xanthorrhiza* Roxb.	Rhizome	—	—	+
15	*Curcuma zedoaria* (Berg.) Roscoe.	Rhizome	—	—	—
16	*Zingiber officinale* Rosc.	Rhizome	—	—	+
17	*Acorus calamus*	Rhizome	—	++	++

Note: Extract was made by maceration in methanol: +: 1–25% inhibition; ++: 26–50% inhibition; +++: 51–75% inhibition; ++++: 76–100% inhibition.

Table 2. Activity of antifungal plant on *Fusarium oxysporum*.

compared to the clove bud. However, the oil content of clove leaf is relatively high compared to the other leaves. The oil content of clove leaves is approximately 2% with the major components which are eugenol 60% and caryophyllene 21% [37]. Clove leaves are considered to be a potential source of secondary metabolite for antifusarium and antiphytophthora. Volatile oil from clove leaves can be obtained from leaves that have already fallen on the ground; therefore, it can be collected throughout the year without disturbing the growth of the tree. In addition, the availability of clove leaves does not depend on the season and can be collected at any time.

4. The antifungal compounds from *Syzygium aromaticum*

Clove bud and leaf contain secondary metabolites that strongly inhibit the growth of *P. palmivora* and *F. oxysporum*. Under continuous extraction with hexane followed by ethyl acetate and methanol, the antifungal compounds mainly present in the hexane extract

suggesting that the active compound is nonpolar compound. Upon extraction of plant material with hexane, most volatile oil components will also present in the extract. Clove oil also demonstrates strong antifungal activity. These findings lead to the hypothesis that the antifungal compound of clove is also the component of clove oil. The major component of clove oil is eugenol. At least two compounds from the extract and the volatile oil of clove are responsible for the antiphytophthora and antifusarium activity. The two compounds were identified as eugenol and eugenol acetate. The activity of eugenol is higher than eugenol acetate.

Observation under scanning electron microscope showed that the hypha of *F. oxysporum* shrank after treated with eugenol (**Figure 1**). Higher magnification showed that the surface of hypha is no longer smooth and the cells may be leaking [38]. A number of mechanisms have been proposed to explain how eugenol acts as antifungal agent. Eugenol alters the membrane and cell wall [39] and induces leakage of protein and lipid from the cells due to the leakage of cell walls [40]. Extensive lesion of the cell membrane reduces quantity of ergosterol [41].

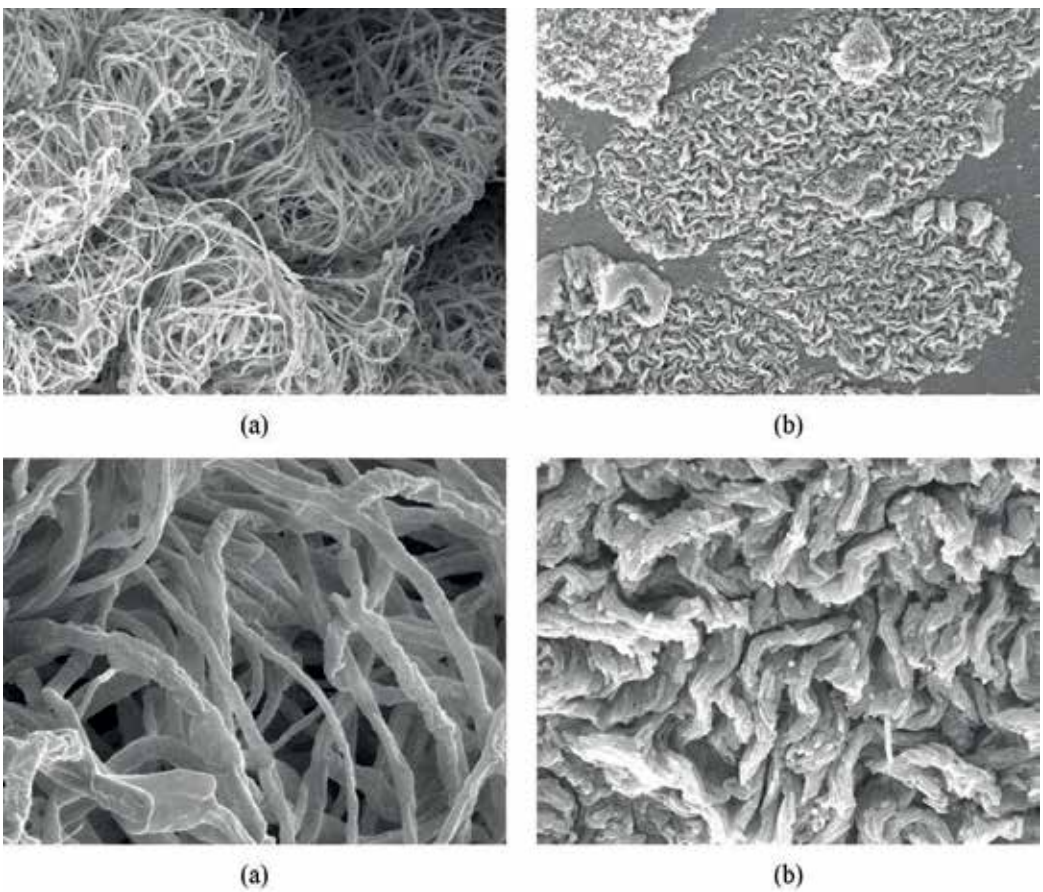

Figure 1. Scanning electron microscopy of normal *Fusarium oxysporum* (a) treated with eugenol (b) at 350× magnification (above) and at 2000× magnification (below).

It was proposed that the inhibition of ergosterol synthesis leads to the damage of cell membrane functionality and integrity [42]. However, the effect of eugenol is not reversed by osmotic support, indicating that its effect does not affect the cell wall synthesis and assembly. Furthermore, eugenol does not bind ergosterol, the main sterol of fungal membrane [43].

Eugenol is suggested to block aromatic and branched chain amino acid synthesis across the cytoplasmic membrane. Eugenol inhibits growth of yeast strain carrying a mutation in gene encoding an enzyme, a tryptophan, phenylalanine, tyrosine, and isoleucine biosynthesis pathway, in a medium supplemented with the related amino acid [44].

There are two approaches to obtain antifusarium from clove. Firstly, the secondary metabolites from clove leaves or buds can be extracted using nonpolar solvent such as hexane, petroleum ether, gasoline, or kerosene. Subsequently, the solvent is removed through evaporation leaving the concentrated extract containing antifusarium and antiphytophthora compounds. Hexane and petroleum ether have relatively low boiling point; therefore, it is easy to evaporate, and while the boiling point of gasoline and kerosene is higher than 100°C, higher temperature or lower pressure is needed to evaporate. By using extraction combined with distillation to recover the solvent, more efficient production system can be developed. Secondly, since eugenol is a component of volatile oil, the oil of clove leaves can be obtained through steam distillation by which the oil will evaporate together with steam, and upon condensation the oil will separate from water and the oil can be collected. To obtain pure eugenol, further separation processes will be needed, such as liquid–liquid extraction, vacuum fraction distillation, and chromatographic techniques.

There are some other plant metabolites having antifungal activity, and the effect is stronger than eugenol. Thymol and other components of volatile oil had been compared, and the results are as shown in **Table 3** [45]. Thymol is the component of volatile oil from *Thymus vulgaris*, herbal medicine commonly used in cough mixture. Most of these compounds demonstrate minimal inhibition concentration (MIC) above 50 ppm. The natural antifungal that demonstrates antifungal activity similar to that of commercially distributed is xanthorrhizol with MIC lower than 10 ppm [17]. Xanthorrhizol is the major component of volatile oil isolated from the rhizome of *C. xanthorrhiza*.

Compound	**Toxicity index**				
	Sclerotium rolfsii	***Rhizoctonia solani***	***Botrytis cinerea***	***Fusarium oxysporum***	***Alternaria solani***
Thymol	100	100	100	100	100
Eugenol	42.2	62.02	17.23	38.14	37.77
Methyl cinnamate	49.65	57.68	16.43	28.16	81.31
Linalool	11.8	11.33	4.45	6.03	0.85
1,8-Cineol	0.0008	0.468	—	—	—

Table 3. Relative antifungal activity of plant component compared to thymol.

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5. Structure requirement of eugenol derivatives for antifungal activity

Eugenol derivatives had been synthesized and their antifungal activities evaluated [43]. Some structures and their antifungal activities are shown in **Figure 2**. It seems that the aromatic, ortho-oxygenation, and the double bond at the terminal of side chain are essential for

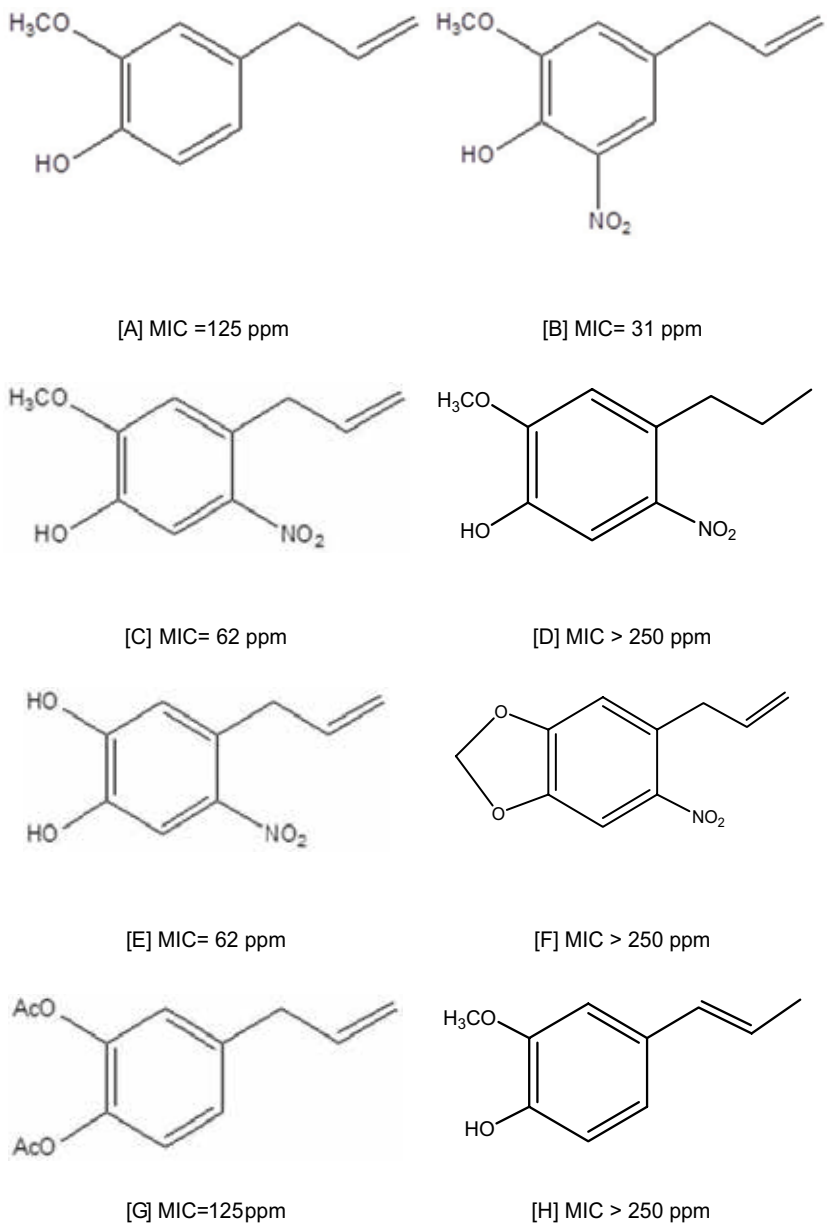

Figure 2. Derivatives of eugenol and their antifungal activities.

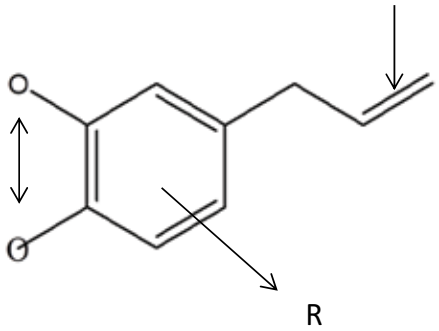

Figure 3. The important sites for antifungal activity of eugenol derivatives.

the antifungal activity. The presence of substituents on the hydroxy phenolic reduces the activity. Compound F by which the orthodioxy is connected by a methine bridge becomes inactive (MIC > 250 ppm). The absence of double bond in the side chain eliminates the antifungal activity; this is shown by compound D with MIC >250 ppm and considered to be inactive. If the position of double bond of the side chain is moved to the middle, the antifungal activity also disappears. This is demonstrated by compound H that is inactive. The presence of nitro substituent attached to the aromatic increases antifungal activity, and the nitro at ortho-position to the hydroxy group gives higher activity than at meta-position (compounds B and C).

Base on the above data, the structure requirement for eugenol derivatives to be active as a fungicide is shown in **Figure 3**.

6. Conclusion

Most extracts from plants that have been used as antifungi or reported as antifungi are also active as antiphytophthora but only few of them that are active as antifusarium. Inhibition of *Fusarium* culture growth needs higher concentration of extract compared to that of phytophthora culture. Clove bud and clove leaves are considered as potential sources for secondary metabolites for antifusarium and antiphytophthora. Clove bud and leaf contain volatile oil with eugenol as the major component. Aromatic moiety, orthodioxy, and double bound at the terminal of the side chain contribute in the antifungal activity of eugenol derivatives.

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Chapter 6

A Molecular Vision of the Interaction of Tomato Plants and *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract

Fusarium oxysporum causes vascular wilt disease in a broad range of crops, including tomato (*Solanum lycopersicum*). Tomato, a major and important vegetable crop, is susceptible to *F. oxysporum* f. sp. *lycopersici* (FOL), a biotrophic pathogen that is the causal agent of tomato wilt resulting in significant yield losses each year. Development of disease in susceptible tomato plants requires FOL to advance through a series of transitions, beginning with spore germination and culminating with establishment of a systemic infection. In addition, many host attributes, including the composition of root exudates, the structure of the root cortex, and the capacity to recognize and respond quickly to invasive growth of a pathogen, can impede the development of FOL. FOL divides into races on the basis of the ability of individual strains to overcome specific genes. This implies the presence of avirulence genes (Avr) in the fungus that is recognized by products of the corresponding genes. In tomato, resistance (R) genes against the wilt-inducing FOL are called immunity genes, and the interaction between these genes will determine the success of the infection.

Keywords: receptors, MAPK cascade, gene expression, effectors, pathogenicity

1. Introduction

Microorganisms have interacted with plants for millions of years. However, for these to be pathogenic, they must have virulence factors, secondary metabolites, and exoenzymes that allow them to access the interior of the plant through the leaves, root or wounds, or natural openings, to establish an interaction of compatibility with the host [1, 2]. One of the pathogens

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that affect a considerable number of plant species is the fungus of the genus *Fusarium*, which causes the disease known as vascular wilt [3]. This genus is made up of a large set of species that possess many biological properties. In addition, it is characterized by the production of fusiform macroconidia that are widely distributed in soil and on organic substrates [4]. The species known as *Fusarium oxysporum* causes large losses to vegetable crops both in open field and in greenhouse production [5]. Special forms (f. sp.) have been assigned according to the specificity of the host, of which about 70 have been described f. sp. [6]. Among these special forms, *F. oxysporum* f. sp. *lycopersici* (FOL) affects the tomato crop (*Solanum lycopersicum*) and is one of the main limiting factors for its production. FOL is divided into physiological races based on its ability to infect specific cultivars [7]. Regardless of biological, chemical, or cultural measures, adequate management strategies to eliminate this pathogen are not currently available once the plants are infected and have *Fusarium* vascular wilt.

The disease development in susceptible tomato plants requires that FOL pass through a series of transitions, beginning with spore germination and culminating in the establishment of a systemic infection [8]. However, to reach this point, FOL requires avoiding the defense mechanisms that activate the plant-pathogen interaction [4]. The protection mechanism in tomato plants requires the perception of the pathogen through receptors of pathogen-associated molecular patterns (PAMPs) located in the plasma membrane, which triggers the basal defense system. This includes the influx of extracellular calcium (Ca) and mobilization of intracellular Ca to the cytosol, generation of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs) as well as calcium-dependent protein kinases (CDPKs) [9], and finally the induction of defense-related genes [10]. To avoid this defense system, FOL has the ability to secrete effectors such as the so-called proteins secreted in the xylem (SIX), which allows the infection to continue [11]. This implies the presence of avirulence (Avr) genes in the fungus, which is recognized by the products of the corresponding genes in the tomato, called R genes [12]. The interaction and compatibility of the Avr genes and R genes will result in the successful FOL infection or the survival of the tomato plant [13].

Despite the importance and necessity of controlling this disease, the molecular mechanisms of pathogenesis in tomato and the genetic basis for host specificity are still poorly understood.

This chapter presents the information necessary to obtain an understanding of fungal pathogenesis at the molecular level, allowing the characterization of actively expressed genes at different stages of plant infection or under various conditions.

2. *F. oxysporum* f. sp. *lycopersici* (FOL)

2.1. Host recognition by FOL

It is known that FOL can produce three types of asexual spores: (i) microconidia, (ii) macroconidia, and (iii) chlamydospores; while the sexual or teleomorphic phase is unknown. FOL can survive saprophytically in soil and organic waste in the absence of a host, either as mycelium or in all types of spores mentioned [14]. Chlamydospores are resistance structures capable of

remaining viable in the soil for several years according to environmental conditions, and this allows this pathogen to be dispersed rapidly with the movement of water, soil, or air [15, 16].

The presence of these reproductive structures of FOL in the development medium of tomato plant allows the plant-pathogen interaction to be initiated with a preinfection state, where the host recognition is carried out, and subsequently the germination of the spores, which will continue with the tissue infection [17].

In this stage, the identification of the host is vital for the initiation of the infection process, and this is done through the release of exudates from the host roots, as these compounds represent a carbon source for the fungus [18]. Its composition includes sugars, polysaccharides, amino acids, aliphatic, aromatic and fatty acids, sterols, phenolic compounds, enzymes, vitamins, plant growth regulators, and other secondary metabolites [19].

The specific compounds that FOL recognizes in its host have not been characterized. To initiate spore germination, it is known that exudates from the root of tomato plants stimulate the germination of FOL microconidia. In addition, a relationship was found between the stimulation of germination and the age of plants. The highest stimulation of germination was observed when the plants were 70 to 90 days old. Changes were also observed in root exudates such as the concentration of phenolic compounds and flavonoids or induced changes in the exudates by the degree of colonization of the arbuscular mycorrhizal fungus Glomus mosseae, modifying the spore germination and the degree of colonization [20, 21].

2.2. Signaling by MAPK

Mitogen-activated protein kinases (MAPKs) are proteins that have been evolutionarily conserved using cycles of phosphorylation and dephosphorylation for signal transduction. Activated MAPK kinase kinases (MAP3Ks) first phosphorylate two serine and/or threonine residues located within the activation loop of MAPK kinases (MAP2Ks). Activated MAP2Ks in turn trigger MAPK activation through dual phosphorylation of a highly conserved activation loop. Sequential activation of this pathway (MAP3Ks-MAP2Ks-MAPK) plays an essential role during the development of FOL. Activation of this signaling pathway will result in the expression of genes and transcripts necessary to regulate the infection process and the development of the disease, such as the expression of pathogenicity, infectious growth, or root attachment, once FOL identifies the host [22, 23].

Recent studies report that the physiological and developmental processes of FOL are regulated by three signaling pathways identified as *Fusarium oxysporum* MAP K (Fmk1), MAP kinase (Mpk1), and high-osmolarity glycerol response (Hog1) and are mediated by MAPKs. Each of these pathways has specific roles; in the case of Fmk1, it has functions related to virulence and fusion of hyphae. Mpk1 is related to characteristics of the cell wall as its integrity and remodeling, the growth and fusion of vegetative hyphae. Finally, Hog1 is linked to osmoregulation responses and stress responses. The three pathways are involved in the pathogenesis of FOL and in the development of the disease [24]. This was demonstrated by using RNA interference (RNAi) to silence these signaling pathways, which caused loss of surface hydrophobicity, reduction of invasion, hypovirulence, conidial size alteration, growth reduction, and a significant decrease in pathogenesis in tomato seedlings [25].

2.3. Pathogenesis and virulence

The concepts of pathogenicity and virulence are often erroneously considered as synonyms. Its definition initially focused on the intrinsic properties of the pathogen. However, currently, the definition of these characteristics considers the contributions of the pathogen and the host in the development of the disease. Based on this, the concept of pathogenicity is described as the ability of a microorganism to cause damage in a host, while virulence is defined as the relative ability of a microorganism to cause damage in a host [26]. Following these definitions, we next describe the genes required for the development of the disease caused by FOL (**Figure 1**).

FOL infection process begins with the germination of spores on the host surface. It begins with the emergence of the germinal tube through the wall of the spore followed by the emergence of ramifications of this structure giving rise to fungal hyphae [27], which adhere to the root and continue with invasive growth and penetration of plant tissue. In this stage of penetration and colonization of the host, FOL requires expression of the Fmk1 gene. It is responsible for the coding of an MAPK, required for root adhesion and penetration, invasive

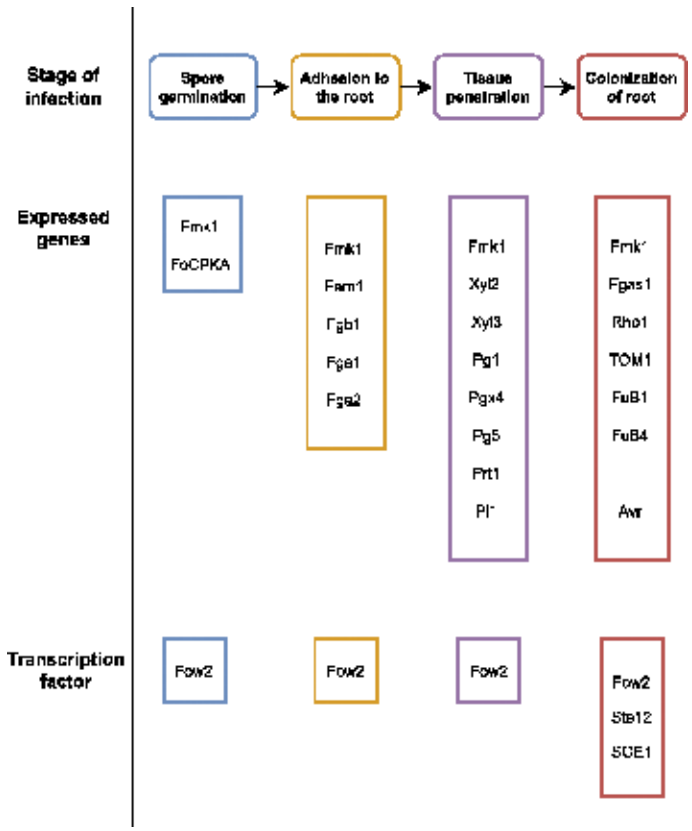

Figure 1. Relationship of genes and transcription factors activated during disease development of FOL.

pathogen growth, as well as secretion of pectinolytic enzymes and fusion of vegetative hyphae [28]. The Fmk1 MAPK cascade critically depends on the transcription factor Ste12, which controls the invasive growth and virulence downstream of the Fmk1 MAPK cascade. However, it is not linked to the regulation of root adhesion, suggesting that activation of Ste12 is independent of the MAPK pathway and that multiple pathways may converge on this transcription factor [29].

The participation of *Fusarium oxysporum* FOW2 gene, a type Zn(II) 2Cys6 transcription factor encoded by the gene designated by the same name, has also been indicated. It is highly conserved in the special forms of FOL, and its expression is essential for pathogenicity [30].

On the other hand, extracellular pH has been shown to act as a key signal for growth, differentiation, and virulence in pathogens [31]. Gene expression in fungi by environmental pH is regulated by a conserved signaling pathway, whose terminal component is the PacC/Rim1p zinc finger transcription factor. PacC is considered to act as a negative virulence regulator in FOL. Expression of this transcription factor is pH dependent, with high transcription levels under alkaline growth conditions and low transcription levels under acidic growth conditions. There was also a clear effect of pH and PacC on the expression of two genes that encode endopolygalacturonases [32].

The genes described related to the pathogenesis of FOL control various processes in the development of the pathogen. Such is the case of the cyclic AMP-dependent protein kinase A of *F. oxysporum* (FoCPKA) gene, which regulates multiple traits related to growth, microconidia formation, germ-tube shape, septation, and branching of hyphae by means of a cyclic adenosine monophosphate-dependent protein kinase A (cAMP) [33]. On the other hand, the Fgb1 gene encodes the β-subunit of the G protein that controls hyphae growth, development, and virulence through pathways dependent and independent of cAMP signals, while the α subunit is encoded by the Fga1 gene, whose silencing reduces the conidiation and pathogenicity of the fungus [34–36].

In a study on the cloning of the Fga2 gene, it was shown that the functions of the two Gα proteins overlap but are distinct. On the one hand, the Fga1 pathway regulates the growth and development of fungi, including morphogenesis and conidiation. In contrast, the Fga2 pathway plays a more crucial role in pathogenicity [37]. In addition, the loss of function of the Fgas1 gene, responsible for the expression of β-1,3-glucosyltransferases, dramatically affects the virulence of FOL, since it is linked to structural alterations of the cell wall [38].

Another gene related to the cell wall characteristics of FOL is Rho1, responsible for the coding of a putative Rho-type GTPase. It plays a crucial role in infection, avoiding recognition by the host defense system since its deletion in FOL leads to changes such as an increase in the amount of polymers in the cell wall, which serve as response activators in plants [39]. The Fow1 gene encodes a protein with high similarity to mitochondrial carrier proteins, which is specifically required for colonization in plant tissue since its absence generates a reduction in the virulence of FOL [40]. Likewise, the involvement of the Fpd1 gene has been described, which has as its possible function the coding for a transmembrane protein whose deficiency reduces the pathogenicity of FOL [41].

2.4. Lithic enzymes

The initiation of FOL infection requires degradation of the host cell wall through the action of a complex of enzymes with lytic activity such as xylanases, cellulases, pectinases, and polygalacturonases, which contribute to the penetration and colonization of the plant [42]. The genes xyl2 and xyl3 are responsible for the coding of xylanases, which degrade xylan. The xyl2 gene is expressed during the final stages of the disease, while xyl3 is present throughout the cycle [43]. The XlnR gene is known to be the main transcriptional activator of the xylanase genes. However, it was demonstrated that it is not determinant in the virulence of FOL, perhaps due to the presence of other xylanase genes whose expression is independent of this transcription factor [44].

The PG1 and PG5 genes are responsible for the expression of extracellular endopolygalacturonases, the latter expressed mostly during the early stages of infection [45, 46]. On the other hand, the characterization of several enzymes with lytic activity, such as PG1, exo-polygalacturonases (PG2 and PG3), an endoxylanase (XYL1), and an endopectate lyase (PL1), has been reported. Coded by genes pg1, pgx4, pg5, xyl2, xyl3, prt1, and pl1, these are expressed during different stages of interaction with the host plant indicating a possible role in the pathogenesis [47, 48]. While the absence of the Fpr1 protein (F-box protein, required for pathogenicity) results in the lack of expression of some enzymes involved in cell wall degradation, this is perceived as the inability of the pathogen to colonize the roots [49, 50].

3. Tomato plant

3.1. Pathogen recognition

Recognition of pathogen infection triggers an immunity system in the plant of two branches [13]. (i) In the first, common molecules are recognized in many classes of microorganisms, including those that are nonpathogenic and called immunity associated to pathogen-associated molecular patterns (PAMP)s or PAMP-triggered immunity (PTI). (ii) The second branch responds to factors of virulence and pathogenesis, so it is designated as effector-triggered immunity (ETI). Both mechanisms are described below (**Figure 2**).

PAMPs are considered as conserved elements by different classes of microorganisms and are essential for survival and pathogenicity [51]. Among the molecules identified as PAMPs for FOL, some components of their cell walls such as chitin, glucan, and glycoproteins are considered in this group due to their interaction with the host [52, 53].

The synthesis of chitin is regulated by the chsV gene and its expression depends on the Fmk1-MAPK signaling pathway. This was demonstrated with mutants of this gene, as they were unable to infect and colonize tomato plants or to grow invasively in tomato fruit tissue. In addition to this, hypersensitivity to defense compounds produced by the host was observed [54, 55].

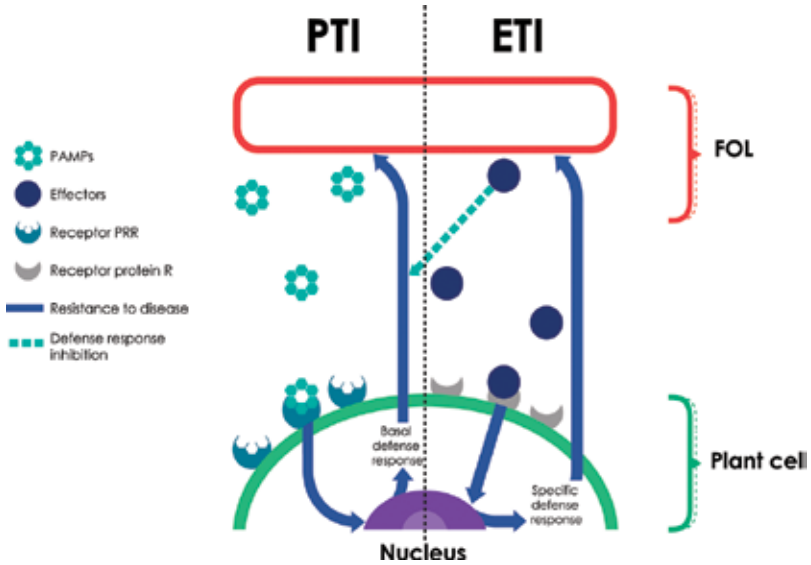

Figure 2. Scheme of activation of the defense system by PTI and ETI in tomato plant.

Glucans are also considered as elicitor molecules, although glucans may be present in the cell wall of both plants and fungi, β-1,6-glucan is specific to the cell wall of fungi, resulting in a potential PAMP [56]. Glycoproteins present in the cell wall of FOL, whose function is the adhesion of hyphae to plant tissue, are encoded by the Fem1 gene and are considered within this group [57]. Each of these elements can be recognized by the plant defense system since it has pattern recognition receptors (PRR) [58]. Plant PRRs are located on the surface of the plasma membrane and can be two types. The receptor-like kinases (RLK) contain a ligand-binding ectodomain, a single-pass transmembrane domain, and an intracellular kinase domain. Or they may be receptor-like proteins (RLPs) typically consisting of a repetitive domain rich in extracellular leucine, a transmembrane domain, and a short cytoplasmic tail [59–61].

To date, chitin has been the most extensively studied PAMPs; the presence of a receptor for chitin in rice cells has been detailed [62], while in *Arabidopsis thaliana*, an RLK-type lysin motif receptor-like protein (LysM RLK1) with an extracellular domain containing three predicted LysM motifs has been detailed. These studies have shown that the binding between the receptor and chitin is specific and direct. However, this interaction is not a simple ligand-binding reaction but could be accompanied by a conformational change of the receptor protein. This allows it to participate in signaling leading to gene induction and defense responses against pathogenic fungi [63, 64]. On the other hand, little is known about the mechanism of recognition of glucans as PAMPs in tomato plants. In soybeans, a glucan-elicitor–binding protein (GEBP) harbored a glucanase domain and a high-affinity glucan binding motif, which makes this protein a powerful tool to release and detect elicitor fragments of the pathogen [65].

3.2. Signaling and defense response

Recognition of pathogen elicitors that are released at the site of infection is rapidly followed by changes in ion flow and the production of reactive oxygen species. These events activate signaling cascades, which lead to the activation of the transcription factors involved in the activation of defense genes [66]. These responses are known to be regulated through complex signaling pathways involving various phytohormones. The FOL-activated signaling network integrates signals shared and mediated by synergistic or antagonistic interactions between salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and ROS [67, 68].

The SA plays an essential role in plant defense signaling since the recognition of FOL-derived components allows the accumulation of this phytohormone, with the subsequent establishment of local resistance in the infected region.

In the same way as the systemic resistance of the whole plant [69], the biosynthesis of SA is regulated by the Arabidopsis defense-related gene (SID2) [70]. This pathway requires the high-affinity protein SABP2, responsible for the conversion of methyl salicylic acid to SA [71], as well as the nonexpresser of pathogenesis-related (PR) genes positive regulator (NPR1) [72], which is regulated by the transcription factors of the TGA and WRKY family [73, 74]. On the other hand, the function of Ca as a second messenger has been characterized in numerous signaling pathways of plants, transporting a wide range of environmental and developmental stimuli to the physiological response [75]. An example is its participation in the regulation of SA levels through the interaction of a Ca/calmodulin with the transcription factor–enhanced disease susceptibility 1 (EDS1), through the activation of the Ca channels for the influx and subsequent mobilization of the intracellular Ca stores [76]. The increase of Ca in the cytoplasm is the first step in the signaling pathway of PAMP-triggered immunity (PTI). This elevation may occur in response to the perception of PAMPs, interactions of the R gene due to phosphorylation events, G protein signaling, and/or cyclic nucleotide increase [77].

The SA is crucial to induce the production of superoxide anion and hydrogen peroxide, by the activation of apoplastic peroxidase, and subsequently NADPH oxidase of the plasma membrane [78], which are connected to each other through the activation of Ca channels, as it has been pointed out that the increase of the cytoplasmic Ca coincides with the concomitant increase of ROS, or by the phosphorylation of proteins [79]. ROS are known for their direct antimicrobial role against pathogens as well as their relation to the activation of second messengers related to the expression of genes related to the production of response proteins [80], such as the peroxidases of class III, which are important due to their involvement in the reinforcement of the cell wall in the site of interaction with the pathogen, through catalysis of the reticulation of cell wall components including glycoproteins, lignin, and suberin [81]. Also, the oxidative burst is associated with the hypersensitivity response or programmed cell death, processes that inhibit the invasion of the pathogen through isolation [82].

Activation of MAPKs is critical in components of basal defense pathways as well as in more specific interactions involving R-gene–mediated resistance. The oxidative burst activates an MAPK cascade that induces the downstream defensive mechanisms regulated by SA, ET, JA,

and ABA. Responses may be ethylene synthesis, ROS production, pathogenesis-related (PR) protein expression, and cell death [83, 84].

On the other hand, jasmonates are involved in the reduction of FOL susceptibility in tomato plants, by increasing the activity of polyphenol oxidase [85]. Whereas in Arabidopsis plants, it has been observed that the response to *F. oxysporum* requires signaling pathways through ET, JA, SA, and the NPR1 gene, although it is independent of the function PAD4 and EDS1 [86]. The interactions between SA and JA signaling are complex, but research indicates an antagonistic relationship between them [87], while jasmonates and ET cooperate to synergistically induce defense genes such as PR1b, PR5, and PDF1.2 [88, 89]. Expression of the ERF1 gene responsive to ET and to JA is a common component in the pathways of both phytohormones [90]. It is suggested that ABA function affects disease resistance by suppressing basal and induced transcription of JA and ET response genes, which clarifies the antagonistic relationship between these hormones [91]. However, ABA has been reported to be involved in the production of callose for the early and efficient construction of papillae at sites of infection to counteract the pathogen [92]. On the other hand, signaling mediated by heterotrimer G proteins has been shown to suppress the induction of SA-, JA-, ET-, and ABA-dependent genes during the initial phase of infection with *F. oxysporum*, whereas at a later phase, it improves the JA-/ET-dependent genes like PDF1.2 and PR4 [93].

The defense responses that are activated by these pathways in tomato plants involve the increase of defense enzymes such as phenylalanine ammonium lyase and peroxidase [94], as well as the synthesis and accumulation of proteins related to pathogenesis, such as chitinases and b 1–3 glucanases. These proteins act synergistically to inhibit the growth of the fungus [95]. Programmed cell death can be induced by α-tomatine, which has a fungicidal action, in addition to its potential role as an activator of tyrosine kinase signaling pathways and the monomeric GTP-binding protein (G protein) that leads to Ca elevation and the ROS burst in FOL cells [96]. The regulation of plant immune responses is mediated by transcription factors of the WRKY family, which are functionally connected by forming a transcriptional network composed of positive and negative feedback loops within a network of partially redundant elements, some of which hold central positions that allow the activation of fast and efficient defense programs [97].

3.3. Effector protein recognition

Once the first line of defense is activated through recognition of PAMPs, FOL employs mechanisms that allow it to suppress such activated responses. During the infection process, it secretes small proteins rich in cysteine (effector or virulence proteins). The function of these proteins is to promote infection and colonization in the host plant, by disrupting various cellular processes such as signal transduction or modifying the proteins in the host plant [98].

The set of these effectors determines the specificity of the host, as well as the ability of the pathogen to manipulate the host immunity [99, 100]. In FOL, these effectors are designated as proteins secreted in the xylem (SIX) and six genes have been reported to encode them [101].

These genes related to pathogenicity are located on a small chromosome within the FOL genome [102].

Recognition of effectors occurs through receptors known as R proteins, which contain at the amino terminus a predicted conserved central domain that functions as a nucleotide-binding site (NBS) and a variable number of leucine-rich repeats (LRR) in the extreme C-terminal [103]. It is considered that this LRR domain of proteins could contribute to the recognition of various ligands derived from pathogens, while the amino-terminal domain determines the specificity of signaling. These receptors are also referred to as NBS-LRR [104–106]. The way in which R proteins activate the signal transduction pathway leading to plant defense is not yet fully understood, but recognition of pathogens is thought to trigger nucleotide-dependent conformational changes that may induce oligomerization, thus providing a scaffold for the activation of downstream signaling components [107].

The perception of the effectors triggers the second branch of the plant defense system or ETI. This is based on the gene-for-gene hypothesis, where resistance to disease is thought to be conferred by R genes, or immunity (I) genes in tomato, but requires the coincidence of avirulence genes (Avr) from FOL [12]. This is why FOL is divided into physiological races based on the ability of the individual strains to overcome the tomato-specific immunity genes (**Figure 3**). Therefore, compatible or incompatible interactions are controlled by three avirulence genes (Avr 1–3) in FOL and the corresponding resistance genes (I-I3) in tomato [108].

The SIX4 protein has been identified as a virulence effector designated as Avr1 gene, and the mature protein is made up of 184 amino acids and contains 6 cysteines in its structure. Its expression was initially reported for race FOL 1 and is required to activate resistance mediated by gene I and I-1. In addition, it is related to the suppression of disease resistance linked to the I-2 gene and the I-3 gene for tomato [109]. The SIX3 protein corresponds to the Avr2 gene, which is made up of 144 amino acids and contains 2 repeated cysteines. It is required for the development of disease symptoms, as demonstrated by its deletion, resulting in a reduction in virulence. This protein can be recognized by the I-2 resistance gene [11].

Expression of the Avr3 gene encoding the SIX1 protein is essential for virulence in tomato. Its structure consists of 189 amino acids and contains 8 repeated cysteines. Its recognition is necessary to activate the resistance through the I-3 gene. A relation between the expression of this gene and Avr1, for the evasion of resistance activated by I-3, has been suggested. The Avr3 gene requires the presence of live plant cells, and its secretion is performed immediately after the penetration of the root cortex [110–112]. Another important effector protein in the pathogenicity of FOL is SIX6. It is made up of 199 amino acids and contains 7 cysteines. It is expressed in early and late stages of infection and its expression suppresses the cellular death triggered by the I-2 protein and requires the presence of live host cells [113]. Close homologs have been found in other special forms for the SIX6 and SIX7 effectors, suggesting that these genes may have a more general role in pathogenicity [114].

The expression of Avr genes in FOL is regulated by the transcription factor SIX Gene Expression 1 (SGE1). Although its expression is not required for the vegetative growth of the fungus, it is essential for the pathogenicity of FOL, by playing an important role during

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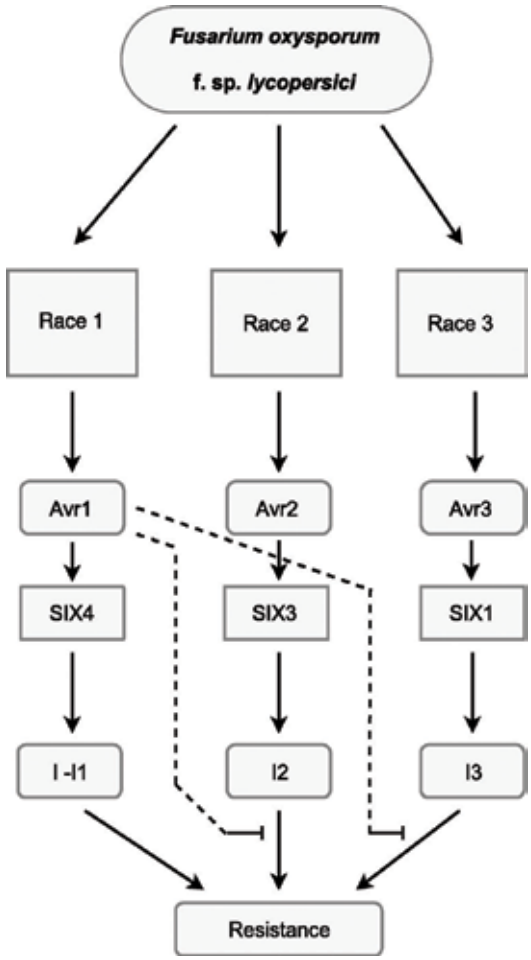

Figure 3. Classification of the physiological races of FOL according to the interaction of Avr genes of FOL and immunity genes in tomato.

parasitic growth, which produces an extensive growth in the plant and leads to the symptoms of the disease [115].

3.4. Overcoming defense response

One of the strategies of FOL to overcome the tomato defense response is the participation of chitinases, through the synergistic action of two proteases that are required for the complete separation of the binding domain of class I and class IV chitinase of tomato, allowing full virulence of FOL [116].

Detoxification of α-tomatine by the action of the enzyme tomatinase in FOL has also been identified. It is encoded by the TOM1 gene, which plays an essential role for the successful infection of the fungus [117]. The role of fusaric acid, which plays an important role in fungal pathogenicity,

has also been described by decreasing the cell viability of the plant. It is directly related to programmed cell death through damage to photosynthetic machinery, increase in protease activity, ROS production, low regulation of antioxidant enzyme activities, and positive regulation in lipid peroxidation, as well as the disorder of the nitrogen metabolism resulting in the collapse of the cell [118–120]. Its biosynthesis requires expression of the FUB1 and FUB4 genes [121], and L-aspartate is suggested as the precursor amino acid in the biosynthetic pathway [122].

If FOL manages to evade the defenses of tomato plants, infection and the development of symptoms of vascular wilting will occur. Effects include leaf detachment and leaf epinasty, followed by slower leaf growth, progressive wilt, defoliation, and inevitably death [123].

4. Conclusion

In this way, it is clear that both FOL and the tomato plant are in a long competition of defensive mechanisms to ensure their survival when the plant-pathogen interaction occurs.

FOL genomic analyzes have revealed the evolutionary characteristics of genes associated with FOL adaptation to their hosts, getting to understand the specificity for these. Molecular biology studies have evidenced the genes that are expressed in FOL interactions with their hosts at different stages of infection. However, a greater level of understanding with regard to the interaction mechanisms is needed. Future efforts will be needed to investigate the role of effector proteins in FOL-host interactions.

A study of elicitor molecules of natural or synthetic origin that are recognized as PAMPs and can trigger a nonspecific defense response is necessary. The obtained knowledge will further improve tolerance to vascular wilt of tomato plants by FOL infection.

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Chapter 7

Analysis of *Fusarium*-Common Beans Pathosystem in Aguascalientes, Mexico

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Abstract

In Mexico, high incidences of *Fusarium* affect common bean (*Phaseolus vulgaris* L.) production, reducing grain yields due to seedling death and crop standing reductions. Production of resistant germplasm could be an appropriate strategy for grain yield increasing. Bean breeding programs need the former analysis of plant-pathogen pathosystem to perform the selection of segregating populations with improved resistance to root rot pathogens and the best agroecosystem adaptation. Here, we report our results on characterization of genetic variability patterns of *Fusarium solani* f. sp. *phaseoli* (FSP) from Aguascalientes, México; the analysis of *P. vulgaris* germplasm reactions to highly and naturally FSP-infested field and controlled conditions; and the identification of genetic basis of resistance to FSP root rot in segregating common bean populations. Significant genetic variability in FSP isolates from Aguascalientes and other regions of México was found. Also, we found high variation on reactions to FSP root rots, resistance was more frequent on black seed-coated beans, and susceptibility was common in pinto beans. Resistance to FSP in BAT 477 seedlings was associated with one quantitative trait loci (QTL).

Keywords: *Fusarium solani* f. sp. *phaseoli* root rots, *Phaseolus vulgaris* L., Aguascalientes, genetic diversity, root rot incidence and severity, genetic resistance, molecular markers

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the second major crop in México. In 2016, approximately 1.63 million hectares were cultivated with common beans and an average grain yield

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of 690 kg ha^{-1} was reported [1]. Grain yields of common bean in México are low since potential yields are estimated to be ≈3 t h^{-1}. Several factors such as biotic (diseases, insect pest, weeds) and abiotic (drought, freeze, low-fertility soils, high temperatures, salinity) stresses reduce common bean production [2]. Drought stress and root rots caused by *Fusarium solani* f. sp. *phaseoli* (FSP), alone or combined, affect bean grain yield in major regions producing common beans in México. Both stresses reduce grain yields due to increase the percentages of seedling death and, consequently, reduce the crop standing (root rots) or reduce growth and development and seed production (water deficits) [3, 4].

Grain yield reductions decrease crop profits. More than 70% of common bean growers use low inputs for production, or in some cases, common bean is a subsistence crop. We consider that production of common bean germplasm with combined resistance to drought stress and diseases could be an appropriate strategy for grain yield improvement because it is a cheap, sustainable and durable strategy for grain yield stabilization [5]. The control of major root rot pathogens includes chemical, cultural and biological strategies, but most of them are not enough efficient to control pathogens or they have poor possibilities to be applied under Mexican bean grower conditions because they are expensive [6].

Mexican common bean breeding programs need the former analysis of plant-pathogen pathosystem to perform the selection of those genotypes with improved resistance to root rot pathogens and the best environmental adaptation. Another challenge is the characterization of pathogenic variability of root rot pathogen populations to identify molecular genetic factors of parasitic capability of the pathogen, since these characteristics affect the variation on reactions of common bean germplasm to the fungus. Then, the development of molecular marker technologies to improve the evaluation and selection of resistant common bean germplasm under marker-assisted selection strategy is needed [7].

This research includes three objectives: (1) to characterize the genetic variability patterns of *Fusarium* isolates from Aguascalientes and other regions of México; (2) to assess the reactions of each root rot pathogen in *Phaseolus* sp. germplasm under field and controlled conditions and (3) to define the genetic basis of resistance to each root rot pathogen in common beans.

2. Materials and methods

Despite the states of Aguascalientes and México, other states do not outstand as bean producers in México that three Mexican northern states (Chihuahua, Zacatecas and Durango) produce 60% of common beans at country while other four southern states (Chiapas, Oaxaca, Veracruz and Puebla) produce 20%, and both groups produce 80% of total beans in Mexico, they are considered by Mexican bean breeders as good locations for germplasm evaluation and/or selection for resistance to drought stress and root rot diseases caused by *Fusarium* sp., *Rhizoctonia solani* and *Pythium* sp., among other diseases such as common blight (*Xanthomonas axonopodis* pv. *phaseoli*) and anthracnose (*Glomerella lindemuthiana*) [8, 9].

Field trials included in this report were conducted in one location of the State of Aguascalientes: Sandovales and one from the State of México: Chapingo. Sandovales is located at 22°09′N, 102°18′W, and 2000 m above sea level and shows dry land conditions with summer rainfall. Annual average precipitations range from 350 to 400 mm, with average temperature ranges from 12 to 18°C. Chapingo is located at 19°28′N, 98°52′W; 2250 m above sea level and has a temperate climate with fresh summer and low variable temperatures (15–18°C) and the annual average precipitations range from 600 to 700 mm [10].

2.1. Variability of FSP isolates from Aguascalientes, México

The procedures for *Fusarium* isolates characterization by using *in vitro*, pathogenicity and AFLP genotype strategies were described when we analyzed the isolates from the State of Aguascalientes [11] and Aguascalientes, México, Guanajuato and Veracruz [4].

2.2. Reactions of common bean germplasm to root rot pathogens under field conditions

Previous works indicated us that soils of Chapingo and Sandovales are highly, naturally and homogeneously infested in most cases by FSP [12, 13]. We divided the characterizations into two groups.

The first group included 6 (experiment I), 75 (experiment II) and 36 (experiment III) (**Table 1**) common bean genotypes under rainfall conditions at Sandovales, Aguascalientes. Experiments were established on June 27 (E-I and E-II) and July 11 (E-III), 2002 under randomized complete block (RCB) design with four replications (E-I), where experimental unit was three rows 5 m-length. The germplasm of E-II was divided into three groups based on color seed coat: 25 pinto seed-type bean genotypes, 25 Flor de Mayo seed-type and 20 black seed beans. Each group of genotypes was randomized in a RCB design with three replications, and where experimental unit was two rows 6 m-length. Finally, germplasm in E-III was randomized on 6×6 lattice design with three replications and experimental unit of 2 rows 6 m in length.

In the second group of experiments, 49 common bean genotypes (**Table 1**) were evaluated under two levels of soil moisture: irrigated and rainfed conditions. Germplasm was randomized in 7 × 7 lattice design with four replications. Two replications were carried out under irrigated conditions, while the other two under rainfed conditions (irrigation was stopped when the most of germplasm initiated flowering and no irrigation was supplied until harvest). Experiments were established in Sandovales and Chapingo, México.

In both groups of experiments, FSP root rot severity ratings were determined at 28 and 56 days after sowing. Five plants were randomly picked off from each experimental unit and damage was evaluated by using the scale described by Abawi and Pastor-Corrales [6]. The scale has nine degrees of damage (1–9) where 1 = no symptoms and 9 = more than 75% of root or stem tissues infected by the pathogen. We took the values 1–3 as a reaction of resistance, while

Experiment I					
Early			Late		
PT Villa	PT Zapata	AZ Tapatío	Tlaxcala 62	FM M38	BY Criollo del Llano
Experiment II					
Pintos		Flor de Mayo		Blacks	
PTD-99036	PTD-99099	FMD-99121	FMD-99009	NGD-99048	NGD-99029
PTD-99004	PTD-99057	FMD-99018	FMD-99022	NGD-99040	NG Sahuatoba
PTD-9903	PTD-99108	FMD-99035	FMD-99001	NGD-99010	NG Altiplano
PTD-99015	PTD-99092	FMD-99004	FMD-99005	NGD-99039	NG Vizcaya
PTD-99008	PTD-99044	FMD-99034	FMD-99010	NGD-99005	NG Otomí
PTD-99002	PTD-99013	FMD-99002	FMD-99007	NGD-99025	NG 8025
PTD-99107	PTD-99100	FMD-99033	FMD-99012	NGD-99028	NG San Luis
PTD-99035	PTD-99068	FMD-99006	FM Sol	NGD-99011	
PTD-99043	PT Villa	FMD-99005	FM 2000	NGD-99004	
PTD-99014	PT Mestizo	FMD-99008	FM M38	NGD-99044	
PTD-99046	PT Bayacora	FMD-99011	FJ Marcela	NGD-99023	
PTD-99034	PT Zapata	FMD-99013	FM Nura	NGD-99012	
PTD-99045		FMD-99019		NGD-99030	
Experiment III					
RAB-608	RJB	SEA 17	SEA 23	G 40068	Tío Canela 75
RAB-609	RAB-632	SEA 18	INB 35	G 40159	DOR 390
RAB-618	RAB-650	SEA 19	INB 36	G 21212	PT Villa
RAB-636	RAB-651	SEA 20	INB 37	G 1977	Apetito
RAB-619	SEA 15	SEA 21	INB 38	SEA 5	FM Sol
RAB-620	SEA16	SEA 22	INB 39	BAT 477	FM 2000
Irrigated-Rainfed Experiment					
G 17427	G 13637	G 842	97RS110	PT Zapata	AZ Namiquipa
G 14645	G 19012	G 4258	DON35	MD 23–24	ICA Quimbaya
G 22923	G 19953A	G 801	DON38	VAX 2	NG Veracruz
G 1836	G 2774	G 18147	BY San Luis	SEA 5	Black Jack
G 17666	G 16054	G 4364	97RS101	TLP 19	NG Huasteco 81
G 1354	G 16054	G 3386	NG Durango	MC 6	
G 6762	G 21137	G 1977	NG 8025	B98111	
G 1688	G 2846	G 3107	G 4523	NG INIFAP	
G 14538	G 847	SEA10	PT Villa		

Prefix indicates seed coat color or commercial type: PT = 'Pinto', AZ = 'Azufrado' (Yellow), FM = 'Flor de Mayo', BY = 'Bayo' (Cream or beige), FJ = 'Flor de Junio' and NG = Black.

Table 1. Germplasm included on field experiments at Sandovales and Chapingo, México.

values of 4–9 indicated susceptibility. Plants were analyzed at laboratory in order to ratify the infection by FSP [11]. Days to flowering and to maturity were registered in each experimental unit in all experiments, and grain yield (kg h^{-1}) registered after physiological maturity.

Data were subjected to analysis of variance (ANOVA). When ANOVA detected significant ($P < 0.05$) differences among treatments, Tukey significant difference values (Tukey LSD, $P = 0.05$) were calculated for mean comparisons. Statistical analysis was performed using Statistical Analysis System version 6.12 and Statistica version 6.0 for Windows.

2.3. Genetic basis of resistance to root rot pathogens in selected common bean cultivars

We selected two common bean genotypes based on their contrasting reaction to FSP under both controlled and field conditions: BAT 477 (resistant) and Pinto UI-114 (susceptible). Crosses between the two parents were carried out under greenhouse conditions at Chapingo, México during 2002. F_1 to F_8 seeds were obtained in successive sowings in different locations of México. Reactions to a highly virulent isolate of FSP were measured in $F_{9:10}$ recombinant inbred lines [3]. A genetic linkage map was built with genotypic data obtained with 30 + 3/+3 AFLP. QTLs associated with resistance to FSP were identified using R software ver. 2.10.1 [14, 15].

3. Results

3.1. Variability of FSP isolates from Aguascalientes, México

Nineteen isolates of *Fusarium* were obtained from different locations of Aguascalientes, although most of them were collected in Pabellón. Ten isolates were FSP and the other nine were *F. oxysporum* f. sp. *phaseoli* (FOP). As controls, isolates from Guanajuato, México and Veracruz were included. FSP and FOP isolates showed a great variability on morphology (**Figure 1**). Most of the isolates showed radial growth of colony, purple color of colony and variation on mycelial production and conidia size and shapes (**Table 2**). Most of the common bean cultivars were susceptible to most of FSP isolates, mainly those from Mesoamerican genetic race. AFLP molecular markers clearly separated FSP isolates from FOP isolates, but pathogenicity patterns were not associated with *Fusarium* species (**Table 3**) [11].

Significant differences were found in morphology, pathogenicity and AFLP genotype among isolates. Isolates from Veracruz, Guanajuato and Aguascalientes grew faster *in vitro* than those from México and showed the largest conidia. The most pathogenic isolates were from Aguascalientes and Mexico. Bean cultivars with Flor de Mayo (Jalisco race) and Pinto (Durango race) seed coat showed the highest frequencies of resistance to the most of FSP isolates (**Table 2**). Isolates from the State of Mexico were genetically different from the other isolates with genetic dissimilarity of >9% [4].

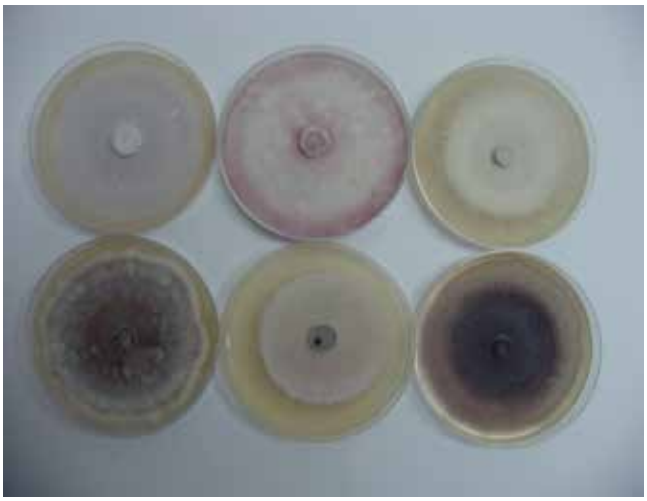

Figure 1. *In vitro* variation of *F. solani* f. sp. *phaseoli* isolates from Aguascalientes, México.

Isolate	Origin	Growth pattern	Color of colony	Conidia (μm)			Aerial mycelium
				Length	Width	L/W	
AGS01	Sta. Rosa	Radial	Pink	1.42	0.48	2.97	+
AGS02	Pabellón	"	Purple	1.49	0.55	2.72	—
AGS03	El Novillo	"	"	1.31	0.53	2.48	+
AGS04	Pabellón	"	"	0.73	0.33	2.20	—
AGS05	Sta. Rosa	"	White	1.32	0.40	3.31	+
AGS06	Pabellón	"	"	1.92	0.91	2.11	+
AGS07	Pabellón	"	Purple	1.22	0.34	3.62	+
AGS08	Sta.Rosa/Loreto	"	White	1.38	0.52	2.68	+
AGS09	´´	"	Dark purple	1.26	0.37	3.45	—
AGS10	Pabellón	Irregular	Pink	1.19	0.46	2.61	+
AGS11	Pabellón	Radial	Purple	1.43	0.34	4.19	+
AGS12	El Molino	"	Dark purple	2.41	0.64	3.81	—
AGS13	Pabellón	"	Purple	1.33	0.32	4.23	—
AGS14	Pabellón	"	White	1.20	0.45	2.67	+
AGS15	Pabellón	"	Pink	1.33	0.40	3.35	+
AGS16	La Luz	"	White	0.86	0.32	2.60	+
Mean				**1.48**	**0.46**	**2.89**	

Isolate	Origin	Growth pattern	Color of colony	Conidia (μm)			Aerial mycelium
				Length	Width	L/W	
VER01	Cotaxtla	Radial	White	1.94	0.72	2.69	+
GTO01	Irapuato	Radial	"	1.27	0.48	2.65	+
MEX01	Texcoco	Irregular	Yellow	1.28	0.47	2.72	+
Mean				**1.50**	**0.56**	**2.69**	

Table 2. Morphological *in vitro* characteristics of *F. solani* f. sp. *phaseoli* isolates from Aguascalientes, México.

3.2. Reactions of common bean germplasm to root rot pathogens under field conditions

Experiment I. The greatest root rot severity was found in Flor de Mayo M38, Pinto Zapata, and Azufrado Tapatío, while Pinto Villa, Tlaxcala 62 and Bayo Criollo del Llano showed the low severity. Tlaxcala 62, Bayo Criollo del Llano and Flor de Mayo M38 were more later than Pinto Villa, Azufrado Tapatío and Pinto Zapata. Pinto Villa, Azufrado Tapatío and Pinto Zapata exhibited the best agronomic characteristics (**Table 4**). A negative association between seed yield and root rot severity at vegetative and reproductive stages was found. Seed yield was negatively associated to days to flowering and days to maturity, while phenology was positively related to harvest index. Harvest index was found to be negatively associated to days to flowering and days to maturity. A positive relationship between root rot severity at vegetative and reproductive stage was found (**Table 5**).

Germplasm seed coat color/type (genotypes)	Genetic race	Resistance (%)	Susceptibility (%)
48 Mexican FSP isolates			
Flor de Mayo (FM Sol, FM Bajío, FM M38)	Jalisco	47	53
Pintos (PT Villa, PT Mestizo, PT Zapata)	Durango	47	53
Bayos (BY Zacatecas, BY Madero, BY Criollo del Llano)	Durango	29	71
Black/Yellow (NG Altiplano, NG Vizcaya, Tlaxcala 62)	Durango/ Mesoamérica/Jalisco	7	12
10 Aguascalientes FSP isolates			
Mesoamérica (BAT 477, TLP 19, SEQ 12, NG 8025, Río Tibagí)	Mesoamérica	32	78
Durango (BY Durango, PT Villa, PT UI-114)	Durango	13	87
Jalisco (BY Mecentral, AZ Tapatío)	Jalisco	10	90

Table 3. Resistance/susceptibility percentages in common bean germplasm classified by genetic races in response to inoculation with *F. solani* f. sp. *phaseoli* isolates.

Experiment	Classification	Genotype	Days to flowering	Days to maturity	Seed yield (kg h^{-1})	Root rot severity
I	Early	PT Villa	44	89	778	4.9
		PT Zapata	42	86	703	6.6
		AZ Tapatío	43	89	719	6.2
		Mean	**43**	**88**	**733**	**5.9**
	Late	FM M38	56	98	547	6.9
		Tlaxcala 62	56	109	597	5.0
		BY Criollo del Llano	56	100	546	5.0
		Mean	**56**	**102**	**563**	**5.6**
		Tukey (P = 0.05)	1	1	218	0.9
II	Pinto-Resistant	PTD-99057	50	97	782	3.5
		PTD-99092	36	88	739	3.5
		PTD-99004	46	96	871	3.6
		Mean	**44**	**94**	**797**	**3.5**
	Pinto-Susceptible	PT Mestizo	38	88	877	5.8
		PTD-99008	43	96	1056	5.8
		PT Zapata	36	88	705	5.7
		Mean	**41**	**91**	**879**	**5.8**
	Flor de Mayo-Resistant	FMD-99033	40	91	594	3.3
		FMD-99019	42	97	747	3.7
		FMD-99004	44	88	884	3.7
		Mean	**42**	**92**	**742**	**3.6**
	Flor de Mayo-Susceptible	FMD-99022	44	92	810	6.0
		FMD-99013	40	88	825	5.9
		FMD-99002	39	90	768	5.9
		Mean	**41**	**90**	**801**	**5.9**
	Black-Resistant	NG Otomí	43	98	1000	2.3
		NG 8025	54	96	855	2.4
		NGD-99023	50	97	1003	2.6
		Mean	**49**	**97**	**953**	**2.4**
	Black-Susceptible	NG Altiplano	51	97	848	3.9
		NGD-99040	46	100	1055	3.9
		NGD-99028	44	99	963	3.8
		Mean	**47**	**99**	**955**	**3.9**
		Tukey (P = 0.05)	3	2	290	1.5

Experiment	Classification	Genotype	Days to flowering	Days to maturity	Seed yield (kg h^{-1})	Root rot severity
III	Resistant	SEA 20	46	93	798	1.6
		RAB 636	43	91	689	2.6
		BAT 477	47	95	1052	2.6
		Mean	**45**	**93**	**846**	**2.3**
	Susceptible	FM 2000	47	101	862	9.0
		SEA 16	43	91	780	5.3
		SEA 15	43	91	688	4.9
		Mean	**44**	**94**	**777**	**6.4**
		Tukey (P = 0.05)	4	5	504	3

Table 4. Phenology, seed yield and root rot severity in common bean germplasm grown in Aguascalientes, México.

Characteristic	Root rot severity (56 d after sowing)	Days to flowering
Experiment I		
Seed yield	−0.25NS	−0.54**
Days to flowering	−0.17NS	
Experiment II		
Seed yield	−0.11NS	0.29**
Days to flowering	−0.10NS	
Experiment III		
Seed yield	−0.07NS	0.02NS
Days to flowering	−0.09NS	
Combined irrigated-rainfed experiment		
Seed yield	−0.25**	0.18**
Days to flowering	0.12*	

*($p \leq 0.05$); **($p \leq 0.01$).

Table 5. Pearson's correlation coefficients (r) among characteristics of common bean grown in experiments conducted at Sandovales and Chapingo, México.

Experiment II. No clear relationship between root rot severity and seed yield was found (**Figure 2a**; **Table 5**). Grain yields ranged from 500 to 1250 kg h^{-1}, but we found a clear differentiation among cultivars by reaction to root rots on the basis of seed coat color. Resistance was more frequent in black beans while intermediate reactions were found in Flor de Mayo germplasm and susceptibility was found in pinto beans. No differences on grain yield were detected between resistant and susceptible genotypes in any seed color type. Resistance was common in bred cultivars, as can be seen in pinto or Flor de Mayo bean types (**Table 4**).

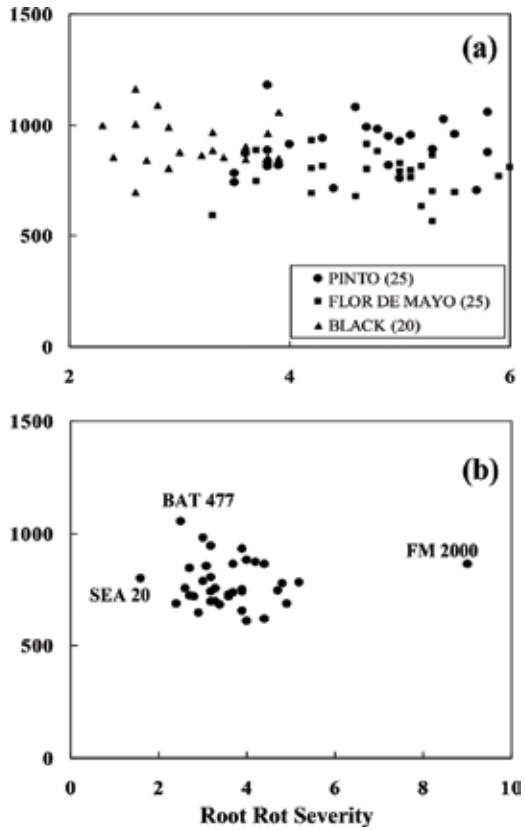

Figure 2. Relationship between root rot severity caused by *F. solani* f. sp. *phaseoli* and grain yield in common beans: (a) experiment II and (b) experiment III.

Experiment III. As found in E-II, no clear relationship between root rot severity and seed yield was detected in this experiment (**Figure 2b**; **Table 5**). All 36 genotypes showed grain yields ranged from 550 to 1100 kg h^{-1}. Here, we found that most of germplasm showed a root rot severity ranged from 2 to 5.5 (intermediate), while grain yield ranged from 550 to 1000 hg h^{-1}. Only three cultivars were clearly different from all other cultivars: BAT 477 (that showed the highest seed yields), SEA 20 (that exhibited the lowest root rot severity) and Flor de Mayo 2000 (that showed the highest root rot severity). No differences can be appreciated on days to flowering or days to maturity or seed yield between resistant and susceptible cultivars (**Table 4**).

Rainfed-irrigated experiment. In Chapingo, germplasm showed later biological cycle and greater seed yields and root rot severity than at Sandovales. In both locations, rainfed conditions reduced seed yields and increased root rot severity (**Table 6**). In this experiment, negative relationship between seed yield and root rot severity was more clear than other experiments and a positive association was found between seed yield and days to flowering and flowering and root rot severity (**Table 5**). The relationship between root rot severity and grain yield exhibited different patterns across locations. In Sandovales, we found a greater variation on root rot severity on the germplasm, while an opposite response was found at Chapingo.

	Days to flowering	Seed yield (kg ha^{-1})	Root rot severity (56 d after sowing)
Experiment Sandovales			
Rainfed	49	622	5.7
Irrigated	48	1141	4.6
Experiment Chapingo			
Rainfed	53	940	6.3
Irrigated	54	1730	5.4
Tukey (P = 0.05)	1	109	0.7
Resistant genotypes			
G 2494	53	982	4.0
G 4258	44	1015	4.1
97RS101	52	1273	4.3
NG 8025	54	1303	4.6
PT Zapata	44	1303	4.6
Mean	**49**	**1175**	**4.3**
Susceptible genotypes			
G 801	52	947	8.1
SEA 5	51	588	7.7
G 14538	58	653	7.1
G 4523	48	1094	7.0
G 14645	46	763	6.7
Mean	**51**	**809**	**7.3**
Tukey (P = 0.05)	2	556	2.4

Table 6. Agronomical characteristics on common bean germplasm under rainfed-irrigated conditions in two locations of México.

An opposite pattern was found in grain yield because higher seed yields were found at Chapingo (50–1880 kg h^{-1} under rainfed conditions and 230–3300 kg h^{-1} under irrigated conditions) than Sandovales (150–1150 under rainfed and 500–2300 kg h^{-1} under irrigated conditions) (**Figure 3a** and **b**). No differences on days to flowering are detected between the two groups of genotypes, but resistant germplasm exhibited greater seed yields than susceptible cultivars (**Table 4**).

3.3. Genetic basis of resistance to root rot pathogens in selected common bean cultivars

Genetic analysis identified one QTL significantly associated with resistance to FSP in BAT 477 growing under controlled conditions. This QTL explained 2.7% of variation in response to the disease and the marker was found at LG 5 [16].

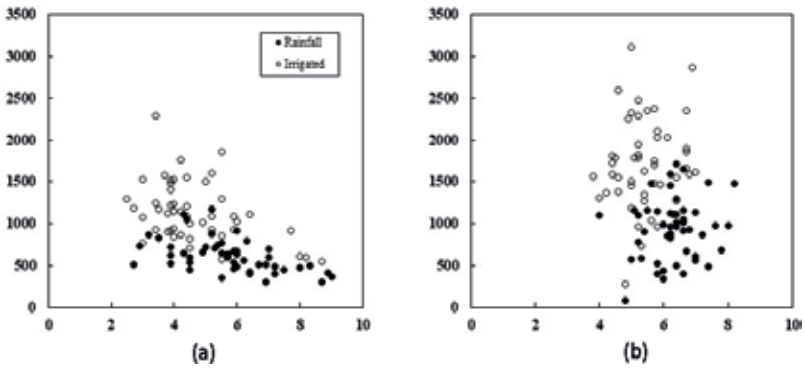

Figure 3. Relationship between root rot severity caused by *F. solani* f. sp. *phaseoli* and grain yield in 49 common bean genotypes under two soil moisture regimes: (a) Sandovales, Aguascalientes and (b) Chapingo, State of México, México.

4. Discussion

4.1. Variability of *Fusarium solani* f. sp. *phaseoli* from Aguascalientes, México

A high morphologic, pathogenic and genetic variability was found in FSP isolates from Aguascalientes, despite the identical host (common beans) and geographical origin. In addition, no relationship among morphology, pathogenicity and genotype was found. Our results indicated the high values of genetic variability in the species due to the presence of heterokaryosis and parasexualism as genetic exchange mechanisms between vegetative compatible isolates. Single members of the same vegetative compatibility group (VCGs) are genetically similar and they are related on basis of genetic lineages [17]. The characterization of VCGs on *Fusarium* isolates from Aguascalientes and other regions of México could clarify the association among *Fusarium* populations and genetic lineages. This research confirmed the diverse and heterogeneous nature on the genus. Host specialization could be useful to establish artificial taxonomic divisions and to perform pathogenic groups and *formae speciales*. However, the host plays an important biological role in selection pressure to the fungus. In addition, the genetic exchange between isolates is supported by the development of VCGs or other strategies for DNA transmission. The evolution of pathogenicity and VCGs contribute to increase in molecular variability. Further research that includes traditional and molecular methodologies will improve the knowledge and understanding of *Fusarium* biodiversity.

The most of common bean cultivars were susceptible to most of FSP isolates, and all isolates were pathogenic to common beans. This result is opposite to Cramer et al. [18]. Most of the resistant germplasm belonged to Mesoamerica of Jalisco genetic races, while susceptible cultivars are classified as Durango race. High frequencies of resistance to other root rot pathogen (*Macrophomina phaseolina*) of common beans were found in Mesoamerican beans [19].

Results suggest that resistance to root rot pathogens in common beans could be operating as a resistance gene cluster that controls similar strategies to defend roots and stems against root rot fungi. Further research could confirm this suggestion. No clear association between host and fungus genotypes was found; this relation was reported in *M. phaseolina*-common beans [20] in contrast with other biotrophic pathogens of common bean as *Colletotrichum lindemuthianum* [21], where a clear formation of genetic lineages based on geographical origin was found.

4.2. Reactions of common bean germplasm to *F. solani* f. sp. *phaseoli* under field conditions

A high variation on reactions to FSP was found in both locations and no immunity was found, while no immunity to root rot pathogens in common bean germplasm was detected previously [8, 22, 23] in Pabellón de Arteaga, Aguascalientes and Chapingo, State of México. No clear association between root rot severity and seed yield or phenology was found in all experiments. However, results indicated that resistance to FSP was more frequent on black beans, while susceptibility was common on pinto beans, which has been found in previous works [20, 22]. Results suggest that black beans from Mesoamerican race could provide resistance to FSP in México. Under rainfed conditions, genotypes as BAT 477 and SEA 20 stood out for their high seed yields and resistance to root rot pathogens. BAT 477 has showed a consistent resistance to root rot pathogens such as *Fusarium*, *Rhizoctonia and Macrophomina* [20, 22, 24].

In both locations, rainfed conditions reduced seed yields and increased root rot severity in common bean germplasm. Navarrete-Maya et al. [23] reported a positive relationship between rain precipitation and *Fusarium* severity in Chapingo. We suggest that low water availability increased physiological stress in the host. Therefore, host defense mechanisms are not efficient to arrest fungal infection or for slow pathogenesis. The relationship between root rot severity and grain yield exhibited different patterns, since a broad variation on root rot severity on the germplasm (Sandovales) or an opposite response (Chapingo). Opposite patterns in grain yields were found, the highest seed yields were found in Chapingo and the lowest in Sandovales. Our data suggested that climate and fungi conditions of Sandovales are more appropriate for common bean germplasm screening for resistance to root rot pathogens under field conditions than Chapingo.

4.3. Genetics of resistance to *Fusarium solani* f. sp. *phaseoli* in common bean cv. BAT 477

Genetic analysis identified one QTL significantly associated with resistance to FSP in BAT 477 growing under controlled conditions. This QTL explained 2.7% of variation in response to the disease and the marker was found at LG 5 [16]. Identification of few QTLs with high effects on explanation of phenotypic variation is important and promising to simplify the

introgression of resistance genes to susceptible germplasm. However, our results indicated a low genetic effect of the QTL detected in BAT 477. Therefore, a more intensive searching of significant QTLs is needed. Genetic map development allows identification and use of genes and genomic regions (QTLs) with economic interest and then develops marker-assisted selection (MAS) strategies [25]. Vallejos et al. [26] performed the first gene map of common beans using morphologic, isozymes and RFLP markers. Then, Schneider et al. [27] identified 16 QTLs associated with *F. solani* f. sp. *phaseoli* resistance in $F_{4:6}$ RILs derived from Montcalm (susceptible) x FR266 (resistant). These QTLs were mainly found on LGs 2 and 5, and seven QTLs explained 64% of disease resistance. Chowdhury et al. [28] identified two QTLs associated with FSP resistance and explaining 50% of phenotypic variance using $F_{2:6}$ RILs from AC Compass (susceptible) × NY2114–12 (resistant), while Román-Avilés et al. [29] identified nine QTLs associated with resistance to FSP in $F_{4:5}$ inbred backcross populations from Red Hawk (susceptible) × NG San Luis (Resistant) and C97407 (susceptible) × NG San Luis, which explained from 5 to 53% of phenotype variation and located mainly at LGs 1 and 7.

5. Conclusions

We found significant genetic variability in FSP isolates from Aguascalientes and other regions of México although no clear association among morphology, pathogenicity or AFLP genotype was detected.

Under field conditions, we found high variation on reactions to FSP root rots; resistance was more frequent on black seed-coated beans, while susceptibility was common in pinto beans. We found a greater variation on root rot severity disease in Aguascalientes when compared with State of México, while an opposite response on grain yields was found across locations.

One QTL with low variance explanation of FSP resistance in BAT 477 was found; therefore, more intensive searching of significant QTLs is needed to improve marker-assisted selection strategies in common beans for México.

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Chapter 8

Fusarium Wilt: A Killer Disease of Lentil

Neha Tiwari, Seid Ahmed and Ashutosh Sarker

Additional information is available at the end of the chapter

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Abstract

Lentil (*Lens culinaris* Medikus subsp. *culinaris*) is an important dietary source of protein and other essential nutrients in South and West Asia, North and East Africa. Lentil crops are vulnerable to a number of diseases caused by fungi, viruses, nematodes, insect pests, parasitic plants and abiotic stresses. Among them, the most significant and serious soil-borne disease is Fusarium wilt (*Fusarium oxysporum* f.sp. *lentis*: *Fol*). Fusarium wilt causes yield loss up to 50% in farmers' fields. The pathogen showed high levels of phenotypic and genotypic diversity in India, Algeria, Syria and Iran. The disease thrives at 22–25°C temperature and affect lentil either at seedling and vegetative or the reproductive stages of the crop. To minimize yield losses, an integrated management strategy comprising resistant/partial resistant cultivars, adjusting sowing time, bio-control and chemical seed treatments is the best approach to reduce the incidence of the Fusarium wilt of lentil. This review covers past achievements in managing the disease, pathogen diversity and identify gaps in managing Fusarium wilt to improve productivity and production of the crop.

Keywords: lentil, *Fusarium oxysporum* f.sp. *lentis*, Fusarium wilt, disease management

1. Introduction

Lentil (*Lens culinaris* Medikus subsp. *culinaris*) is a cool season, diploid (2n = 2X = 14) self-pollinating grain legume with genome size of approximately 4 Gbp [1]. It is an ancient crop originated in the Near East and after that rapidly spread all through the Mediterranean Basin, Central Asia and later to the New World including Latin America. It is one of the oldest grain legumes domesticated about 10,000 years ago [2–4]. Production and consumption of lentil involve more than 100 countries. The total world lentil production is about 4.8 million tons from an estimated area of 4.5 million ha with an average yield of 0.11 t/ha [5]. The cultivated *L. culinaris* sub spp. *culinaris* includes two physio-morphological cultivated lentil types: small

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seeded (microsperma) and large seeded (macrosperma) [6]. Lentil is recognized as one of the most nutritious pulse crops ranking next to chickpea among cool-season food legumes. It contains 57–60% carbohydrate, 24–26% protein, 3.2% and 1.3% fiber. It is also a rich source of minerals containing calcium (69 mg per 100 g), phosphorus (300 mg per 100 g) and Iron (7 mg per 100 g) of seed [7, 8]. Lentil seed contains lysine, an essential amino acid, found only at low levels in cereal protein. Lentil is a valuable human food, mostly consumed as dry seeds as well as used as fodder, and generally grown as a crop rotation after cereals to enrich the soil by their nitrogen fixing ability [9]. In South East Asia, lentil mostly grows on residual soil moisture after post rainy season under rainfed conditions. The inclusion of lentil as a crop rotation can benefits the succeeding crops by improving the soil health through biological nitrogen fixation and carbon sequestration. The amount of nitrogen fixed by plants varies from 0 to 192 kg total N/ha around a mean of 80 kg total N/ha [10]. This estimate of N fixation is similar to the quantities fixed by chickpea and dry bean.

Since 1970s there have been significant achievements in national and international lentil programs in developing phenologically adapted, stress resistant and high-yielding cultivars [11]. During the past three decades, different national agricultural systems released more than 90 improved cultivars from germplasm developed by the International Centre for Agricultural Research in the Dry Areas (ICARDA) [11]. Therefore, the current review covers past achievements in managing the disease, pathogen diversity and identify gaps in managing Fusarium wilt to improve productivity and production of the crop.

2. Production and constraints

In the global lentil scenario, India ranked first in the area and second in the production with 39% and 22% of world area and production respectively. Canada ranking first in production (41.2%). The highest yield is recorded in Croatia (0.3 tons per ha) followed by New Zealand (0.25 tons per ha) [5] (**Table 1**).

In many countries, lentil is cultivated as a rainfed crop and affected by several biotic (fungi, viruses, nematodes, insect pests and parasitic plants) and abiotic stresses (terminal drought, heat stress, cold, waterlogging and low soil fertility). Biotic stresses caused by pathogenic fungi include Fusarium wilt (*Fusarium oxysporum* f.sp. *lentis: Fol*), Ascochyta blight (*Ascochyta lentis*), Anthracnose (*Colletotrichum truncatum*), Stemphylium blight (*Stemphylium botryosum*), Rust (*Uromyces viciae-fabae*), Collar rot (*Sclerotiun rolfsii*), Root rot (*Rhizoctonia solani*), and Botrytis gray mold (*Botrytis cinerea*) [12, 13]. It is also known that lentil is susceptible to several species of *Orobanche* and *Phelipanche* prevalent in the Mediterranean region [14]. Till now, only *F. oxysporum* f.sp. *lentis* has been reported as the cause of Fusarium wilt of lentil but recently *F. redolens* was found associated with lentil wilt in Italy [15].

Among the biotic stresses, Fusarium wilt is a serious disease in reducing lentil yield in India, West Asia, North Africa and East Africa [16]. Fusarium wilt can cause yield losses up to 50% of the production to complete yield loss if severely affected. The disease appears at seedling stage (early wilt) or during the reproductive stage (late wilt) [17, 18]. The pathogen can survive in the soil as chlamydospores which can remain viable for many years [19] making crop rotation as a control option ineffective.

Rank	Area			Production			Yield	
	Country	Area	% to world	Country	Prod	% to world	Country	Yield
1	India	1.80	39.8%	Canada	1.99	41.2%	Croatia	0.286
2	Canada	1.22	26.9%	India	1.10	22.8%	New Zealand	0.247
3	Turkey	0.24	5.4%	Turkey	0.35	7.1%	Armenia	0.226
4	Nepal	0.21	4.6%	Australia	0.24	4.9%	China	0.208
5	Iran	0.17	3.7%	Nepal	0.23	4.7%	Egypt	0.206
6	Australia	0.16	3.6%	Bangladesh	0.16	3.3%	Canada	0.163
7	Bangladesh	0.12	2.8%	USA	0.16	3.2%	Iraq	0.157
8	Syria	0.11	2.5%	Ethiopia	0.14	2.8%	USA	0.149
9	U.S.A	0.10	2.3%	China	0.13	2.6%	Australia	0.147
10	Ethiopia	0.10	2.2%	Iran	0.08	1.8%	Lebanon	0.146
	World	4.52		World	4.83		World	0.107

Source: FAOSTAT 2015
http://faostat3.fao.org/home/index.html

Table 1. Global ranking in area, production and yield: (area—million hectare, production—million tons, yield—tons/hectare).

Fusarium wilt epidemics depend on crop stages (seedling or adult flowering), environment and crop variety [20, 21]. Fusarium wilt is part of a disease complex under field conditions. In India, 12 fungal pathogens were identified where *Fol* is the dominant pathogen (30.8%), followed by *Rhizoctonia bataticola* (17.5%) and *Sclerotium rolfsii* (15.7%) [22]. The prevalence of wilt-root rot complex and their associated pathogens were reported by Chaudhary et al. (2010) from India and the main pathogens associated with plant mortality were *Fusarium oxysporum* f.sp. *lentis* (62.0%), *Rhizoctonia bataticola* (25.2%) and *Sclerotium rolfsii* (9.8%) [23]. In India, under natural conditions wilt incidence can reach 50–78% [21, 24] and cause up to 100% yield loss if the crop is affected at the seedling stage [17]. In Czechoslovakia yield losses can reach as high as 70% [25]. In South and Northwest Syria disease incidence can reach up to 29% [26–29]. Moreover, field experiments indicated that the percentage seed yield loss per unit change in wilt incidence was 0.89 [30]. The disease incidence due to lentil wilt in Pakistan was recorded as 5–10% and may result in 100% crop loss under favorable conditions [31, 32]. Recently, the first report of *Fusarium nygamai* causing wilt disease on lentil in Pakistan is also reported [33]. Presence of the nematode significantly increased Fusarium wilt incidence. It causes significant reduction in shoot length, root length and nodulation in both susceptible and resistant cultivars [34].

3. Fusarium wilt pathogen

Fusarium oxysporum is a pathogenic fungus commonly found around the world. It is a soil borne ascomycete causing Fusarium wilt, on many economically important crops. The pathogen comprises of over 120 known strains and each of which is specific to unique host plant in which it causes disease. *F. oxysporum* strains infect and kills many commercially harvested crops and

legumes. The spores of *F. oxysporum* survive in a dormant stage in the soil for several years and are easily spread in water, it can infect vegetative cuttings, and can transmit to other individuals. Scientists around the world proposed developing *F. oxysporum* as a universal model for the understanding of fungal virulence [35]. *Fusarium oxysporum* infects its host by entering through the root and grows in the plant xylem. It blocks the vascular system and prevents transport of water and nutrients to the plant that causes wilting, discoloration, and eventually death of the plant.

The pathogen Fol affecting lentil crop was first reported from Hungary [36] and later from many countries including India [37], USA [38], Czechoslovakia [39], USSR [40], Brazil [41], France [42], Argentina [43], Bangladesh [44], Turkey [45], Syria [26, 46], Myanmar and Pakistan [47], Nepal [48], Ethiopia [49], Egypt [50], Italy [51]. In India, Fusarium wilt is known to limit the production of lentil in the states of Uttar Pradesh, Madhya Pradesh, Himachal Pradesh, Bihar, West Bengal, Assam, Rajasthan, Haryana and Punjab [22].

Wilt appears in the field as patches at both seedling i.e. early wilting and adult stages i.e. late wilting. Early wilting is characterized by sudden drooping and drying of leaves and seedling death (**Figure 1**). The roots are healthy but having reduced proliferation and nodulation and no internal discoloration of the vascular system. Late wilting appears from flowering to late pod-filling stage and sudden drooping of top leaflets of the affected plant and dull green foliage followed by wilting of the whole plant or in the individual branches [45]. The pod filling stage of the plant is severely affected and eventually a huge yield loss occurs. The disease thrives at 22–25°C temperature, with warm and dry soil conditions [52].

A culture of *Fol* display hyaline, septate and much branched mycelium. On media the growth pattern varies from fluffy to appressed and also vary in color from no color to pink. The pathogen is known to produce three kinds of asexual spores; micro conidia, macro conidia and chlamydospores [53]. Microconidia are usually single celled, ovoid or kidney-shaped and hyaline. Macroconidia are usually two to seven celled, long with pointed apical cell and notched basal cell. Chlamydospores are single celled, oval or spherical shaped and thick walled, formed singly in macroconidia or apical or intercalary in the hyphae [53]. In laboratory, the culturing of infected plant tissue should be done with caution because other saprophytic *Fusarium* spp. may be present that appears similar to *Fol*.

Figure 1. Lentil wilt disease: (a) lentil plants infected by wilt disease in field; (b) cross section showing internal discoloration of tap root in wilted lentil plant; (c) Fusarium wilt symptoms on artificial inoculated lentil plants.

4. Pathogen diversity

The pathogen can interact with specific host which results in breakdown of plant resistance within very short duration of time [54]. Therefore, it is important to determine the pathogen genetic diversity in *Fol* population, which can be used by plant breeders for disease resistance and can also help in studying its epidemiology, taxonomy, and detection [55]. The amount of genetic variation can be evaluated by molecular markers techniques like Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR) and Inter-Simple Sequence Repeat (ISSR).

The genetic diversity of *Fol* was studied by different researchers in many countries. Pouralibaba (2005) has studied the pathogenic diversity based on growth media (Czapecs Agar, PDA and Lentil Extract Agar) and a set of host differentials (ILL590, Gachsaran and Moghan lentil genotypes) using 13 isolates collected from Iran and Syria. The results showed that the difference among pathogen population was not related to different aggressiveness properties with no virulence patterns [56]. In other studies, Iranian isolates of *Fol* were grouped into 10 using RAPD markers and to six groups using ISSR markers [57]. In a recent study, 101 *Fol* isolates from five countries in Ilam provinces of western showed low level of genetic variability using Simple Sequence Repeat (SSR) markers [58]. In Algeria, all isolates were in one Vegetative Compatibility Group [59].

In India, 43 cultural and morphological groups were grouped into three clusters based on their aggressiveness of lentil genotypes [60]. Datta et al. (2011) showed varying degree of genetic diversity ranging from 54% in case of RAPD to up to 35% with ITS markers of *Fol* collected from different agro-ecologies in India where isolates from north region fall in same cluster, whereas isolates from north east regions and eastern region fall in different group [61]. In Syria, three major groups of *Fol* were identified using RAPD, SSR and ISSR markers [62].

5. Race concept in *Fusarium oxysporum* f.sp. *lentis*

In order to devise strategies for conferring resistance against disease, it is important to have knowledge of pathogen variability and prevalence of particular races in the target environment. The pathogen populations are primarily characterized by its virulence analysis on cultivars carrying differential resistance genes. Many researchers have studied the pathogen variability based on their grown on different solid media and on the basis of their pathogenicity [63, 64]. Later, Pandya et al. (1980) has evaluated the line (Pant-406) against seven races proposed by Kannaiyan and Nene [64], and found it immune to race 5, resistant against races 3 and 6, and partially resistant against race 4 [65].

Belabid et al. (2004) has reported that all the 32 Algerian isolates of *Fol* under study represent a single race but differ in their aggressiveness on the susceptible line on the basis of virulence and vegetative compatibility [66]. In India, on the basis of disease reactions against seven lentil differentials, the isolates were grouped into three clusters [60]. In an another study based on genetic variability, the *Fol* isolates collected from north eastern Indo-Gangetic plains revealed two sub-populations groups [61]. Sallam and Abdel-Monaim (2012) have collected 10 isolates

of *Fol* from different locations at Minia, Assuit and New Valley governorates, which were varied in their virulence [67].

Altaf et al. (2014) have characterized 15 *Fol* isolates (Fol-1 to Fol-15) collected from nine district of Pakistan on the basis of their pathogenicity and morphology [68]. Recently, Pouralibaba et al. (2016, 2017) have identify seven pathotypes (1–7) of *Fol* on the basis of their different pattern of virulence on lentil genotypes. Fifty-two *Fol* isolates originated from Iran, Syria and Algeria were used in the study. The results suggest that the pathogen 7 was virulent on all the accessions under study and there was no correlation found between the pathotype and the geographical origin of the isolates. The study was further confirmed by analyzing histopathology pattern of infection on resistant/susceptible varieties by pathotypes 1 and 7, which suggests that lower disease index was measured with plants inoculated with pathotypes 1 but not with pathotype 2 [69, 70]. Further studies are required to identify region specific pathogenic races using differential lines for conferring resistance against them in the respective agro-climatic regions.

6. Host ranges

Due to the presence of high mutations and variations among the pathogen populations limit the effectiveness of natural resistance in the host plants against the pathogens [71]. Therefore, it is important to access the variability among the pathogen and regarding its host resistance for a successful breeding program. It is also important to replace the low yielding genotypes and disease susceptible varieties with those of high yielding and disease resistance ones.

The forma specialis of lentil has a very limited host range and can induce disease of lentil only under natural conditions. Khare (1980) and Taheri et al. (2010) studied the host range of *Fol* by inoculating it on plants such as cowpea, french bean, bengal gram, lathyrus, mungbean, urdbean, pea, soybean, tomato and eggplant (Solanaceae), melon (Cucurbitaceae) or red gram which results in no infection [53, 72]. Recent host range studies on soybean, chickpea and tomato did not result to infection [73].

7. Fusarium wilt management options

Different Fusarium wilt management are used by lentil growers in different countries. These include cultural, biological, chemical, host plant resistance and an integration of two or more control options.

7.1. Chemical control

Several fungicides have been tested against the *Fol* in different parts of the world. The study reveals that the systemic fungicides found to be superior to non-systemic fungicides in inhibiting the fungal mycelial growth in plates as well as in pot seed treatment. Benomyl (76.6%) showed the most positive results against the pathogen followed by thiophanate methyl (73.0%) whereas non-systemic fungicides viz. captan (67.8%) and dithane M-45 (62.3%) were the least efficient in reducing the fungal growth compared to the systemic fungicides [74]. On the other hand, Kasyap et al. (2008) has found much reduced fungal growth with captan (88.3%) [75].

Maheshwari et al. (2008) tested the effect of seven fungitoxicants against *Fol*. The results suggest that carbendazim was the most effective (5.6 mm) followed by captan (9.9 mm) and hexaconazole and diniconazole for reducing the fungal growth [76]. Several studies were carried out for determining the concentration of the fungicides to control the growth. The results suggest that the best fungus control was observed at highest fungicidal concentration (100 ppm) with benomyl followed by thiophanate methyl, second most effective at 100 ppm concentration [74, 77]. In Syria, seed treatment with benomyl-thiram did not affect Fusarium wilt incidence [78].

7.2. Biological control

Biological control is known to be the best and effective method, against soil-borne pathogens. This method has many advantages such as environment friendly, cost effective and extended plant protection. Many fungal and bacterial species like *Pseudomonas*, *Trichoderma* and *Streptomyces* have antagonistic effect on Fusarium wilt of lentil. Among them *Trichoderma* species are been extensively used as bio-control agent against soil and seed-borne diseases [74]. A study revealed that the seed treatment with *Gliocladium virens* + *P. fluorescens* + carboxin and *Bacillus subtilis* + carboxin + *T. harzianum*/*T. viride*/*G. virens* have been found more effective in controlling Fusarium wilt incidence in lentil [79, 80]. In the recent study, two species of *Trichoderma* were employed against highly virulent isolate of *Fusarium* responsible for lentil wilt. The results revealed that *T. harzianum* was highly effective in controlling wilt disease in comparison to other isolate, when applied as a soil drench [74].

In an experiment conducted by Garkoti et al. (2013) observed significant reduction in disease incidence and maximum grain yield in field trials using 'Pant L-639' a popular cultivar against lentil wilt with *T. harzianum* + *Pseudomonas fluorescence* [81]. Similarly, in another report the result suggest that the disease severity was reduced with increased plant height with the combination of *T. harzianum* + *S. vermifera* [82]. Likewise, El-Hassan and Gowen (2006) has evaluated the formulation and delivery of the bacterial antagonist *Bacillus subtilis* against Fusarium wilt of lentil. The result reveal that the seed treatments with formulations of *B. subtilis* on glucose, talc and peat significantly enhanced its biocontrol activity against *Fusarium* compared with a treatment in which spores were applied directly to seed [83]. Additionally, several studies have also proved the importance of the organic material in reducing the disease incidence caused by plant pathogen like bacteria [84], fungi [85] and nematode [86] species.

7.3. Cultural practices

The cultural control generally depends on date and depth of sowing and manipulation of agronomic practices [68, 87]. It is reported that delay sowing usually lowers the wilt incidence whereas compared with early sowing (end of July), late sowing resulted in low yield [88]. The most suitable dates vary according to the different production regions. Use of clean seed for sowing and use of fungicidal seed treatment can reduce contaminating inoculum sources. To prevent the crop from various diseases a proper depth (10–12 cm) of seed planting should be used [89]. Intercropping/mixed cropping is being suggested for reduced wilt incidence and increased crop yield. Haware (1982) suggested that deep ploughing and removal of infected trash can reduce inoculum levels of Fusarium wilt of chickpea [90]. Soil solarization is another way to minimize the disease incidence [91]. In order to control the lentil wilt pathogen, chemical amendments (Mn and Zn) and foliar application on lentil wilt is also recommended. The

study suggests that the application of Zn and Mn salts at 80 ppm concentration on presoaked seeds of lentil has shown promising results on the control of wilt disease [92].

7.4. Fusarium wilt resistant cultivars

The initial step in managing the disease is to develop a reliable and reproducible disease screening techniques, so that a large number of germplasm (cultivated and wild relatives) can be evaluated in wilt sick plot and in greenhouse. The varietal resistance is a major goal of lentil improvement programme currently running at the International Centre for Agricultural Research in the Dry Areas (ICARDA). In order to identify the resistant variety of Fusarium wilt, screening under field and controlled conditions (green house and laboratory conditions) has been suggested [93, 94]. The systematic utilization of resistant source for wilt from cultivated accessions such as 'ILL 5883', 'ILL 5588', 'ILL 4400' and 'ILL 590' has resulted in the development of a wide spectrum of Fusarium wilt resistant varieties at ICARDA. Some of the prominent wilt resistant varieties in Syria ('Idleb 2', 'Idleb 3', 'Idleb 4' and 'Ebla 1'), Lebanon ('Talya 2', 'Rachayya' and 'Hala'), Turkey ('Firat 87' and 'Syran 96'), Ethiopia ('Adaa', 'Alemaya', 'Assano', 'Alemtena' and 'Teshale'), Iran ('Kimiya') and Iraq ('IPA 98') [95]. In India, several wilt resistant varieties are released such as 'L 4147', 'Pant L 406', 'Pant L 4', 'Pant L 639', 'Priya', 'Seri', 'JL 3', 'Noori', and 'VL 507' under national program [65, 96, 97].

The lentil germplasm can be screened under natural condition with natural inoculum of *Fol* in field. Wilt sick plot (WSP) is the most common method used to screen disease resistant plants under natural conditions. The WSPs have been developed by ICARDA, and NARS. The advantage of this method is that, large number of genotypes can be screened. Bayaa et al. (1997) has screened a core collection of 577 lentil germplasm accessions from 33 countries. The result reveals that the most resistant accessions came from Chile, Egypt, India, Iran and Romania and also emphasize the relative uniformity of disease pressure in WSPs [19].

Different inoculation methods have been used to for the infectivity of wilt in chickpea but in lentil very limited work has been conducted [12, 98, 99]. The inoculum density of about 10^6 conidia ml^{-1} is generally been used to establish the pathogen [100]. Wild species are an invaluable source for disease resistance. The wild germplasm of lentil was evaluated for resistance against biotic and abiotic stresses was done at ICARDA [101]. The crosses were made between the wild lentil (*L. culinaris* ssp. *orientalis*) and the cultigen has resulted in high-yielding selections under dryland conditions. Similarly, another study was done to screen the 221 accessions representing five species/subspecies, showed resistance in ILWL 113 (*L. culinaris* ssp. *orientalis*) from Turkey and ILWL 138 (*L. ervoides*) from Syria [102]. In India, seventy accessions representing four wild species/sub-species were evaluated and the donors for *Fol* resistance were identified in all species. The wild accessions of lentil (one of *L. culinaris* ssp. *orientalis* (ILWL76), five of *L. odemensis* (ILWLs 35, 39, 153, 237, 300), eleven of *L. ervoides* (ILWLs 40, 41, 42, 133, 204, 251, 258, 261, 271, 280 and 299) and six of *L. nigricans* (ILWLs 22, 26, 31, 37, 38, 430) can provide an important source of alien genes for disease resistance [103].

7.5. Genetic of Fusarium wilt resistance

The most economical means to control the Fusarium wilt of lentil is through the development of resistant varieties [12]. Due to the evolution of new races and co-existence of more than one

pathotypes, it is difficult to develop the resistant cultivars. Hence, the knowledge of about the inheritance and genetics of wilt resistance is important to develop resistant or moderately resistant cultivars. The studies in genetics of resistance to Fusarium wilt will eventually help to produce more resistant lentil cultivars [104]. Resistant or moderately resistant lentil cultivars (OPL 58, DPL 61 and DPL 62) significantly reduced wilt incidence and severity of root rot, and increase grain yield [31]. Very limited studies are available on the genetics and inheritance pattern of wilt resistance in lentil. Kamboj et al. (1990) has reported five independent genes to confer resistance to Fusarium wilt in lentil [105]. Eujayl et al. (1998) has also recorded the monogenic inheritance in 'ILL 5588' for wilt resistance and designated the gene as Fw. A study based on allelism test, identified 2 genes each of duplicate genes and complementary genes imparting resistance in the variety PL 234, JL 446 and PL 286, respectively [104]. However, Abbas (1995) has reported that only one dominant gene is controlling the wilt resistance found in the crosses made at ICARDA [106].

Stevenson et al. (1995) has explained that the plant root exudates and the difference in resistance in genotypes depend on the amount of root exudates and their antifungal compound [107]. Another study reveals that the root exudates release considerable amounts of organic substance in soil including the amino acids and sugars and the amino acid (Glycine and phenylalanine) were found to have an inhibitory effect upon the spore germination of pathogen [108]. Iftikhar et al. (2005) analyzed the presence and involvement of antifungal compounds in wilt resistance. The result suggests that the phenolics have an important role in imparting resistance against wilt disease because only wilt-resistant lines produced this compound [109]. Similarly, in another study the potential of the lines to produce phytoalexins influences their resistance to fungal infections [73, 110]. Similarly, a peptide with a molecular mass of 11 kDa, was isolated from dry seeds of red lentil has exhibited antifungal activity against *Fusarium oxysporum* [111]. These selected lines serve as a reliable source of disease resistance and can be used in Fusarium wilt resistance breeding programs.

7.6. Modern breeding approach

The classical plant breeding is based on recombination breeding approach by selecting the desirable plants on the basis of their phenotypic characters. However, this approach is less precise and time consuming when dealing with quantitative traits which are highly influenced by environment and genotype-environment (GE) interaction [112]. Therefore, it is important to integrate modern biotechnological tools such as genetic engineering and marker assisted selection (MAS) in lentil breeding program to mainstream new genetic variability in the cultivated gene pool.

In early 1980s, the first genetic linkage map of lentil was constructed using morphological and isozyme markers [113, 114]. Later, Eujayl et al. (1998) has reported first comprehensive linkage map with 177 RAPD, AFLP, RFLP, and morphological markers was developed using inter specific recombinant inbred lines (RIL) population of a single cross of *L. culinaris* × *L. orientalis* [104, 106]. Hamwieh et al. (2005) added 39 SSR and 50 AFLP markers to the comprehensive Lens map constructed by Eujayl et al. (1998), comprising 283 genetic markers covering 715 cM. They have constructed first genomic library from a cultivated accession, ILL5588 using the restriction enzyme Sau3AI (*Staphylococcus aureus* 3A) and screened with (GT)10, (GA)10, (GC)10, (GAA)8, (TA)10, and (TAA) probes. This study reveals that only SSR59-2B

was closely linked with Fw at 19.7 cM [115]. In an another study, a set of 122 functional SSR markers have been developed using a genomic library enriched for GA/CT motifs for utilization in the lentil breeding program [116].

As lentil has a narrow genetic base an inter-varietal linkage maps were developed by utilizing diverge parents from the wild and cultivated species but these maps have low recombination rate and the map size is also small. QTLs responsible for many traits can be identifying by intra specific mapping population and desirable gene of interest can be tagged. First intra specific lentil map was developed by Ford et al. (2003) through RAPD and ISSR markers [117]. Bi-parental mapping populations derived from the most divergent parents are always better for developing recombinant inbred line and through that a dense mapping or fine mapping can be done from the population developed through the cross of resistant and susceptible parents. These maps are useful to identify genes and major QTLs responsible for the variation of the trait of interest.

Gene cloning can help to characterize the function of the gene or QTLs responsible for the wilt and the knowledge of the genes cloned in lentil can facilitate the development of functional markers for the marker assisted selection. Resistant genes for different functions have cloned in lentil [118]. Using functional genomics approaches, genes expressing differentially in contrasting lentil genotypes can be identified.

Focusing towards the natural defense of host plant may reduce the impact of the pathogen on productivity. However, our poor knowledge about the molecular interaction between the crop and the pathogen limits support for breeding disease-resistant varieties. Due to the development of sequencing technologies, several genes coding transcription factors (TFs) and candidate defense genes (CDGs) from lentil are identified [112]. The full sequence of candidate defense genes like a β-1,3-glucanase (GLU1) (CV793598), a pathogenesis-relate (PR) protein from the Bet v I superfamily (AY792956), a disease resistance response protein 230 (DRR230-A) from pea (AJ308155), another disease resistance response protein (DRRG49-C) from pea (J03680), a pathogenesis-related 4 (PR4) type gene (DY396388) and a gene encoding an antimicrobial SNAKIN2 protein from tomato (HQ008860) are available in NCBI Genbank [112]. A partial sequence of translation elongation factor-1α (TEF-1α) (KR061303 and KR061304) from *Fusarium nygamai* infecting lentil were also deposited in Genbank [33]. These candidate genes and TFs should be further biologically characterized and can help us in decoding the defense pathways and pathogen recognition.

7.7. Integrated management of Fusarium wilt of lentil

Integration of two or more disease management option can reduce the impact of any disease affecting crops. The expected benefit in opting this strategy is improved and sustainable control of disease. The use of biocontrol agents in combination with chemical control can act as one of the strategies in controlling some soil-borne diseases. Therefore, some researchers have used the combination of *Bacillus megaterium* with carbendazim, which provided an effective control of Fusarium crown and root rot of tomato [119]. Similarly, the combination of soil amendments and biological control agents such as *Trichoderma* spp., have been shown to increase disease control and horticultural productivity [120]. Nowadays the use of organic amendments to improve soil properties, plant health and yield has expanded [121].

In Syria, the effect of different control options on disease parameters and yields were conducted as field experiment. The control options are changes in sowing dates, host plant resistance and fungicide seed treatment and the disease parameters like wilt onset, duration, per cent terminal wilt and areas under the disease progress curve were considered. The results revealed that the lentil genotypes had a greater effect on the onset and duration of Fusarium wilt than planting date or fungicide seed treatment. The percent terminal wilt and areas under the disease progress curve was observed lowest during November plantings for all lentil genotypes [78]. Therefore, different individual control options should be recommended to mitigate the effect of Fusarium wilt on lentil yield include manipulation of sowing date, fungicide seed treatment, biological control agents and host resistance [52].

8. Future directions

Knowledge about the pathogen has improved since it was observed, but still few challenges remain. A region specific race of the pathogen is needed. Since there are potential differences in the reaction of lentil cultivars to different races of the pathogen, so information about the distribution of races will be of great importance for breeding programs and the development of resistant genotypes. Along with this, a standardized set of host differentials is required to correlate pathogenicity with DNA techniques. A robust screening techniques for resistant to the pathogen is also required. With the lack of host-pathogen interaction studies, management remains elusive and additional research is needed in this area. Marker assisted selection (MAS) offers great opportunity for improved efficiency and effectiveness in the selection of plant genotypes with a desired combination of traits. Through marker assisted selection, disease resistance can be evaluated in the absence of the disease and in early stages of plant development. Implementation of markers for routine use in lentil breeding programs is currently very limited, integration of the markers within the breeding program to ensure that cost effective utilization of the technology is achieved. Establishment of a tight linkage between a molecular marker and the chromosome allocation of the gene(s) governing the trait to be selected in a particular environment is required. The information from multiple population-specific genetic maps can be integrated to produce high-density consensus structures utilizing the sequence-linked genetic markers which enables the identification of bridging loci between maps. This will further assist in the identification of more closely linked markers for Fusarium wilt resistance in lentil that can be effectively used in breeding and there is a need to develop and map more functional markers like EST-SSRs and SNPs on such maps to enhance their relevance in lentil genetics and breeding. The study based on SNP markers is still limited in lentil due to the lack of available sequence data. For effective variety development marker assisted selection is very imprint that requires much attention in lentil breeding program. Comparative genomics and synteny analyses with closely related legumes can play an important role in enhancing the knowledge of the lentil genome and can provide the genes and selectable markers for use in MAS. Transgenic and non-transgenic approaches including RNAi technology and virus-induced gene silencing (VIGS) can be explore to understand the molecular mechanisms of host resistance in lentil. Additional refined genetic materials are required in order to apply advanced genomic tools such as transcriptome profiling and

map-based gene cloning of lentil. Germplasm with wilt resistance and drought tolerance are key areas of emphases since the later pre-dispose the crop to *Fusarium* infection. In addition to host plant resistance, integrated management of Fusarium wilt is very important to narrow the yield gap due to Fusarium wilt in many countries.

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Chapter 9

Fusarium Species Complex Causing Pokkah Boeng in China

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Additional information is available at the end of the chapter

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Abstract

Sugarcane is one of the most important crops for sugar production in sugarcane-growing areas. Many biotic and abiotic stresses affected the sugarcane production which leads to severe losses. Pokkah boeng is now playing a very important role due to its economic threats. Currently, the occurrence and rigorousness of pokkah boeng disease have been spread like wildfire from major sugarcane-growing countries. Pokkah boeng is a fungal disease that can cause serious yield losses in susceptible varieties. Infection of the disease is caused either by spores or ascospores. It may cause serious yield losses in commercial plantings. However, there have been many reported outbreaks of the disease which have looked spectacular but have caused trade and industry loss. *Fusarium* species complex is the major causal agent of this disease around the world, but some researchers have documented the increased importance of *Fusarium.* Three *Fusarium* species have been identified to cause the sugarcane pokkah boeng disease in China. Moreover, *Fusarium* may be accompanied of its mycotoxin production, genomic sequencing, and association with nitrogen application in China. Many studies on disease investigations, breeding of disease-resistant varieties, and strategy of disease control have also been carried out in China.

Keywords: sugarcane, pokkah boeng, *Fusarium* species complex, nitrogen, secondary metabolism

1. Introduction

Sugarcane is a major crop in Southern China, and it is the third biggest sugarcane producer in the world. Sugarcane is one of the most important crops grown commercially in the tropical and subtropical region. Sugarcane belongs to the genus *Saccharum* L. composed of hybrids [1, 2]

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derived from *Saccharum officinarum* (noble clones), *S. sinense* (Chinese clones), *S. barberi* (North Indian clones), and *S. spontaneum*. This species has C4 photosynthesis, resulting in a vigorous biomass accumulation under tropical conditions, but it also implies a less growth in temperate regions. It grows well in deep, well-drained soils of medium fertility of sandy loam soil textures with a pH range from 6.0 to 7.7. It plays a major role in the economy of sugarcane-growing areas. Sugarcane can be affected from different organisms with various factors such as environmental and physiological disorders and nutritional deficiencies.

Many biotic and abiotic stresses affected the sugarcane production and are known to be one of the oldest cultivated plants in the world. Improving sugarcane production will greatly help in economic prosperity of the farmers and others associated with sugarcane cultivation. Large numbers of sugarcane pathogens have been recorded all over the world. One of the current major diseases affecting sugarcane and sugar production is pokkah boeng. It is caused by *Fusarium* species complex, a destructive fungal disease in sugarcane-growing regions.

Fusarium is a devastating phytopathogenic fungi belonging to Division: Ascomycota, Class: Sordariomycetes, Order: Hypocreales and Family: Nectriaceae. The fungal genus *Fusarium* is composed of a large number of species that can be pathogenic on plants. Within the genus the following 16 sections have been recognized: Eupionnotes, Macroconia, Spirarioides, Submicrocera, Pseudomicrocera, Arachnites, Sporotrichiella, Roseum, Arthrosporiella, Gibbosum, Discolor, Lateritium, Liseola, Elegans, Martiella, and Ventricosum. However, many *Fusarium* species are abundant in fertile cultivated and rangeland soils rather than in forest soils [3]. *Fusarium* species are causal agents of various diseases affecting many economically important cereals, crops, etc. Airborne *Fusarium* species are rarely found in the cultures obtained from soil or the roots of plants.

Fusarium species can grow on a variety of substrates and have efficient dispersal mechanisms owing to their worldwide distribution. Plant debris in soils plays a very important role as nutrient reservoir for *Fusarium* species to continue living in soils as saprotrophs [4]. *Fusarium* spp. also produce gibberellic acid [5], fusaproliferin, and beauvericin [6]. Fusaproliferin and beauvericin have been found to be toxic to insects [7, 8]. The pathogens are difficult to control by conventional strategies such as the use of resistant host cultivars and synthetic fungicides.

Pokkah boeng disease on sugarcane has been recorded in almost all countries where sugarcane is grown commercially. It normally appears during periods of hot humid conditions when the cane is growing rapidly. This disease was originally described in Java in 1896, denoting a malformed or distorted top. The temperature, light, and fertilizer regimes are optimized for maximal plant growth, but these conditions may also be favorable for pathogens. Walker and Went (1896) were the first ones who describe the pokkah boeng disease on sugarcane. Generally, it appears that slowly growing fungi, which are less efficient than quickly growing fungi at escaping competition by entering specific niches, have a higher prevalence of enmity against competing fungi. Geh [9] first reported the presence of the disease in Malaysia. It may cause substantial damage to the crop and not severe except in very susceptible varieties.

Pokkah boeng is a reemerging disease of sugarcane—which has been found recently to cause major yield losses—in most sugarcane-producing regions, including South Africa, Malaysia,

India, and China [10–14]. Pokka boeng disease of sugarcane has associated with several diseases of sugarcane such as sett rot, root rot, and wilt [15]. The pathogen is transmitted by air currents, and airborne spores will colonize the leaves, flowers, and stems of the plant [16]. Pokkah boeng causes serious yield losses in commercial plantings. Reported outbreaks of the disease, while looking spectacular, have caused economic losses. The fungus was reported to occur systemically in all plant parts of sugarcane.

Pokka boeng diseases are dependent upon the environmental conditions, quality of setts, and handling of the plants, e.g., exposing sugarcane plants to stress either from water stress, temperature, pH, or soil nutrition. Hail damage can cause cane plants to be easily susceptible to diseases due to the bruised stalks and broken leaves, giving the diseases access to the damaged setts. Some of the favorable conditions for disease development included drenched conditions of the soil, lack of cultural practices that result in the growth of weeds, constant cultivation of same variety in the field, and existence of susceptible varieties in the surroundings. It is very important for a farmer to prevent and control such pests and diseases to avoid losses. *Fusarium* species complex can produce many kinds of toxic secondary metabolites known as mycotoxins, which can easily enter humans and animals through food and feed because of their resistance to milling, processing, and heating [17].

The taxonomy of *Fusarium* species complex (FSC) is based on phylogenetic, biological, and morphological species concepts [18, 19]. Species in the FSC produce a wide range of mycotoxins that contaminate food and are harmful to human and animal health. *Fusarium* species are common and can survive for long periods in soil. The nature of *Fusarium* disease is that they often become a problem after plant stress occurs. It is now well known that *Fusarium* causes two different diseases, one in stalk and the other in leaves/spindle, and two different species, namely, *F. sacchari* and *F. verticillioides*, respectively, were associated with these diseases. Conventional field-based screening for resistance to pests and diseases is a key component of the breeding program prior to release of a commercial cultivar [20].

Several control measures may be implemented to minimize potential sugarcane yield loss caused by pests and diseases, but an integrated approach is often recommended. Good farming practices are essential but do not guarantee eradication of infections. The planting of resistant cultivars is recommended as the best and most economical approach for controlling pests and diseases, having the least impact on the environment and increasing productivity without the need for other inputs, such as costly chemical applications or labor. Breeding sugarcane that is resistant to multiple pests and diseases is difficult due to the complex genome of sugarcane [21]. Additional genome-scale comparative and functional studies are needed to elucidate the evolution and diversity of pathogenicity mechanisms, which may help inform novel disease management strategies against *Fusarium* pathogens.

2. Manifestation of pokkah boeng

The initial symptoms were easy to recognize the disease since they attack the top parts and are chlorotic areas at the base of young leaves. Heavily infected plants showed a malformed or

damaged top, and stalk may occur in highly susceptible varieties. The base of affected leaves is often narrower than that of normal leaves. Ladder-like lesion on the spindle leaves pronounced yellowing, wrinkling of the spindle, twisting or tangling appearance of the spindle, marketing red stripes, and shortening of the leaves accompanied the malformation or distortion of the young leaves. The most advanced and serious stage of pokkah boeng is a top rot phase. Leaf infection sometimes continued to downward and penetrates in the stalk by way of a growing point. The young spindles are killed and the entire top dies. Leaf sheaths may also become chlorotic and develop asymmetrical necrotic areas of reddish color.

The reddish tissue form ladder-like lesions, often with dark edges. These lesions sometimes break through the surface of the rind. Occasionally, the pathogen also attacks the spindle, and from there it moves down the terminal portion of the stalk causing top rot. The pathogen makes its entry into the host tissues through any sort of injury made by insects or borers or natural growth cracks, etc. The severity of symptoms varies with the susceptibility of a variety and with the congenial environmental conditions and governs the development of the causal organism. During fungal penetration and growth inside the plant, *Fusarium* proteases and mycotoxins act in a kind of strategic cooperation during spike and core colonization by featuring complementary roles during the host defense suppression and the intracellular colonization of spikelet.

3. Mode of transferal

The pathogens of pokkah boeng disease are transmitted by the movement of spores through airflow. For spores to take off, it depends on the environmental situation that requires different strategies to disperse. Fungal species that dispersed by rain splash are based on the "puff" and "tap" mechanisms that will cause the dry spores to become airborne, and usually the spores are curved like *Fusarium* species.

The growth of sugarcane is the most important factor in the biological control and prevention and land and natural environmental factor. The processes for controlling are limited, and there is an increasing need for novel and environmental strategies to control diseases of sugarcane. There will be four sections in this chapter, including *Fusarium* species complex (FSC) and their distribution, comparative genomics of *Fusarium* species complex (FSC), FSC and nitrogen, and sugarcane resistance to FSC.

3.1. *Fusarium* species complex (FSC) and their distribution

Fusarium is a genus of filamentous fungi that includes many toxin-producing plant pathogens of agricultural significance and opportunistic human pathogens. The *Fusarium* collectively represents the most important group of fungal plant pathogens, causing various diseases on nearly every economically important plant species. Besides their economic importance, species of fusarium also serve as key model organisms for biological and evolutionary research. It is the most common and significant pathogen which spread pokkah boeng disease all over the world. Pokkah boeng disease of sugarcane can drastically reduce crop yield and quality. *Fusarium* species produce a number of secondary metabolites that are dependent on different

physiological responses in plants and animals. It also produces a variety of other compounds such as other mycotoxins, pigments, antibiotics, and phytotoxins.

Fusarium species are commonly identified based on their micro- and macroscopic features. But these features are mostly unstable and render the taxonomy of the group problematic. The presence of different taxonomic systems for the genus also contributes to this problem. A number of molecular tools have been used to circumvent these limitations and also to characterize *Fusarium* isolates in terms of their genetic diversity, population biology, and phylogeny. In the studies presented here, *Fusarium* strains isolated from agricultural soils and plant tissues were characterized using different DNA-based tools.

Fusarium fujikuroi (formerly *Gibberella fujikuroi*) species complex (FFSC) members cause important diseases in gramineous crops. The FFSC becomes compatible with the species concept of *F. moniliforme* as described by Snyder and Hansen or section Liseola as defined by Wollenweber and Reinking. *Fusarium fujikuroi* is known to produce a broad spectrum of secondary metabolites.

To recognize and define species in the FFSC, various operational species concepts have been applied. However, a variety of genetic, ecological, and biological traits and properties may be used for this purpose. Only morphological species recognition (MSR), biological species recognition (BSR), and phylogenetic species recognition (PSR) have contributed significantly to the classification of *Fusarium* species in the FFSC. Of these, the MSR was the most widely used and has dominated *Fusarium* taxonomy since its establishment in 1809. The MSR also takes into account physiological characters such as growth rates at different temperatures, host associations, and secondary metabolite production. The majority of the current GFC species definitions and descriptions are based on such polyphasic or integrative taxonomic approaches that incorporate various types of data. Till now, two species of FFSC have been identified to cause sugarcane pokkah boeng disease in China.

3.1.1. Fusarium verticillioides

Fusarium verticilloides is the most commonly reported fungal species infecting sugarcane. *F. verticillioides* is the accepted species, which was also known as *Fusarium moniliforme*. It can able to produce the chemical agent fusaric. Among the *Fusarium* species, *F. verticillioides* is the most prominent *Fusarium* species in China. It is regulated by the fumonisin biosynthetic gene cluster (*FUM*), responsible for transport proteins. In our previous study, a total of 101 isolates were recovered from the sugarcane plants affected by pokkah boeng, which were collected from the major sugarcane-producing areas (Guangxi, Yunnan, Guangdong, Fujian, Hainan) in China throughout 2012 and 2013. More than 90% of the isolates (94 isolates) belonged to F. verticillioides, which was closely related to *F. sacchari,* using the morphological observation and the phylogenetic tree of rDNA-ITS region sequence amplified using fungus-conserved ITS1 and ITS4 primers.

Fusarium verticillioides causes seedling decay, stalk rot, and mycotoxin contamination in sugarcane. This destructive disease occurs virtually everywhere that sugarcane is grown worldwide. Airborne spores (conidia) arising from fungal growth on plant debris or current growth on silks or leaves may cause infection. *F. verticillioides* (teleomorph *Gibberella moniliformis*) is a filamentous fungus that produces two types of conidia—macroconidia and microconidia. The fungal colony of the *F. verticillioides* isolate (CNO-1) appeared to be pale in color but became

orange at the top as it aged, while it was initially white at the bottom which later changed into a yellow color. The fungus is distributed throughout the world but predominant in humid tropical and subtropical regions and also present in the temperate regions.

3.1.2. Fusarium proliferatum

Fusarium proliferatum is grouped in FFSC and can be found on a wide host range as well as pathogenic on various agricultural crops. *F. proliferatum* is a common pathogen infecting numerous crop plants and occurring in various climatic zones. It occurs worldwide as a moderately aggressive pathogen of multiple plant species. *F. proliferatum* is well documented as a fumonisin-producing species, and some strains can produce large quantities of fumonisins, a group of polyketide-derived mycotoxins. *F. proliferatum* causes diseases on a remarkably wide range of plant species, including asparagus, banana, date palm, fig, mango, pine, and sorghum. *F. proliferatum* causing sugarcane pokkah boeng disease was firstly detected in 2012 in China. *F. proliferatum* in sugarcane is important for resistance, for estimating the evolutionary risk of the pathogen, and for planning the agricultural management practices.

During winter or in dry periods, *F. proliferatum* survives in the soil and on plant debris. It also produces other mycotoxins, including beauvericin, enniatins, fusaric acid, fusarin, fusaproliferin, and moniliformin. *F. proliferatum* can be distinguished from other species of the FFSC by analysis of molecular markers. Most recent assessments of fungal pathogens have used multilocus markers to detect populations. The ability of strains and species from geographically separated locations to recombine poses the danger of introducing virulence or toxigenic genes into local pathogen populations. The most commonly observed in human infections are *F. proliferatum*. However, members of FFSC are increasingly identified in especially invasive and disseminated infections in hemato-oncological patients. Many environmental *Fusarium* species and the human infections they cause have a worldwide distribution. The knowledge of the genetic structure of the *F. proliferatum* populations might be useful in order to establish effective strategies for controlling the disease.

3.1.3. The other members of Fusarium fujikuroi species complex (FFSC)

Other FFSC, viz., *F. sacchari*, *F. verticillioides*, *F. proliferatum*, and *F. subglutinans*, have been isolated from sugarcane. The fungus *F. sacchari* grows on decaying plant material and produces a large number of conidia that are spread by wind and rain. The stem borer *D. saccharalis* was shown to carry the fungus from plant to plant in different locations and provide access of conidia in the wind and rain to the inner stem, through their damage made to the stalk. Sugarcane infestation by the stem borer *E. saccharina* is a major problem in the sugar industry. The lepidopteran's infestation of sugarcane by boring the stalk rind permits *Fusarium* species access to the stem tissue. As a result, *E. saccharina* infestation is usually associated with *Fusarium* infection, which can cause stem rot in sugarcane.

3.1.4. Fusarium oxysporum species complex (FOSC)

Fusarium oxysporum is one of the most economically important pathogens in the genus, but members of this species complex are generally considered to be non-toxigenic. *F. oxysporum* comprises over 120 known strains or "special forms," each of which is specific to a unique

plant host in which it causes disease. From a traditional taxonomic point of view, *F. oxysporum* isolates are differentiated from each other based on the pathogenicity as *formae speciales*, but this has been shown to be an unreliable approach. Vegetative compatibility groups (VCG) have been useful in the FOSC to characterize strains with similar pathogenic properties, and their genetic basis is an active area of research on the toxigenic species and mycotoxins in FOSC.

Fusarium oxysporum can spread short distances by irrigation water and contaminated agricultural machinery and via air or long distances by infected seeds and planting material. This prevents transport of water and nutrients to the rest of the host, causing wilting, discoloration, and ultimately death of the plant. Some strains of *Fusarium oxysporum* are pathogenic to different plant species; they penetrate into the roots and provoke the vascular system, causing severe damage on many plant species of economic importance.

Fungal growth initiated with white mycelium which subsequently turned pale violet. Ten isolates were recovered from the single-spore cultivation. The mycelia were floccose, sparse, or abundant. The microconidia were oval, elliptical, or kidney shaped and with 0 septate, while the macroconidia usually had three septa. The apical cell was tapered and basal cell was foot shaped. The morphological features and sporulation pattern were consistent with the description of *Fusarium oxysporum* (Leslie et al., 2006). The pairwise alignment and phylogenetic tree based on three genes (rDNA-ITS, GenBank Accession No. KU863663; pgx4, KU863663; tef, KU933831) and other reference sequences from GenBank also showed that our isolate gx3 belonged to *Fusarium oxysporum*, close related to FFSC.

3.2. Comparative genomics of *Fusarium* species complex (FSC)

Comparative genomics allows investigating many questions of evolutionary and functional significance of sequence features. By associating the species-specific genes with the unique characteristic of that species, researchers can find the potential relationship between genotype and phenotype. Various forward and reverse genetic methods have been developed to explore the repertoire of *Fusarium* genes contributing to disease formation, mycotoxin production, and sporulation. Phylogenetic tree is another application of comparative genomics to infer evolutionary relationship and to estimate diverge time based on the sequence similarity.

The whole genome of three fungal isolates (CNO1, YN41, and BS2–BS6) from the *Fusarium* species complex (FSC) that caused pokkah boeng disease of sugarcane was sequenced by Illumina and PacBio platforms. The genome coverages ranged from 100× to 200×. The newly sequenced genomes, along with five previously sequenced isolates (*F. fujikuroi* IMI58289, *F. verticillioides* 7600, *F. mangiferae*, *F. circinatum* FSP34, and *F. oxysporum* 4287), were selected based on their incidence in geographical locations, taxonomy/species, host isolation, toxin production, and pathology. Overall, the eight sequenced genomes were comparable in size and structure. The sizes of the eight sequenced genomes ranged from 41.9 to 61.4 Mb with approximately 48.0 of GC content (from 47.3 to 48.3). The CDS (protein-coding genes) ranged from 10,522 to 17,753. The gene density ranged from 284 to 356 per Mb.

The development of genomics is allowing the incorporation of new tools and resources to address the important new challenges for agriculture. The commercial sugarcane cultivars used today resulted from crosses of *S. officinarum* and *S. spontaneum*. However, the reproductive

biology and complex genome of sugarcane complicate breeding of genetically improved varieties by conventional means. The global relationship between the linear sequence of nucleic acid bases in the DNA of the gene and the sequence of amino acids in the protein encoded by the DNA of *S. officinarum* and *S. spontaneum* associated these chromosome exchanges occurred through recombinations between the chromosomes of the two species.

A comparative genomics approach was effective in resolving the genetic relationship among fungal species and isolates. The *Fusarium fujikuroi* species complex (FFC) causes a wide spectrum of devastating diseases on diverse agricultural crops, like sugarcane. This is in part due to the complexity of the sugarcane genome which is probably the most complex of all plant crops. Sugarcane complex genome structure is due to a number of interesting developments that resulted in the sugarcane varieties grown.

FFC species can produce structurally diverse secondary metabolites (SMs), including the mycotoxins fumonisins, fusarins, fusaric acid, and beauvericin and the phytohormones gibberellins, auxins, and cytokinins. *Fusarium*-induced crop diseases as well as mycotoxin contamination problems result in significant economic losses to world agriculture every year. The major discoveries contributed by genomic analyses of *Fusarium* focused on plant pathogenicity, production of mycotoxins, and other secondary metabolites. A key theme is the finding that a *Fusarium* genome is compartmentalized into core and adaptive regions that encode functions associated mostly with primary growth versus adaptation to specific niches (e.g., virulence on specific hosts, growth in specific environments). This genome compartmentalization should enable functional studies focused on the development of improved means for controlling *Fusarium* diseases and toxin contamination.

Secondary metabolites are very important in mediating interactions between fungus and host plant. The genes encoded the secondary metabolite often involve particular types of key enzymes, including polyketide synthase (PKS), non-ribosomal peptide synthetase (NPS), and terpenoid synthase. These key enzymes are clustered along with various combinations of additional enzymes for further metabolite catalyzing and with transporters and transcription factors that are essential for the regulation of most of the clustered genes. Based on the fungal SM analyses by antiSMASH software, some SM biosynthetic gene clusters were shared in all *Fusarium* species complex causing sugarcane pokkah boeng, including aflatrem, fusaric acid, fusarubin, asperfuranone, bikaverin, acetylaranotin, fusaridione A, equisetin, nivalenol/deoxynivalenol/3-acetyldeoxynivalenol, and fujikurins. Ustilagic acid was only produced in *F. oxysporum* 4287. The gene clusters for azaphilone, fumonisin, and apicidin biosynthesis were only available in *F. proliferatum.* Gibberellin acid was only produced in *F. verticillioides* CNO1, but not in *F. proliferatum* YN41 because the genome of *F. proliferatum* YN41 lacked one of the key genes (p450) in GA biosynthetic gene cluster, which regulated GA production.

The genotype *F. verticillioides* isolates exposed the event of non-toxigenic strains and confirmed that their phenotype was likely the deletion of genes which are requisite for fumonisin biosynthesis. Some *Fusarium* secondary metabolite gene clusters exhibit a discontinuous distribution that does not correlate with phylogenetic relationships of species. For example, the intently distributed fumonisin and gibberellin gene clusters are present in some but not all species of the *F. fujikuroi* and *F. oxysporum* species complexes. Along with genes responsible for the

biosynthesis of the secondary metabolite, genes with regulatory and transport functions are usually also present in these clusters.

The *Fusarium* comparative genomics highlighted the existence of lineage-specific chromosomes that are enriched for transposable elements and encoded genes that are pathogenicity related. These lineage-specific chromosomes play significant roles in adaptation to changing environments among this species complex. The well-defined genetic model system, will help to redefine the control strategies for pathogens with such genetic pathogenicity; qualitative genes are more often inherited dominantly and are also normally found clustered together in certain chromosome arms. These genes have major effects and are expressed throughout the life of a plant, tending to produce a plant completely resistant to one or more strains of a particular pathogen.

Modern sugarcane cultivars were derived from the interspecific crosses among a few clones of *S. officinarum*, *S. barberi*, and *S. sinense* and the wild relatives of *S. spontaneum* and *S. robustum*. The wild species has played an important role in the development of adaptation or tolerance to varied abiotic and biotic stresses. After the initial interspecific crosses, sugarcane breeders concentrated their attention on intercrossing of the hybrid derivatives. The direct contribution of the chromosomes to pathogenicity is indicated by the fact that they encode known virulence factors such as effector proteins, necrosis-inducing peptides, and a large array of enzymes targeting plant substrates but lack genes involved in primary metabolism. Effectors are "secreted proteins and other molecules which allow plant-associated organisms to modulate plant defense circuitry and enable colonization of plant tissue" [22]. *F. verticillioides*, *F. proliferatum*, and several other species can produce a variety of mycotoxins (e.g., trichothecenes or fumonisins) that are associated with plant disease.

3.3. *Fusarium* species complex and nitrogen source

Nitrogen is one of the most important nutrients for crop growth and production. It is a major component in chlorophyll, which is the most important pigment needed for photosynthesis, as well as amino acids, the key building blocks of proteins. Nitrogen accelerates growth, gives vitality to plants, and promotes dark green color in leaves due to better chlorophyll synthesis. Sugarcane is the world's largest sugar crop and an economically important crop in China. The symptoms of sugarcane pokkah boeng tend to develop during periods in which high concentrations of nitrogen are applied.

Fungi are able to respond to quantitative and qualitative changes in nitrogen availability through complex regulatory mechanisms. The source of nitrogen has been isolated from sugarcane (*Saccharum* spp.) as beneficial interaction that promotes plant growth. Nitrogen is the most essential factor having direct effect on cane growth, sugarcane yield, and juice quality. However, nitrogen application at high rates exceeding sugarcane plant utilization has adverse effect on cane quality. The beneficial plant microorganism association has unique features that remain to be characterized. This living microorganism, which has potential for the development of plant growth by civilizing the nutrient condition of the plant and inadequate supply of nitrogen, decreases the plant metabolism and growth.

Nitrogen availability has significant effects not only on physiological and morphological characteristics of the fungus but also on the biosynthesis of secondary metabolites, such as mycotoxins in *Fusarium* species complex. It is difficult to determine exact N requirements of sugarcane crop. The use of organic nitrogen base may reduce the disease outbreaks and improve antagonistic to pathogens on certain fungi and microorganisms.

On the other hand, it has been observed that in some plants as the N content is increased beyond sufficient levels, the amount of antifungal compounds decreases. Nitrogen fixation is a biological process that reduces molecular N_2 into ammonia (NH_3), which can be easily absorbed by plants. During this adaptation, nitrogenase plays a very important role in catalysis. Strains with nitrogenase activity were identified on the basis of their phenotypic and 16S rDNA sequence analysis and concluded that isolates had potential for regulation of plant growth. To synthesize the secondary metabolites of nitrogen molecules, ammonia plays the vital role in plant growth and development.

Nitrogen supply can bang plant-pathogen interactions through consequence on pathogen virulence. The well-established virulence factor of *Fusarium oxysporum* was found to repress the capacity of the fungal species to penetrate cellophane membrane through the nitrogen source. Nitrogen Utilization may be either regular or impartial with phosphorus and potassium so that nitrification can take place properly. Due to swift mobility of nitrogen, its effect is quite visible in the form of rapid growths due to its presence and rapid retardation in growth and of the crop due to its deficiency. The profuse of nitrogen can enhance the production of young, luscious growth, an expanded vegetative period, and tardy ripeness. The N requirements vary with climate, crop growth, cane yield pattern, irrigation frequency and distribution, land preparation, soil types, and soil behavior. The sustainability of prospective crops is strongly reliant on minimization of fertilizer inputs that can be achieved by enhancement of plant-associated nitrogen fixation. The various applications of nitrogen have been allied with increase in yield. Similarly, the heavy use of nitrogen can promote lodging which can reduce potential yield. In exacting, the nitrogen accessibility directly modulates the regulation of nitrogen source.

Biofertilizers are based on effective strains of microorganisms in sufficient numbers, which are useful for nitrogen fixation in plants and synthesis of growth-promoting substances like hormones, vitamins, and auxins. Besides being essential as a source of cheap protein for human nutrition and animal feed, symbiosis with rhizobia is essential in crop rotation to maintain soil fertility. Poultry manure and other animal waste products were used as a source of supplemental nitrogen long before inorganic nitrogen fertilizer came into popular use. The utilization of BNF for agricultural purposes has long been the dynamic force behind N-fixation research. The environmental benefits from using biological N-fixation are seen to be associated with the proxy of chemical-based technologies with a biological system. Some of the main benefits provided through crop rotation include the prevention of soil erosion, increased soil microorganism diversity, decreased pest prevalence, and increased field fertility. The importance of field fertility in the process of growing crop is immense. The process of BNF can be defined as the reduction of dinitrogen to ammonia by means of a prokaryote. BNF is accomplished by a wide variety of prokaryotes; some can accomplish this as free living organisms, while others require a symbiotic association with plants.

The secondary metabolism, also called specialized metabolism, is part of the metabolism of fungi which is not essential for direct survival; such gene will rely on regulatory mechanisms for biosynthesis and their perpetual relations with the nitrogen regulation of other pathways in *Fusarium*, a paradigmatic model fungus for secondary metabolism. The screening of plant genotypes for their enhanced ability to acquire nitrogen by BNF can reduce the use of expensive nitrogen fertilizers in several important cash crops like sugarcane. In fact, the condition of biologically fixed nitrogen plays a key role in crop production in world cultivation. To understand the role of biological nitrogen fixation, more research work is needed for improving efficiency of nitrogen in order to reduce the use of synthetic fertilizer for production.

Fusarium species having plant growth-promoting activities are exploited for growing agricultural needs. The *Fusarium* spp. range in their pathogenicity, but they can produce mycotoxins in sugarcane. Such *Fusarium* includes the *Gibberella fujikuroi* species complex especially *F. verticillioides* and *F. proliferatum*, which can cause stalk root. Under alternating drought/wet conditions, *F. verticillioides* produces toxic fumonisins and under warm in certain crop variety. The effects of nitrogen sources on *F. verticillioides* will lead to pigmentation variation derived from bikaverin, fusarubins, and carotenoids. The gene expression in *F. verticillioides* was established to analyze processes modulated by different sources of nitrogen and to identify new regulatory mechanisms. Historically, a small amount of N fertilizers was recommended at planting to aid in early season fall growth as well as a mid-season N fertilization application in early spring. Excess N can lead to prolonged vegetative growth and reduced sucrose concentration mainly due to increased moisture in stalks. Crop response to immunization with symbiotic nitrogen has established their important role in supplementing nitrogen to the plant, allowing a sustainable use of nitrogen fertilizers. Today, nitrogen source of applications on sugarcane tends to be a complicated issue due to previous research showing contradicting results. However, economic factors and soil reaction must be considered while selecting the forms of fertilizers.

3.4. Preventive and control measures

Sugarcane is a highly industrious crop which suffers from numerous diseases caused by different organisms and factors such as environmental and physiological disorders and nutritional deficiencies. Historically, planting susceptible varieties in a large area encouraged the outbreak of a certain diseases in a particular period of time. Several control measures may be implemented to reduce potential sugarcane yield loss caused by pests and diseases, but an incorporated approach is often recommended.

Disease control in sugarcane is based on an integration of legislative control, resistant cultivars, and other management procedures. Short-term spraying options are available, but their economic viability may not be sustained. Machine harvest can also transmit disease. Many sugarcane diseases are also managed through the use of disease-free planting material supplied through Cane Protection and Productivity Boards. The genetical resistant cultivars is the most cost-effective method to control the disease, and the presence of genetic variations against pokkah boeng and its associates is well documented. Because of the more serious disease

problem, a progressive effort to socialize and conduct integrated management for controlling the disease.

Several control measures may be implemented to reduce potential sugarcane yield loss caused by pests and diseases, but an incorporated approach is often recommended. To remove and destruct of infected plants on the first appearance of the disease in case of pokkah boeng established that frequent breakdown of varietal resistance against pokkah boeng is due to the appearance of new pathotypes matching the resistance of cane genotypes.

Successive ratoons are characterized by reductions in cane yield due to systemic diseases or physical damage to stools, and the number of ratoons obtained from a single harvest also depends on genotypic and environmental factors. Ratoon productivity has been proved to increase with proper management involving timely agricultural operations, proper nutrition management and integrated pest management, and maintenance of adequate plant population. A number of ratoon management practices currently in use, such as inter-row ripping, burning of crop residues at harvest, harvesting under wet conditions, and using heavy infield transport, were found to be contrary with the substantial, chemical, and biological properties of the soil. The incidence was figured out as five grades (**Figure 1**).

Based on the disease severity index (DSI) of pokkah boeng disease of sugarcane, the resistance of sugarcane against pokkah boeng was classified into five levels from 0 to 5. Level 0 was defined as highly resistant (HR) with DSI≦1.0, Level 1 as resistant (R) with DSI ranged from 1.1 to 5.0, Level 2 as moderately resistant (MR) with DSI from 5.1 to 10.0, Level 3 as moderately susceptible (MS) with DSI from 10.1to 15.0, Level 4 as susceptible (S) with DSI from 15.1 to 20.0, and Level 5 as highly susceptible (HS) with DSI > 20.0.

The disease severity index (DSI) was calculated as follows:

$$\mathrm{DSI} = \left[\frac{\textit{Sum of all numerical grades}}{(\textit{Total number of plants counted} \times \textit{maximum grade})}\right] \times 100 \quad (1)$$

Conidial suspensions of the isolates (CNO-1 and YN41, 10^6 conidia mL^{-1}, 100 μL) were dripped into the young spindle of 89 sugarcane germplasm, and the symptoms were observed on the inoculated plants in 6–8 days post-inoculation, respectively. Our results showed that 34 of 89 tested clones (38.2%) were susceptible to both CNO1 and YN41, 32 clones (36.0%) susceptible to CNO-1 but resistant to YN-41, 14 clones (15.7%) susceptible to YN41 but resistant to CNO-1, and only 8 clones (9.0%) resistant to both CNO-1 and resistant YN41. Both these resistant clones included CP84-1198, GT94-40, GT05-3846, ROC1, ROC27, YC58-14, YC64-173, and YT94-128. Moreover, our results also showed that CNO-1 had higher infection than YN-41 by this inoculation with a success of up to 89.8%.

Chemical control is often expensive and has downstream unconstructive effects on the environment. Nine compounds were tested at three concentrations (100, 50, and 10 ppm) for their ability to inhibit mycelial growth of *Fusarium* species complex. Two antibacterial compounds including copper 8-hydroxyquinoline and validamycins had no effect on the mycelial growth of *Fusarium* species complex at 10 ppm and partially inhibited mycelial growth at the concentration of 50 and 100 ppm. In addition, two compounds, including thifluzamide and chloroisobromine

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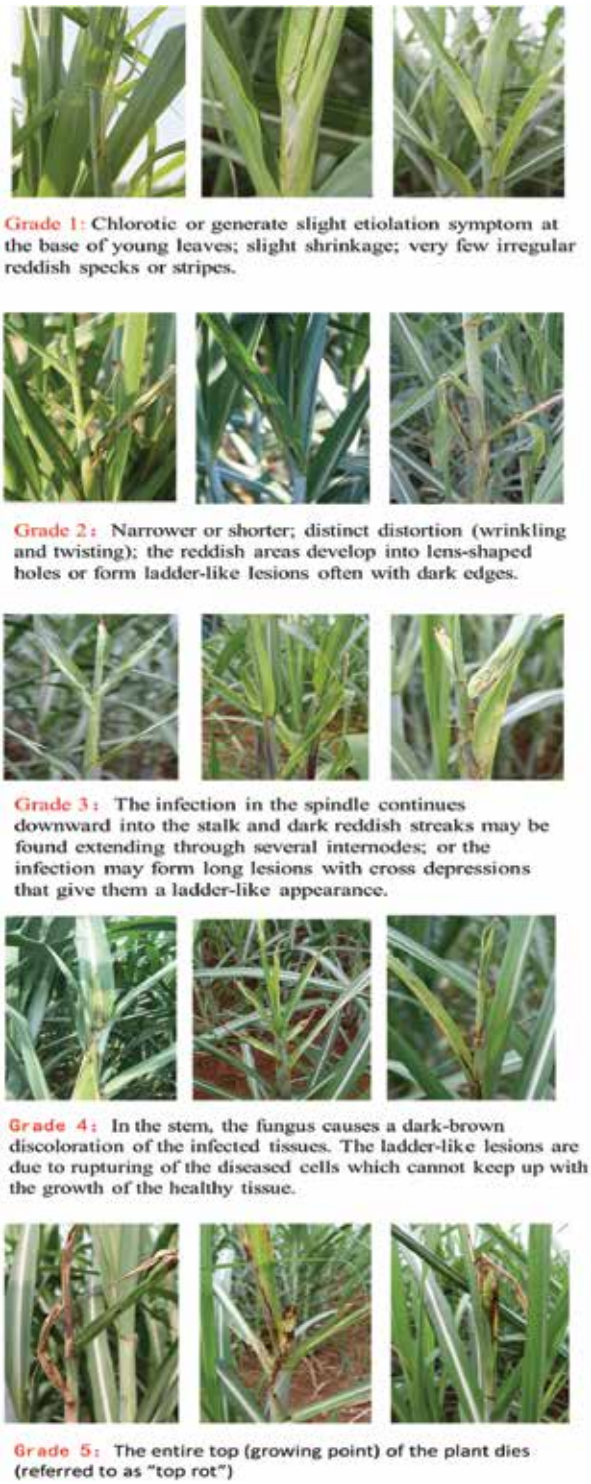

Figure 1. Symptoms of sugarcane pokkah boeng disease in China.

cyanuric acid, had great effect on inhibition of fungal growth at 10 ppm rather than at 50 and 100 ppm.

In the field test, spraying of different fungicides like Bavistin or Blitox or copper oxychloride or carbendazim is efficient for reducing the pokkah boeng disease. Planting of healthy seed, the use of resistant varieties, and following the integrated disease management practices are the best ways to prevent disease incidence. The use of resistant cultivars is particularly useful, as it reduces the use of harmful chemicals which can disturb the balance of nature and result in other pests becoming a problem. Furthermore, *Fusarium* spp. prevalence in the soil can be affected considerably by crop rotation practices. Although the use of resistant varieties is the best means of control, some strains have been found to overcome resistance, and the once-resistant varieties were reported to be susceptible.

Host plant resistance shows major advantages compared to chemical, biological, and cultural control components for management programmes. However, it needs to be supported with additional management practices to ensure durability in the field. Biological control of plant pathogens is an attractive alternative to the strong dependence of modern agriculture on chemical fungicides, which cause environmental pollution and development of resistant strains. The endophytic bacterial community associated with sugarcane harbors multiple genera with potential for plant growth promotion and disease control.

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Chapter 10

Biological Control of *Fusarium oxysporum* in Tomato Seedling Production with Mexican Strains of *Trichoderma*

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Abstract

The problems and limitations of the control of diseases caused by phytopathogens through the use of fungicides, make the biological control present as an alternative method in the production of tomato plants in greenhouse, which is limited by the incidence of *Fusarium oxysporum* Schlechtend.:Fr., being the most worldwide destructive disease. The objective of the present investigation was to evaluate the effect of three Mexican strains of the genus *Trichoderma* against *F. oxysporum* on the production of tomato seedlings under greenhouse conditions, as well as to determine the antagonistic effect of the strains used. The *Trichoderma harzianum* strain had the highest antagonistic activity (81.50%) and the highest growth rate (1.25 cm/day), proving to be the most aggressive strain to control *F. oxysporum*. in addition the results of the interaction of the dual cultures paired, presented a visible overgrowth zone with hyphae of *Trichoderma* spp. Seeds inoculated with *T. harzianum* showed a survival of 84% and a mortality of 16%, lower than the control group, which present a mortality of 58%; however, the treatment inoculated with *F. oxysporum* had the highest incidence of "disease" with 83%, the lowest survival (17%) and a decrease of the green biomass with respect to the control.

Keywords: antagonistic, greenhouse, phytopathogen, incidence, mortality

1. Introduction

The need to reduce the use of fungicides in phytosanitary control makes it necessary to develop technologies that allow easy, economical and effective ways to obtain products from

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endogenous microorganisms with sufficient quality and quantity to their application in the crops areas [1]. In the soil there are microorganisms with antagonistic capacity, the most studied in the world is *Trichoderma* spp [2]; due to its ubiquity, its ability to isolate and present rapid growth on a large number of substrates [3, 4].

In the last 10 years, research work has been carried out, and native species of *Trichoderma* spp. have been isolated, selected and evaluated, with the potential to establish a biological control against different diseases, which have proposed several mechanisms of innovation for the implementation of this fungus with satisfactory results [5–7]. These mechanisms of action may act synergistically on various phytopathogens such as *Septoria triticii* in wheat, *Sclerotinia sclerotiorum* in soybean and lettuce, *Rhizoctonia solani* in soybean, *Sclerotium rolfsii* in cucumber cohombro, *Fusarium oxysporum* in tomato and *Pythium splendens* in beans [8, 9].

The genus *Trichoderma* presents several mechanisms by which they easily move to the phytopathogen, but the most important is based on three types: (a) Direct competition for space or nutrients [10–13], (b) Production of antibiotic metabolites, whether of a volatile or non-volatile nature [14, 15] and (c) Direct parasitism of some species of *Trichoderma* spp [16, 17].

The fungus *F. oxysporum* Schlechtend.: Fr. cause root and neck rot in tomato plants (*Lycopersicon esculentum* M.), causing severe losses that affect the quality and quantity of the production [18]. The most noticeable symptoms produced by using *F. oxysporum* occur in the transplantation of seedlings and at the beginning of flowering [18]. If a transverse section of the stem is made, it is possible to observe a vascular necrosis of brown color, particularly on the smaller lateral roots; which accelerates foliage wilting; after the plant dies and the fungus fructifies on the surface of the stem, under conditions of a humid environment [19]. The vascular wilt of the tomato by *F. oxysporum* was first described by Masse in 1885, on the Isle of Wight and Guernsey, located in the English Channel. In the year 1899, the disease was already in the United States of America, causing severe losses in the areas dedicated to growing tomato in the north of the state of Florida. In 1940 they reported that the disease was disseminated throughout the world and *F. oxysporum* was given greater importance [20].

The tomato (*L. esculentum* M.), is grown in all types of soils for family and commercial use [21]; for the 2016 year, occupied the first place with a total area planted of 4734 million hectares with a production of 163 million tons [22]. To date China is the first producer with 50 million tons, followed by India with 18 million tons, the United States with 12 million tons and Mexico is in the tenth position with 3282 million tons [23]. In Mexico the statistics of the Sistema de Información Agropecuaria [24], reported that in the 2016 year, 52,374 thousand hectares of tomato were planted with a production of 2875.164 tons, with a value of 15,735 million of pesos. While data from the Sistema Producto, they indicated that exports amounted to 20 billion pesos, with the United States and Canada being the main buyers; where the main producers were Sinaloa with 867,832.04 tons, San Luis Potosí with 196,011.25 tons and Michoacán with 169,768.98 tons [25]. Tomato production under greenhouse conditions during 2016 represented 26.2% of the national production, with average yields of 171.82 tons/ha, where Puebla ranked 14th with 75,219.09 tons of tomato [24].

In Mexico, few investigations have been carried out for the biological management of phytopathogens with soil origin through the use of native strains of *Trichoderma* spp [26]. Biocontrol of phytopathogenic fungi and biofertilization using the genus *Trichoderma* is a method used in various crops in different parts of the world; however, the use of commercial strains presents difficulties with their persistence in the soil, due to factors such as the genetics of the isolates, the environmental conditions and others characteristic of phytopathogenic species [27]. For the aftermentioned, the objective of this research was to characterize three native *Trichoderma* strains from the municipality of Tetela de Ocampo, Puebla-Mexico and to evaluate its antagonistic effect on the incidence of root and neck rot in tomatoes caused by *F. oxysporum* in the production of tomato seedlings in greenhouse.

2. Materials and methods

2.1. Strains

Strains native from the state of Puebla-Mexico were used, Th-T4 (3) from *Trichoderma harzianum*, Tav-T7 (2) from *Trichoderma atroviridae*, Tv-T3 (1) from *Trichoderma viridae* and the strain (Fo-A) from *Fusarium oxysporum*, which belong to the Centro de Recursos Genéticos del Centro de Agroecología del Instituto de Ciencias-BUAP and are in culture medium PDA (Potato Dextrose Agar).

2.2. Rate of development and speed of growth

The rate of development and rate growth of Th-T4 (3), Tav-T7 (2), TV-T3 (1) and (Fo-A) strains, was determined in Petri dishes (4.5 cm in diameter) in culture medium (PDA), incubated at room temperature for 7 days; the growth rate was measured every 24 h until the culmination of the total colonization of the strains, the macroscopic morphological characteristics of the colonies were recorded in texture, density, aerial mycelium and color. The rate of development and growth rate were determined using the following formula [28]:

$$TD = VCF - VCI/\text{Number of days} \quad (1)$$

2.3. Antagonistic activity of the strains of *Trichoderma spp.* on *F. oxysporum in vitro*

To evaluate the antagonistic activity of *Trichoderma* spp., Cherif and Benhamou technique was used [29]. For each of the treatments that were performed in Petri dishes with PDA (Agar, Dextrose and Potato) culture medium, was place at one end of the Petri dish a 5 mm diameter agar disc with mycelium of the pathogenic fungus, in this case *F. oxysporum*, due to its slow growth was allowed to develop for 2 days and then another 5 mm disc with mycelium of *Trichoderma* spp. was inoculated at the opposite end, (natives) at a distance of approximately 5 cm between them [30, 31]. The controls consisted of mycelium of the pathogens and

the antagonist, separately cultured in Petri dishes with PDA medium described above. Petri dishes were incubated at a temperature of 26°C. To measure the inhibitory effect of antagonistic fungus to the pathogen, measures colony diameter was recorded every 24 h; until standoff and formation of an inhibition zone between the colonies was formed. Ten repetitions were considered for each comparison, in this case will be three strains of *Trichoderma* spp., a single strain of *F. oxysporum*, evaluating a total of 30 experimental units. The percentage growth inhibition are calculated using the formula given by [32]:

$$PICR = [(\mathbf{R1} - \mathbf{R2})/\mathbf{R1}] \times \mathbf{100} \quad (2)$$

where:

PICR: Percent inhibition of radial growth.

R1: Diameter of token.

R2: Diameter of organism tested.

Additionally, the strain was compared respect to the antagonistic capacity according to the scale proposed by Bell [33] in 1982 (**Table 1**).

2.4. Greenhouse antagonism tests

Seeds of tomato (*L. esculentum* Mill) var. Ramses were used, these were sterilized in 20% (v/v) sodium hypochlorite solution for 20 min, followed by three 5-minute washes in sterile distilled water. Subsequently the sowing was carried out in disinfected trays of 27 × 17 × 4 cm, containing 60 g of sterilized vermiculite in an autoclave at 121°C for 1 h. In each tray 100 tomato seeds were sown. Three strains of *Trichoderma* spp. were selected in the laboratory for their antagonistic response [Th-T4 (3), Tav-T7 (2), Tv-T3 (1)] plus two commercial strains (Perkins-C21 and Tricovel-25) and a control inoculated with *F. oxysporum* without *Trichoderma* spp. plus an absolute control; for a total of seven treatments, where each tray with 100 tomato seeds represented an experimental unit. The inoculations were carried out at the time of planting the seeds in the trays with 1 mL of a suspension of *F. oxysporum* at a concentration of 5×10^6 conidia mL^{-1} per well. The trays were installed in a culture chamber at 25 ± 2°C, were irrigated with 80 mL of distilled water per tray every 2 days. After 15 days, 1 mL of the five strains of *Trichoderma* spp. was applied at a total concentration of 83×10^4 conidia with a viability percentage of 96%.

Class	Characteristics
1	Overgrowth of *Trichoderma* that colonized the entire medium surface and reduced colony pathogen.
2	Overgrowth *Trichoderma* colonized at least 2/3 of the medium surface.
3	*Trichoderma* and pathogen colonized medium to medium (more than 1/3 and less than 2/3).
4	Pathogenic fungus colonized at least 2/3 of the medium surface and resist invasion by *Trichoderma*.
5	Overgrowth of the pathogenic fungus that colonized the entire surface of the medium.

Table 1. Class and characteristics of ***Trichoderma*** spp. antagonistic capacity.

Scale of severity in root of plants			
Code	**Description**	**Code**	**Description**
a	Circular dry injury.	A	Healthy plant.
b	Light necrosis at the base of the root, lesion greater than 0.5 cm.	B	Plant with some leaves with loss of turgor.
c	Dry necrosis approx 1.5 cm, suberized, adventitious roots.	C	Plants with all leaves with loss of turgor.
d	Root presenting lesions with necrosis of 2 cm.	D	Plants with withered leaves. Planta sana.
e	Lesions with necrosis of 2 cm, roots begin to detach.		
f	Lesions with necrosis of 2 cm, large amount of roots are released.		
g	It gives off epidermis leaving vascular tissue, loss of 50% of roots.		
h	Detachment 80% roots expose tissue loss of epidermis, necrotic roots.		

Table 2. Severity scale of ***F. oxysporum*** attack on tomato culture.

The variables evaluated were incidence and severity of disease at 30 days after sowing, both for the radical part and for the aerial part (**Table 2**) using the scale proposed by Amaro-Leal [34]. The percentage of mortality and survival of tomato seedlings at 45 days, as well as height, stem thickness and total dry biomass were evaluated.

The results obtained were subjected to an analysis of variance (ANOVA) and test of separation of means by Tukey ($p < 0.05$), sing the SPSS version 17 (Statistical Package for Social Sciences) to determine differences between treatments.

3. Results and discussion

3.1. Growth rate and rate of development

The Tv-T3 (1) and Th-T4 (3) strains of *Trichoderma* spp. presented a cottony texture with abundant density and abundant mycelium, a dark green and white coloration, whereas the Tav-T7 (2) strain presented a regular density, regular mycelium and a green/yellow coloration. For the *F. oxysporum* strain in this case (Fo-A) presented a velvety texture with a regular density and mycelium of pink white color, as described by Guzman [35], in addition its mycelium is formed by septate hyphae and the conidiophores present clusters of macroconidia where chlamydospores are observed.

T. harzianum presented the highest growth rate with a mean of 1.25 cm/day, followed by *T. viridae* with 0.75 cm/day, with the *T. atroviridae* strain being the lowest growth rate with 0.64 cm/day. For *F. oxysporum* the growth was 0.83 mm/day, results similar to those found by Amaro-Leal [36], with a speed between 70 and 73 mm/day in PDA, results similar to those of the present investigation.

Code	Texture	Density	Aerial mycelium	Color	Form	Growth rate (cm/day) *	Development rate(mm/day) *
Tav-T7 *T. atroviridae*	Cottony	Regular	Regular	Green/yellow	Radial	0.75 b	4.85c
Th-T4 *T. harzianum*	Cottony	Abundant	Abundant	Green/white	Radial	1.25 a	5.69a
Tv-T3 *T. viridae*	Cottony	Abundant	Abundant	Green/white	Radial	0.64 c	5.04b
(Fo-A) *F. oxysporum*	Velvety	Low	Regular	White/pink	Radial	0.83 b	3.80d

*Different lowercase letters indicate significant differences with the Tukey test (P = 0.05)

Table 3. Macroscopic characterization of the colonies of Mexican strains ***Trichoderma*** spp. and ***F. oxysporum*** in culture PDA.

The highest development rate was 5.69 mm/day for *T. harzianum*, followed by *T. viridae* at 5.04 mm/day and *T. atroviridae* at 4.85 mm/day; with respect to strain of *F. oxysporum* Fo-A had a rate of 3.80 mm/day, where a significant difference (p = 0.023) occurs among strains of *Trichoderma* spp. (**Table 3**).

3.2. Confrontation of *Trichoderma spp.* on *F. oxysporum in vitro*

The results of the percentage of inhibition of *Trichoderma* spp. strains on *F. oxysporum* by the dual culture method are shown in **Figure 1**, the Mexican strains of *Trichoderma* spp. inhibited the growth of the pathogenic fungus, where they presented a percentage of inhibition of radial significant growth (PIRG) [p = 0.056] at the Fo-A strain of *F. oxysporum*, with *T. harzianum* which showed higher antagonistic activity, with an average value of 81.50% (PIRG), followed

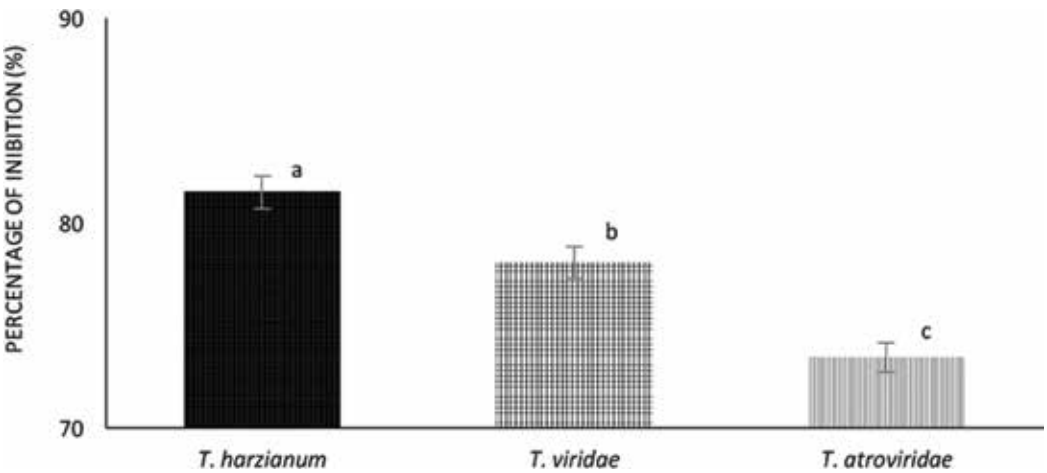

Figure 1. Percentage of radial growth inhibition (PICR) in replicates of *Trichoderma* spp., on *F. oxysporum* in dual culture. *Different letters in the columns mean statistical differences in percent inhibition with Tukey's test (p < 0.05).

by *T. viridae* with (PIRG) 79.61% and finally *T. atroviridae* with (PIRG) 73.41%. Reports of the percentage inhibition of *Fusarium* by *Trichoderma* show values from 22.5 to 86.44% [37]. The values obtained from inhibition are higher than those obtained by Michel [38], who at evaluating the antagonistic effect of native *Trichoderma* spp., on mycelial growth and reproductive potential of *F. oxysporum* and *Fusarium subglutinans*, presented inhibition of 47.6 and 73.0%, respectively. Snyder and Hansen [39], reported a percentage inhibition of 77.8% for *F. oxysporum,* when compared with *Trichoderma viridae* isolates, results lower than those reported in the present investigation.

The results of the interaction of the most representative paired cultures are presented in **Figure 2**. The *F. oxysporum* Fo-A strain was given 2 days advantage because of its slow growth compared to *Trichoderma*; the days after the first contact between hyphae, the behavior was determined, which was very heterogeneous and highly significant (P = 0.0001). Most of the *Trichoderma* isolates showed a visible overgrowth zone with the hyphae of *F. oxysporum*; the greater the area of overgrowth, the greater the aggressiveness of the antagonistic fungus [29].

In this sense, Michel [36], reported antagonism 1, 2 and 3 of *F. subglutinans* and *F. oxysporum*, similar results in the present investigation.

3.3. Greenhouse antagonism tests

The treatment inoculated with *F. oxysporum* showed symptoms of the disease in the root and aerial part (**Table 4**), presenting the highest values in incidence and severity, this in comparison with the other treatments evaluated in this study. These results coincide with that observed by Kim [40], who point out the damage caused by *Phytophthora* sp., at the root and crown of the stem of chile plants under greenhouse conditions, similar results in this research.

In the present investigation, the lowest incidence and severity was obtained in the treatment based on *T. harzianum* with 6%, presenting slight dry circular lesions in the root and without symptomatology in the aerial part. *T. harzianum* has the ability to produce enzymes such as cellulases, β-1,3-glucanase and chitinases, which degrade the cell wall of phytopathogens [41].

Treatments based on *T. harzianum*, *T. atroviridae* and *T. viridae*. used in this research work, present antagonistic efficacy against *F. oxysporum* with a survival ranging from 62.7 to 76.4% in comparison to the control treatment (**Figure 3**), which had a survival rate of 46%; while

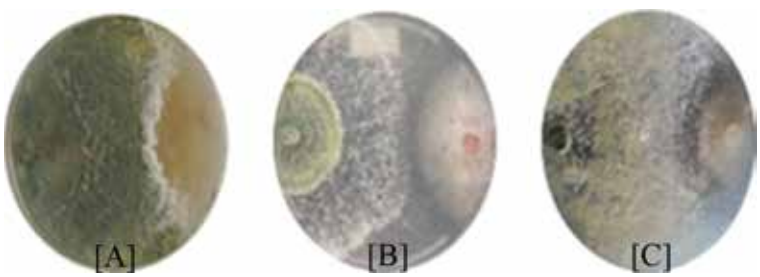

Figure 2. Antagonistic capacity, according to the scale of Bell for *Trichoderma* spp. on *F. oxysporum*. (A and B = *T. harzianum* and *T. atroviridae* overlapping *F. oxysporum*, has a type II interaction, covers 2/3 of the surface of the medium, stops its growth and can overgrow it, and C = *T. viridae* presents an interaction of type I, where it covers the entire surface of the medium and stops its growth).

	Root damage		Damage in aerial part
Treatment	**% incidence**	***Severity scale**	**% incidence**
Witness	28	e	11
F. oxysporum	70	h	58
T. viridae	13	b	10
T. atroviridae	15	b	13
T. harzianum	6	a	4
Perkins-C21	24	e	14
Tricovel-25	26	e	14

Table 4. Percentage of incidence and severity caused by ***F. oxysporum*** at 30 days after sowing in tomato plants (***L. esculentum*** Mill).

tomato plants inoculated with only *F. oxysporum* achieved the lowest survival with 26.3%. Michel [38] performed antagonistic studies with native isolates obtained from tomato crops planted in Tlayacapan, Morelos-Mexico, with which it confronted *Trichoderma* spp. against *Alternaria solani* and *Phytophthora infestans* achieving a range of inhibition percentage from 16.3 to 85.5%, results that are similar to those obtained in this study.

For the variables height, stem thickness and dry biomass of each treatment, showed significant differences (p = 0.043) among the strains of *Trichoderma* spp.; being the treatments based on Mexican strains *Trichoderma* spp. those that presented better results in the evaluated

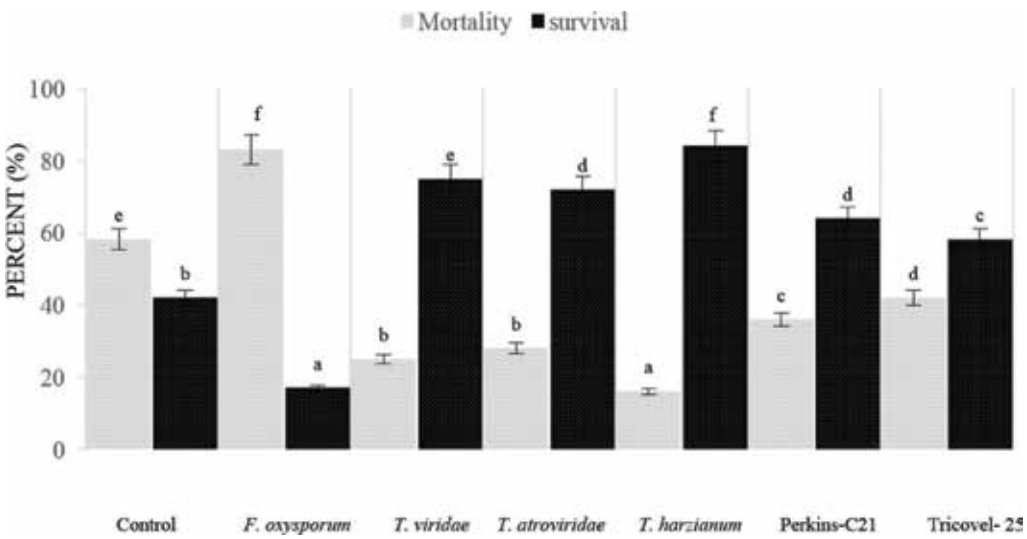

Figure 3. Percentage of mortality and survival caused by *F.* oxysporum, evaluated 30 days after tomato planting (*L. esculentum* Mill). *Different lowercase letters indicate significant differences for % of mortality and survival with Tukey test ($p < 0.05$).

Identification key	Root (cm)	*	P. aerial (cm)	*	Total height (cm)	*	Stem diameter (mm)	*	Weight (green)	*	Weight (dry)	*
Witness	4.53	c	9.04	d	13.57	b	0.20	d	0.19	b	0.02	b
F. oxysporum	3.70	c	8.96	d	12.66	b	0.10	c	0.18	b	0.02	b
T. viridae	9.25	ab	12.18	abc	21.43	a	0.80	a	0.62	b	0.05	b
T. atroviridae	9.04	ab	12.38	abc	21.42	a	0.60	b	0.48	b	0.04	b
T. harzianum	10.58	a	11.05	c	22.64	a	1.00	b	0.97	b	0.18	a
Perkins-C21	8.30	b	13.81	a	20.12	a	0.30	a	0.83	b	0.07	b
Tricovel- 25	7.81	b	12.73	b	20.54	a	0.30	d	0.66	a	0.06	b

*Different lowercase letters indicate significant statistic differences with the Tukey test (P = 0.05)

Table 5. Mean values of height, stem diameter and dry biomass of tomato plants (***L. esculentum*** Mill) under greenhouse conditions.

variables, highlighting the treatment based on *T. harzianum*, which showed an average height of 22.64 cm/plant, an average stem thickness of 1.00 mm and an average dry biomass of 0.18 g; denoting a significant increase in comparison with the control, which showed an average height of 13.57 cm/plant, an average stem thickness of 0.20 cm and an average dry biomass of 0.02 g; while the treatment inoculated with the *F. oxysporum* strain Fo-A presented the lowest averages of height (12.66 cm/plant), 0.10 mm of stem thickness and 0.02 g in dry biomass (**Table 5**). Romo [42], mention that *Trichoderma* spp. has antifungal properties, thanks to the production of substances such as: trichodermine, dermadina, sequisterpeno, suzukacillina, alamethicina, trichotoxina, acetaldehyde, as well as extracellular enzymes such as β-1,3 glucanase, chitinase and cellulase that degrade The host cell walls and allow the penetration of the antagonist's hyphae, reducing its propagation in the root.

Figure 4. Significant statistic differences of each of the treatments in green weight. (a) *T. viridae,* (b) *T. harzianum,* (c) *T. atroviridae*, (d) *Perkins*-C21, (e) *Tricovel*-25, (f) control and (g) *F. Oxysporum*.

Harman [43], argues that *T. harzianum* stimulates the growth of plants by producing metabolites that promote developmental processes, which allow greater root development and absorbent hairs, which favors the mobilization of nutrients in the soil, thus improving nutrition and water absorption; also accelerates the decomposition of organic matter and minerals [44].

The native strains of *Trichoderma* spp. presented higher biomass (green), emphasizing the treatment based on *T. harzianum*, which showed a root height of 10.58 cm and a green biomass of 0.97 g; denoting a significant increase in comparison with the control "**Figure 4**", which showed an average root height of 4.53 cm, and a green biomass of 0.19 g, while the treatment inoculated with the *F. oxysporum* strain Fo-A, presented the lowest root height averages, with 3.70 cm and 0.18 g in green biomass.

4. Conclusions

Plants affected by *F. oxysporum* reduce their growth due to the pathogen's ability to colonize roots, which prevents proper nutrition of the seedling and leads to death, causing losses in the producer in the first stage of tomato crop.

The evaluation of Mexican strains of *Trichoderma* spp. and its antagonistic effect on *F. oxysporum* on tomato seedlings (*L. esculentum* Mill) was determinant to verify their potential for biological control in a crop of great importance in the economy and the country's food.

The *T. harzianum* strain presented the highest growth rate with a mean of 1.25 cm/day, proving to be the most aggressive strain to control *F. oxysporum* with a development rate of 3.80 mm/day.

The three native strains of *Trichoderma* spp. present inhibition of radial growth on the Fo-A (*F. oxysporum*) strain, with *T. harzianum* being the most antagonistic (81.50%), in addition, the results of the interaction of the paired dual cultures to *F. oxysporum*, was very heterogeneous and highly significant statistic differences, where the *Trichoderma* isolates showed a zone of visible overgrowth with the *F. oxysporum* hyphae, which shows more aggressiveness on the part of the antagonistic fungus.

The efficacy shown by the native strains of *Trichoderma* spp. evaluated in this study against *F. oxysporum*, applied to tomato seedlings (*Lycopersicon esculentum* Mill), showed that *T. harzianum* obtained higher height, greater stem thickness and greater production of dry biomass, likewise, the treatment inoculated with *F. oxysporum* obtained the highest incidence (83%) and the lowest survival (17%) of germination in greenhouse conditions.

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Fusarium species are ubiquitous environmental fungi and can cause severe invasive infections in plants. They are crop pathogens, and consumption of such infected crops can cause diseases in humans and animals. Furthermore, they act as spoilage organisms in stored products, such as wheat, sorghum, rice, and corn (maize). *Fusarium* species are mycotoxin producers and contaminate food and grains. Therefore, their eradication and management have economic importance as they can cause enormous economic and agricultural production losses. Despite the fact that the genus *Fusarium* Link (1809) has been known for over 200 years, new scientific information is being revealed by rapid advancements and breakthrough findings of interdisciplinary studies.

This book presents an introductory overview of an update to the scientific knowledge about *Fusarium*. It discusses various aspects of *Fusarium*, such as its genetic diversity, root rot incidence and severity, genetic resistance, molecular markers, mycotoxins, diseases caused by *Fusarium,* and their management and the biological control of these phytopathogens. Furthermore, it also elaborates upon new plant secondary metabolites that are effective against *Fusarium* and the molecular interaction between *Fusarium* and the plant.

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