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The toxigenic fungi contaminating wheat in Tunisia and the development of biological control tests

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Dedication

I humbly dedicate this thesis:

To my dear parents,
who have given me far more than an education: they have instilled in me values, inner strength, and the unwavering belief that anything is possible when guided by love and perseverance.
May these words reflect my boundless gratitude, deep affection, and eternal appreciation for their countless sacrifices, patience, and steadfast faith in me.
It is thanks to them that I am who I am today, and who I will become tomorrow.

To my husband Selim and my beloved daughter, my treasure Serena,
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Abbreviations

3-ADON: 3-acetyl-deoxynivalenol
15-ADON: 15-acetyl-deoxynivalenol
ANOVA: Analysis of variance
DON: Deoxynivalenol
FAO: Food and Agriculture Organization
ITS1: Internal Transcribed Spacers.
QTL: Quantitative Trait Locus
NaClO: Sodium hypochlorite
nm: nanometer
PCR: Polymerase Chain Reaction
PDA: Potato Dextrose Agar
TEF1-α: Translation elongation factor1-alpha
ZEN: Zearalenon
dNTPs: Deoxynucleotide triphosphates
Taq: Taq DNA Polymerase
UV: Ultraviolet
HPLC: High-Performance Liquid Chromatography
DAD: Diode Array Detector
LOD: Limit of Detection

Summary

Durum wheat (*Triticum turgidum L. var. Durum Desf.*) is one of the most important cereal crops worldwide and plays a crucial role in Tunisian agricultural systems. However, this cereal faces major threats, notably fungal diseases caused by pathogens of the *Fusarium* genus. Among these, Fusarium Head Blight (FHB) and Fusarium Crown Rot (FCR) stand out for their considerable impact on crop yield, grain quality and stubble quality. These diseases not only cause production losses, but also the accumulation of mycotoxins, toxic secondary metabolites to human and animal health, thus compromising the marketability and the safety of the foodstuff.

The geographical distribution of *Fusarium* species is influenced by climate, temperature, and humidity, affecting their prevalence, pathogenicity, and mycotoxin production. Climate change may alter these dynamics, highlighting the need for in-depth studies on their regional diversity and crop interactions.

The first objective of this thesis was to study the geographical distribution of *Fusarium* species responsible for FHB and FCR of wheat in Tunisia, as well as their ability to produce mycotoxins. Focusing on the country's main cereal-growing areas, this research made it possible to map the presence of the various species and relate their toxigenic profile to local agro-ecological conditions (Chapter II).

Secondly, the impact of infection by *Fusarium culmorum*, one of the main agents responsible for these diseases, was analyzed on nine commercial wheat varieties commonly grown in Tunisia. Experimental trials, considering artificially inoculated and non-inoculated plots, were set up to study various agronomic parameters, as well as the incidence and severity of these diseases, mycotoxin accumulation in stems and grains, and fungal contamination of tissues. Analysis of the correlations between disease severity and these parameters provided a better

understanding of the effects of this pathogen on production and the quality of crops and residues (Chapter III).

Finally, this study also aimed to select *Trichoderma* strains isolated from different Tunisian cereal-growing regions for their potential as biological control agents against *Fusarium culmorum* and as bio-stimulant on wheat plants. Sampling was carried out across several regions for identification and characterization. The antagonistic activity was evaluated *in vitro* through dual culture and volatile compound assays, while bio-stimulant effects were assessed on wheat growth parameters, including a seed coating trial to test their impact under both normal and pathogen-stress conditions. (Chapter IV).

In conclusion, this thesis provides valuable insights into the dynamics of *Fusarium*-induced diseases affecting wheat crops in Tunisia. It also explores innovative solutions, such as the use of biocontrol agents, to mitigate these diseases and promote more sustainable agricultural practices in response in the face of current environmental challenges.

Sintesi

Il grano duro (*Triticum turgidum L. var. Durum Desf.*) è una delle colture cerealicole più importanti a livello mondiale e svolge un ruolo cruciale nei sistemi agricoli tunisini. Tuttavia, questo cereale è esposto a numerose patologie fungine, fra le quali molto importanti sono quelle causate da patogeni del genere *Fusarium*. Tra queste, "Fusarium Head Blight" (FHB) e "Fusarium Crown rot" (FCR) si distinguono per il loro notevole impatto sulla resa del raccolto, sulla qualità della granella e sulla qualità delle stoppie. Queste malattie non solo causano perdite di produzione, ma anche l'accumulo di micotossine, metaboliti secondari tossici per la salute umana e animale, compromettendo così la commerciabilità dei raccolti.

La distribuzione geografica delle specie di *Fusarium* è influenzata dal clima, dalla temperatura e dall'umidità, con effetti sulla loro distribuzione, patogenicità e produzione di micotossine. I cambiamenti climatici possono alterare queste dinamiche, evidenziando la necessità di studi approfonditi sulla loro diversità regionale e sulle interazioni con le colture.

Il primo obiettivo di questa tesi è stato quello di studiare la distribuzione geografica delle specie di *Fusarium* coinvolte nella FHB e nella FCR del grano in Tunisia, nonché la loro capacità di produrre micotossine. Concentrandosi sulle principali aree cerealicole del Paese, questa ricerca ha permesso di mappare la presenza delle varie specie e di mettere in relazione il loro profilo tossigenico con le condizioni agro-ecologiche locali (Capitolo II).

In secondo luogo, è stato analizzato l'impatto dell'infezione da *Fusarium culmorum*, uno dei principali agenti responsabili di queste malattie, su nove varietà commerciali di grano comunemente coltivate in Tunisia. Le prove in parcelle inoculate e non inoculate sono state utilizzate per misurare diversi parametri agronomici, l'incidenza e la gravità di queste malattie, l'accumulo di micotossine negli steli e nei chicchi e la contaminazione fungina dei tessuti. L'analisi delle correlazioni tra la gravità delle malattie e questi parametri ha permesso di

comprendere meglio gli effetti di questo patogeno sulla produzione e sulla qualità delle colture e dei residui (Capitolo III).

Infine, questo studio mirava anche a selezionare ceppi di *Trichoderma* isolati da diverse regioni cerealicole tunisine per il loro potenziale impiego come agenti di bio-controllo contro *Fusarium culmorum* e per il loro effetto bio-stimolante su piante di frumento. Sono stati effettuati campionamenti in diverse regioni per l'identificazione e la caratterizzazione. L'attività antagonista è stata valutata *in vitro* mediante coltura doppia e saggi di composti volatili, mentre gli effetti biostimolanti sono stati valutati sui parametri di crescita del frumento, compresa una prova di rivestimento delle sementi per testare il loro impatto in condizioni normali e di stress da patogeno. (Capitolo IV).

In conclusione, questa tesi fornisce preziose indicazioni sulle dinamiche delle malattie indotte da *Fusarium* che colpiscono le colture di grano in Tunisia. Inoltre, esplora soluzioni innovative, come l'uso di agenti di bio-controllo, per controllare queste malattie e promuovere pratiche agricole più sostenibili in risposta alle attuali sfide ambientali.

Résumé

Le blé dur (*Triticum turgidum L. var. Durum Desf.*) est l'une des cultures céréalières les plus importantes au monde et joue un rôle crucial dans les systèmes agricoles tunisiens. Cependant, cette céréale est confrontée à des menaces majeures, notamment des maladies fongiques causées par des agents pathogènes du genre *Fusarium*. Parmi celles-ci, la fusariose de l'épi (FHB) et la pourriture fusarienne du collet (FCR) se distinguent par leur impact considérable sur le rendement des cultures, la qualité des grains et la qualité des chaumes. Ces maladies entraînent non seulement des pertes de production, mais aussi l'accumulation de mycotoxines, des métabolites secondaires toxiques pour la santé humaine et animale, compromettant ainsi la commercialisation des cultures.

La répartition géographique des espèces de Fusarium est influencée par le climat, la température et l'humidité, qui affectent leur prévalence, leur pathogénicité et leur production de mycotoxines. Le changement climatique peut modifier ces dynamiques, soulignant la nécessité d'études approfondies sur leur diversité régionale et leurs interactions avec les cultures.

Le premier objectif de cette thèse était d'étudier la répartition géographique des espèces de *Fusarium* responsables de la (FHB) et de la (FCR) du blé en Tunisie, ainsi que leur capacité à produire des mycotoxines. En se concentrant sur les principales zones céréalières du pays, cette recherche a permis de cartographier la présence des différentes espèces et de mettre en relation leur profil toxigène avec les conditions agroécologiques locales (chapitre II).

Deuxièmement, l'impact de l'infection par *Fusarium culmorum*, l'un des principaux agents responsables de ces maladies, a été analysé sur neuf variétés de blé commerciales couramment cultivées en Tunisie. Des essais sur des parcelles inoculées et non inoculées ont été utilisés pour mesurer divers paramètres agronomiques, l'incidence et la gravité de ces maladies, l'accumulation de mycotoxines dans les tiges et les grains, et la contamination fongique des

tissus. L'analyse des corrélations entre la gravité de la maladie et ces paramètres a permis de mieux comprendre les effets de ce pathogène sur la production et la qualité des cultures et des résidus (chapitre III).

Enfin, cette étude visait également à valoriser les souches de *Trichoderma* isolées dans différentes régions céréalières tunisiennes pour leur potentiel en tant qu'agents de lutte biologique contre *Fusarium culmorum* et en tant que biostimulants sur les plants de blé. L'échantillonnage a été effectué dans plusieurs régions à des fins d'identification et de caractérisation. L'activité antagoniste a été évaluée *in vitro* par des tests de double culture et de composés volatils, tandis que les effets biostimulants ont été évalués sur les paramètres de croissance du blé, notamment par un essai d'enrobage des graines afin de tester leur impact dans des conditions normales et dans des conditions de stress pathogène (chapitre IV).

En conclusion, cette thèse fournit des informations précieuses sur la dynamique des maladies induites par *Fusarium* qui affectent les cultures de blé en Tunisie. Elle explore également des solutions innovantes, telles que l'utilisation d'agents de lutte biologique, pour atténuer ces maladies et promouvoir des pratiques agricoles plus durables en réponse aux défis environnementaux actuels.

General Introduction

Wheat (*Triticum* spp.) is an essential staple food and one of the world's most important crops. It represents a mainstay of food security for around 40% of the world's population (Bockus et al., 2010; Moore et al., 2015). Alongside rice and maize, wheat ranks among the three dominant crops in terms of production and consumption, being widely used as a staple food in many regions (Shiferaw et al., 2013; Erenstein et al., 2022). Its special position as a food crop is explained by the diversity of its by-products and its status as the world's most traded cereal (FAO, 2024).

In addition, wheat plays a central role in global food security, constituting a major source of calories for billions of people. Its importance exceeds that of other crops such as rice and maize in terms of agricultural exports (OECD-FAO, 2023).

In the Mediterranean context, agriculture continues to play a pivotal role in Tunisia's economy and society, providing approximately 12.85% of total employment in 2023(TRADING ECONOMICS, 2024). Cereal crops, particularly durum wheat and barley, remain central to the national agricultural system, occupying nearly one-third of the country's arable land and significantly contributing to food security (Thabet, 2024). In the 2023/2024 cropping season, cereal production increased by 25%, reaching 660,000 tons, with durum wheat accounting for 88% of the total harvest (Milling, 2024). Cereals are fundamental to the Tunisian diet, providing 54% of the population's caloric intake and 64% of its protein requirements. On average, each Tunisian consumes 181 kg of cereals per year, with durum wheat accounting for 51% and soft wheat 41% of this total (Melki *et al.*, 2024). This underscores the vital importance of these crops not only for food security but also for the overall economy.

Wheat culture can be infected by many diseases caused by plant pathogens, and which diminish the quality and quantity of the harvest. These include Fusarium Crown Rot (FCR) and Fusarium Head Blight (FHB), which are two of the most destructive and damaging fungal diseases worldwide including Tunisia, transmitted through soils and residues of the previous crops (Burgess et al., 2001; Tunali et al., 2008; Shikur Gebremariam et al., 2018).

Diseases caused by *Fusarium* species represent a major threat to cereal production worldwide, not only for yield losses, but also since these pathogens produce a range of potent mycotoxins that can be accumulated in the grains, posing a danger to human and animal health (Logrieco and Moretti, 2008). Many *Fusarium* species can be implicated and are recognized for their high pathogenicity and ability to cause considerable economic losses (Smiley et al., 2005; Dyer et al., 2009, Haidukowski et al., 2021).

The correct identification of each *Fusarium* species is important since each species has its own toxigenic profile and can have a specific toxicological risk.

Fusarium culmorum is the main *Fusarium* species causing FCR worldwide, including Tunisia. This species can produce a range of mycotoxins, among which the most important are zearalenone and type B trichothecenes, including deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives 3- and 15-acetyl-deoxynivalenol (3-AcDON and 15-AcDON) and 4-acetyl-nivalenol (Desjardins, 2006).

The geographical distribution of *Fusarium* species is influenced by both highly worldwide variable environmental factors such as climate, temperature and humidity, and agronomic factors such as crop rotation, tillage and fungicide use, which explains the variations of their impact on the wheat crops observed from one region to another (Leonard and Bushnell 2003; Xu and Nicholson 2009). Due to climate change, the prevalence and distribution of these pathogens is expected to evolve, necessitating a better understanding of their regional diversity and pathogenicity. In particular, the study of interactions between *Fusarium* species, their toxigenic characteristics and environmental conditions is essential to characterize their specific impacts on wheat. In Tunisia, *Fusarium* species have been widely documented as significant contributors to wheat diseases (Khemir et al., 2020; Gargouri et al., 2003). The potential yield loss is considerable, with studies reporting losses of up to 44% in bread wheat and up to 56% in durum wheat (Chekali et al., 2024).

A second major issue concerns the interaction between FCR and FHB, and the potential translocation from stem to kernels of the main Type B trichothecenes (Winter et al., 2013). This translocation, if confirmed, could exacerbate the health risks associated with grain contamination, particularly in certain wheat varieties.

Due to the environmental impact of the fungicides to control both FCR and FHB, an alternative strategy frequently investigated is the potential use of biological control. Among promising biocontrol agents, *Trichoderma* species stand out for their efficacy and low environmental impact (Gwa and Nwankiti, 2017). These fungi act through a variety of mechanisms, including the production of antifungal metabolites, mycoparasitism and competition for nutrients (Mukherjee et al., 2011). In Tunisia, the identification and valorization of local strains of

Trichoderma spp. could provide a sustainable solution for protecting wheat crops against the main species causing FCR.

Objectives of the thesis

This thesis, carried out under Tunisian Italian co-direction, aimed to study the *Fusarium* species associated to wheat diseases in Tunisia and to develop biological control agents effective against them. The research activities were carried out at the Plant Protection Laboratory of the Tunisian National Institute of Agronomic Research (INRAT), at the Department of Soil, Plant and Food Sciences (Di.S.S.P.A.) of the university of Bari "Aldo Moro", and at the Institute of Food Production Sciences (ISPA-CNR) Italy.

The specific objectives of this research were:

(i) **Geographical distribution of *Fusarium* species involved in Fusarium Head Blight and Fusarium Crown Rot of wheat in Tunisia and their mycotoxin accumulation.**

(ii) **Investigation of the relationship between Fusarium Crown Rot, grain infection and DON mycotoxin contamination in nine wheat varieties in Tunisia.**

(iii) ***Trichoderma* spp. as bio-control agent against *F. culmorum***

Chapter I: Introduction

I. Durum wheat: Origin, History, and Global Importance

I.1. Origin and history of wheat

The wheat plant, one of the first domesticated cereals, occupies a prominent place in the history of agriculture. Its domestication took place around 10,000 years ago in the Fertile Crescent region of the Near East, which today encompasses parts of Turkey, Iraq, Syria and Iran (Zohary and Hopf, 1988). This crop, which originated in this geographical area, gradually spread westwards, reaching the Mediterranean regions, where it adapted perfectly to local climatic conditions and soils. Durum wheat (*Triticum turgidum* L. var. *Durum*), a particular subspecies of tetraploid wheat, is particularly well represented in these regions, where it has been traditionally cultivated for millennia.

Wheat is part of a polyploid complex of plants belonging to the *Triticeae* tribe, and its genetics are varied. There are diploid ($2n = 2\times = 14$, AA), tetraploid ($2n = 4\times = 28$, AABB) and hexaploid ($2n = 6\times = 42$, AABBDD) species (Giménez et al., 2015). Of the various forms grown, hexaploid soft wheat (*T. aestivum*) accounts for most of the world production, while durum wheat (*T. turgidum*) makes up a smaller, but significant share in Mediterranean regions and beyond.

The history of this crop has been marked by continuous exchanges between peoples and territories, contributing to its expansion into Europe, North Africa and, later, America and Asia. Around 7,000 years ago, durum wheat spread across Italy and North Africa to reach the Iberian Peninsula (MacKey, 2005; Moragues et al., 2006).

I.2. Socioeconomic and global importance of durum wheat

Nowadays, wheat is an essential crop throughout the world, both for its role in diet and for its economic implications. In socio-economic terms, wheat is a major source of calories and protein for human populations, and its by-products are ubiquitous in many cultures. World wheat production is dominated by *Triticum aestivum*, used primarily to make bread and other bakery products, but durum wheat is also crucial in specific areas, notably in the manufacture of pasta, couscous, bulgur and certain types of traditional bread.

Durum wheat is particularly appreciated in Mediterranean countries, where it is transformed into traditional food products for mass consumption. These products are made using artisanal methods, but industrial production of pasta and couscous, for example, has also become increasingly important in these regions. Although the industrial version of these foods has become common place, there is still a widespread practice of domestic preparation, preserving an ancient culinary tradition (Ranieri, 2015).

From an economic point of view, wheat is a major cash crop in many countries, with a direct impact on agriculture, trade and the food industry.

The large areas under wheat cultivation are a source of direct employment for farmers and farm workers, as well as for those involved in grain processing, such as flour mills and pasta factories. Worldwide, a large proportion of wheat production is used for human consumption, but a significant proportion is also destined for animal feed (Braimoh et al., 2021).

Because of its key role in food security, wheat is considered a strategic crop. The growing demand for wheat-based products, particularly in developing countries, makes this cereal an essential commodity for feeding the world's population. Durum wheat also plays an important role in the agricultural economies of the Mediterranean, North Africa and the Middle East, where the production of pasta and other wheat-based products is a constantly expanding sector. Wheat is thus a factor of economic growth and social stability for these countries, contributing to job creation and food security (Giraldo et al., 2016).

In sum, wheat, with its long history and indispensable role in the global diet and economy, remains a fundamental crop for ensuring food security and sustaining agricultural economies around the world.

I.3. Durum wheat cultivation areas

Durum wheat (*Triticum turgidum* L. var. *Durum* Desf.) is a highly prized wheat variety worldwide, particularly for its production of pasta, couscous, bulgur and other essential food products. The crop occupies an important place in global agriculture, with around 13 million hectares cultivated worldwide (Giraldo et al., 2016). While durum wheat is often seen as a minor crop worldwide, it remains fundamental in Mediterranean regions, where it has historically been a staple food (Prat, 2016).

The main durum-growing areas are concentrated in three major geographical basins: the Mediterranean region, the northern plains between the United States and Canada, and the desert areas of the southwestern United States and Mexico (Ranieri, 2015). Although durum wheat production also extends to countries such as Russia, Kazakhstan, Australia, India and Argentina, the majority of global production is located in these three main areas (FAO, 2012). Durum wheat differs from common wheat in its preference for semi-arid environments and a hot, dry Mediterranean climate, which limits its growing areas.

I.2. The dynamics of durum wheat production and use

I.2.1. The growth of global wheat production

Since the 1960s, global wheat production has grown considerably, from 222 million tonnes in 1961 to around 780 million tonnes in 2022. This growth has been driven largely by technological advances in genetic improvement and better agricultural practices (FAOSTAT, 2024). Although cultivated areas have not undergone a major expansion in recent decades, yields have continued to grow, making it possible to meet growing demand.

I.2.2. Use of wheat: human and animal food

In 2022, 69% of global wheat production was destined for human consumption, while 19% was used for animal feed, and 12% for other uses (FAOSTAT, 2022). This breakdown clearly shows wheat's central place in global diets, particularly in developed countries, where demand for wheat for animal feed continues to grow, especially in the European Union (Prat, 2016). In developing countries, wheat remains an essential food, with fast-growing demand fueled by urbanization and diet diversification (Kabbaj et al., 2017).

I.3. The strategic role of wheat in the global economy

I.3.1. A rapidly expanding global trade

The global wheat trade is of vital importance to global food security. In 2023, global wheat trade was estimated at around 212 million tonnes, confirming wheat’s status as one of the most heavily traded agricultural commodities worldwide (FAO, 2024). This trade is marked by price volatility influenced by climatic, geopolitical and economic factors. The growing dependence of importing countries on world markets is a key factor in this dynamic. In 2020, Russia, the European Union, the United States and Canada were the world's leading wheat exporters, accounting for a large proportion of global exports (Kabbaj et al., 2017).

I.3.2. Growing demand in the face of demographic growth

Demand for wheat is directly linked to global population growth. According to the United Nations (2022), the world population is projected to reach approximately 8.5 billion by 2030, increasing demand for cereals, much of which will be wheat. At the same time, urbanization and the shift in diets towards increased consumption of wheat-based products, such as pasta and bread, are expected to reinforce this upward trend (FAO, 2022).

I.4. The main world producers and consumers of wheat

I.4.1. Dominant producer countries

Global wheat production is dominated by a few large countries. China, India, Russia, the United States, Canada and France represent the world's leading wheat producers, with yields constantly increasing thanks to advanced agricultural practices (Giraldo et al., 2016). In 2020, total wheat production was estimated at 761 million tons (FAO, 2022), a figure that continues to evolve thanks to genetic improvements and agricultural yields.

I.4.2. Importing countries and increasing dependence

Global wheat imports are particularly crucial for countries such as Egypt, Morocco, Algeria and Tunisia, where production deficits are significant. Egypt is the world's largest importer of wheat, accounting for around 10 million tons a year (FAO, 2022). This growing dependence of importing countries on global wheat exports makes these nations particularly vulnerable to fluctuations in world prices and geopolitical events disrupting trade (Prat, 2016).

I.5. Future challenges for wheat production

I.5.1. Climate change and population growth pressures

Wheat production faces several challenges, not least climate change, which is affecting yields in many producing regions, particularly in semi-arid areas (Ranieri, 2015). At the same time, the growing demand for wheat, linked to population growth and urbanization, is putting additional pressure on global agricultural systems, particularly in developing regions where food security could be at risk (FAO, 2009).

Extreme climatic events, such as prolonged droughts and heat waves, can reduce productivity and disrupt global supply chains. This has a direct impact on importing countries, which are increasingly dependent on international markets to meet their food needs, making global food security more vulnerable to disruption.

I.5.2. The importance of durum wheat in the Mediterranean basin and in Tunisia

Durum wheat (*Triticum turgidum* L. var. *Durum*) is an essential crop in the Mediterranean basin, where it represents one of the main agricultural resources. A staple food in many regions, it is a crucial source of carbohydrates, fiber, protein and vitamins. In Tunisia, wheat occupies an area of around 0.6 million hectares, with annual production of around 1.135 million tonnes (FAOSTAT, 2022). On a global scale, the Mediterranean basin, which encompasses Southern Europe, Western Asia and North Africa, is the leading durum wheat-growing area, accounting for almost 60% of the world's area dedicated to this crop (Prat, 2016; Ranieri, 2015). Among producing countries, the European Union plays a key role, contributing around 22% of global production, with leaders such as Italy, France, Greece and Spain, while more than half of global production is concentrated in the SEWANA region (Prat, 2016).

In this region, durum wheat is generally grown under a winter cycle, making it particularly vulnerable to climatic variations and rainfall. Depending on weather conditions, yields can vary considerably, ranging from 14 to 20 million metric tons (MTM) per year (International Grains Council).

Wheat production in North Africa and Southern Europe is frequently disrupted by drought, leading to growing import dependency. Indeed, these regions have to import over 5 million tons of wheat every year to meet local demand (Ranieri, 2015). Among the main Mediterranean producers, Italy stands out as the biggest contributor, closely followed by Turkey and France. On the other hand, production in North African countries such as Tunisia, Morocco and Algeria is more modest, mainly due to recurrent drought, which limits yields. Moreover, the quality of durum wheat produced in these countries varies considerably according to climatic conditions and specific market needs (Ranieri, 2015).

Since the 1960s, the introduction of modern durum wheat varieties has considerably altered farming practices, replacing traditional varieties adapted to local conditions. Although more productive, these new varieties are less resistant to extreme weather conditions and fungal diseases such as Fusarium Head blight (FHB), which threatens grain quality and food safety. This disease, often associated with conditions of high humidity and heat, can lead to contamination by mycotoxins, rendering crops unfit for human and animal consumption (Legrand et al., 2017; Rawat et al., 2016; Shah et al., 2018). The mass adoption of modern cultivars has thus contributed to reducing the genetic diversity of durum wheat, to the detriment of local varieties which, until the late 1960s, dominated cultivation in these regions (Giraldo et al., 2016).

II Biotic and abiotic stresses affecting wheat cultivation

Despite advanced development of wheat cropping systems, various biotic and abiotic stresses compromise yields (William et al., 2011). Diseases caused by fungal pathogens represent the main biotic constraints to wheat production. Fusarium Head blight, and FCR caused by *Fusarium* species, along with Septoria Tritici Blotch (STB), are among the most widespread and dangerous diseases worldwide, determining yield losses and drastic deterioration of grain quality because of mycotoxin contaminations. Interestingly, both are caused by hemi-biotrophic pathogen species (Singh et al., 2011a; Pretorius et al., 2012; Castroagudín et al., 2016; Abu Sadat and Choi, 2017). In addition to diseases, wheat production is also affected by climatic hazards, responsible for significant yield declines every year.

II.2. Fusarium Crown Rot in wheat

II.2.1 Economic importance

Fusarium Crown Rot is a fungal disease that causes significant economic losses in many cereal-growing regions of the world. In Tunisia, the disease has reduced durum wheat yields by over 29% (Chekali et al., 2013). In the USA, losses reached up to 35% of yields (Smiley et al., 2005), while in Iran, they varied between 18 and 44% between 1999 and 2004 (Saremi et al., 2007). In Australia, annual economic losses have been estimated at 79 million Australian dollars (Murray and Bernan, 2009).

II.2.2 Disease-causing agents

Crown rot is caused by several pathogenic species belonging mainly to the *Fusarium*, *Microdochium* and *Bipolaris* genera. The most common *Fusarium* species are *F. culmorum, F. pseudograminearum* and the species belonging to the Fusarium incarnatum- equiseti Species Complex (FIESC; Munkvold et al., 2021). Other pathogens, such as *Bipolaris sorokiniana*, *Microdochium majus*, *M. nivale* and certain oomycetes like *Pythium aristosporum* and *P. heterothallicum*, are also involved (Acharya et al., 2011; Ünal and Dolar, 2012).

II.2.3. Symptoms

Symptoms of crown rot appear as the browning of roots and stem bases, accompanied by necrotic lesions. In wet weather, a pinkish-white mycelium may form on the crown and early nodes. Stems often show brownish or reddish streaks, and affected ears dry out prematurely, without producing seeds (Scherm et al., 2013).

***Figure 1**: Fusarium Crown Rot (FCR) symptoms: (A) browning on the stem base :(C) reddish-pink discoloration on the basal nodes (Scherm et al., 2013)*

***Figure 2.** Fusarium Crown Rot (FCR) on durum wheat in Tunisia caused by Fusarium culmorum: a) Browning symptoms at the stem base; b) Healthy stem (personal source).*

II.2.4 Distribution of causal agents and disease

The geographical distribution of *Fusarium* species responsible for crown rot is closely linked to climatic conditions and cultural practices. As example, *F. culmorum*, historically predominant in the cold climates of northern and central Europe, has been largely replaced by *F. graminearum*, a phenomenon attributed to corn/wheat rotation and climate change (Scherm et al., 2013). This disease affects many cereal-growing regions around the world. In Tunisia, it causes significant losses in durum wheat yields, while in Australia and the USA, it is also a major source of economic loss. The prevalence of crown rot is therefore highly dependent on climatic conditions and local farming practices (Murray and Bernan, 2009; Smiley et al., 2005).

Figure 3: *Fusarium Head Blight (FHB) symptoms: (A, B) head blight symptoms; (C) brown/purplish discoloration below head; (D–F) orange sporodochia on spikelets. (Scherm et al., 2013)*

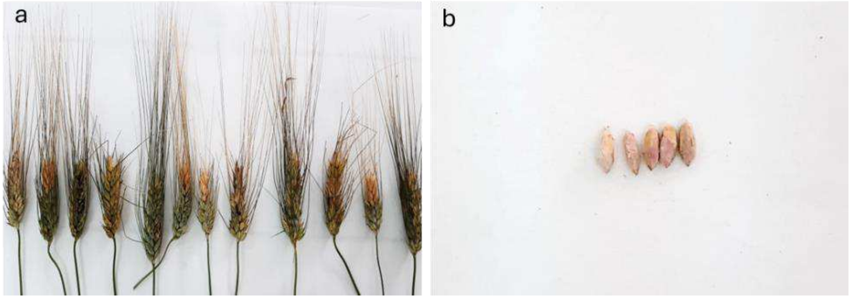


Figure 4. *Fusarium Head Blight on Tunisian durum wheat: a) Symptoms on wheat spikes; b) Mycelial growth of Fusarium spp. on kernels (personal source).*

II.1 Fusarium Head Blight in wheat

II.1.1 Economical importance

Fusarium Head Blight causes considerable yield losses and degrades grain quality through mycotoxin contamination. In Europe, this disease causes yield losses of 10-30% (Logrieco et al., 2002). In England, 11% of wheat production was rejected in 2008 due to mycotoxin contamination (Brown, 2011). In the USA, economic losses were estimated at $2.7 billion between 1998 and 2000 (Nganje et al., 2004). In China, outbreaks in 2008, 2010 and 2012 also caused significant losses (Qiu et al., 2014).

II.1.2. Disease-causing agents

Fusarium head blight is caused by a complex of species belonging to *Fusarium* genus, the most common being *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae* and *F. langsethiae*

(Munkvold et al., 2021). However, other *Fusarium* species can also sporadically be isolated from the wheat kernels.

II.1.3. Symptoms

Fusarium Head Blight symptoms appear as small brownish spots on the glumes and rachis. These lesions spread, leading to discoloration and partial or total drying of the ears. Orange sporodochia may appear on infected spikelets, while grains show reduced size, wrinkled texture and pinkish or brownish coloration (Scherm et al., 2013).

II.1.4. Distribution of causal agents and disease

The geographical distribution of the agents responsible for FHB is strongly influenced by climatic conditions and cultural practices, such as crop rotation and tillage. For example, *F. culmorum* has historically predominated in cold climates, while *F. graminearum* is better adapted to temperate and humid climates, a trend accentuated by climate change (Scherm et al., 2013). This disease is ubiquitous in the world's main cereal-growing regions. In Europe, it mainly affects wetlands, while in China, recurrent epidemics in 2008, 2010 and 2012 underline its growing impact in Asia. In the United States, FHB remains a major concern, especially in the central and northern States (Nganje et al., 2004).

II.3. THE *FUSARIUM* GENUS

The *Fusarium* genus comprises ubiquitous fungi widely distributed in a variety of environments, including soil, plants, air and water. With some 300 species identified to date, these fungi are particularly diverse and include numerous phytopathogens responsible for serious diseases in over 100 plant species. Their economic impact is considerable, causing worldwide agricultural losses running into billions of euros a year. In addition, some species are also recognized for their role as opportunistic pathogens in humans and animals, mainly in immunocompromised individuals (Aoki et al., 2014; Leslie and Summerell, 2006; O'Donnell et al., 2015).

II.3.1. Taxonomy and classification

The genus *Fusarium* has been first described in 1809 by Link and owes its name to the Latin word fusus, which refers to the fusiform or bananiform shape of its spores. In 1821, Fries introduced the genus *Gibberella* as a teleomorph (sexual stage) of many *Fusarium* species. This genus belongs to the phylum Ascomycota, subphylum *Pezizomycotina*, class *Sordariomycetes*, order *Hypocreales*, and family *Nectriaceae*.

Some *Fusarium* species have teleomorphs that were defined with different taxonomic genus names: *Albonectria*, *Calonectria*, *Gibberella*, or Nectria. However, changes to the International Code of Nomenclature (Hawksworth, 2011) prohibited application of both anamorphic and teleomorphic names to a single species and required that existing teleomorph-anamorph names be unified. As a result, most *Fusarium* researchers conserved the genus name *Fusarium* (Geiser et al., 2013). Because of varying species concepts and numerous taxonomic revisions, the number of toxigenic *Fusarium* species is unclear. A book, “Toxigenic *Fusarium* species: Identity and Mycotoxicology” by Marasas et al., (1984) was seminal in combining information on the association of *Fusarium* species with their toxins and toxicoses. The book included 24 species, but the authors considered only 13 to be mycotoxigenic. Together, this book and a companion book by Nelson et al., 1983 began to clarify erroneous nomenclature and unsubstantiated reports of toxin production. However, additional uncertainties about toxigenicity of species have resulted from the many novel species that have been described since these 1983-1984 publications. In 2006, two books were published that represented updates to Nelson et al., (1983) and Marasas et al., (1984). First, “The *Fusarium* Laboratory Manual” by Leslie and Summerell. (2006) encompasses primarily morphological and biological species concepts. This book recognized 70 species and included detailed information on multiple taxonomic systems and species concepts, as well as information on pathogenicity and toxigenicity. Second, “*Fusarium* Mycotoxins: Chemistry, Genetics, and Biology” by Desjardins (2006) reviewed verifiable mycotoxin production among the species. Desjardins’ book lists 45 toxigenic species. These books, along with other recent publications, have de-emphasized the section concept, and in some cases such as Geiser et al., (2021) it has been replaced by the concept of multispecies lineages known as Species Complexes. A recent reassessment (O'Donnel et al., 2018) of the *Fusarium* strains examined by Marasas et al., (1984) highlights the evolution of species concepts and their relationship to mycotoxin production. O’Donnell et al., (2018) proposed a different taxonomy approach based on phylogenetic species concept.

According to the phylogenetic species concept, a species consists of the smallest group (clade) of individuals that share a fixed set of characters. For *Fusarium* and other fungi, DNA sequence is most frequently used to differentiate phylogenetic species (Leslie and Summerell, 2006). Differences in sequences among species are indicative of genetic isolation of individual species. This isolation can be assessed by Genealogical Concordance Phylogenetic Species Recognition (GCPSR), wherein phylogenetic analysis of sequences from multiple loci resolves members of the same species into a clade that excludes members of other species (Taylor et al., 2000). GCPSR has helped overcome limitations of morphological species concepts, e.g., some species that are morphologically indistinguishable have distinct DNA sequences. Because GCPSR can resolve species that are morphologically the same, there tend to be more phylogenetic species of *Fusarium* than morphological species. GCPSR has contributed to estimates of over 50 species in the *Fusarium fujikuroi* Species Complex, 34 in the *Fusarium incarnatum-equiseti* Species Complex45 species in the *Fusarium oxysporum* Species Complex, 50 species in the *Fusarium sambucinum* Species Complex, 80 species in the *Fusarium solani* Species Complex, and 20 species in the *Fusarium tricinctum* Species Complex (Achari et al., 2020; Xia et al., 2019; O'Donnel et al., 2018; O'Donnel et al., 2015; Lee et al., 2015; Sarver et al., 2011; O'Donnel et al., 2009). Numerous *Fusarium* species have been identified capable of inducing FHB, namely *F. avenaceum, F. culmorum, F. graminearum, F. langsethiae, F. poae,* and *F. sporotrichioides*, (Xu and Nicholson 2009). This complex of *Fusarium* species varies over time and geography and can be integrated by other less frequent species such as *F. acuminatum, F. cerealis (*syn. *F. crookwellense), F. chlamydosporum, F. equiseti, F. oxysporum,* and *F. tricinctum* (Xu and Nicholson 2009).

II 3.2. *Fusarium* life cycle

The life cycle of *Fusarium* pathogens is illustrated, using that of *F. culmorum*. These pathogens survive in the soil, mainly in the form of chlamydospores, throughout the winter and the cropping cycle (Bai and Shaner, 1994). These structures constitute the primary inoculum, capable of infecting seedlings and causing diseases such as seedling blight and crown rot (Arsan et al., 2011).

Spores (ascospores and conidia) are then dispersed by wind (Fernando et al., 1997) or rain splash, facilitating their spread from leaf to leaf. Three main sources of inoculum favor the development of fusariosis:

- Infected crop residues, particularly lignified tissues such as nodes, are a major and long-lasting source of inoculum. These residues can harbor pathogens for more than two years, especially if they remain on the soil surface (Champeil et al., 2004; Audenaert et al., 2009).
- Sensitive previous crops, such as maize, wheat and barley, increase the risk of contamination. Deep down, only chlamydospores survive for several years thanks to their thick walls, enabling them to withstand unfavorable conditions (Caron, 1993).

- Infected seeds: when grains are colonized, the mycelium penetrates deep into their tissues, reducing or inhibiting germination, resulting in losses during emergence (Xu and Nicholson, 2009).

In addition, certain asymptomatic host plants (Agrostis, Bromus, Medicago, Festuca, etc.) can act as pathogen reservoirs (Leonard and Bushnell, 2003). *Fusarium* wheat diseases are often caused by numerous *Fusarium* species, with varying biological and ecological characteristics that influence their development and ability to produce mycotoxins (Picot et al., 2011). These variations depend on environmental factors (temperature, humidity) and influence the distribution and dominance of species in a given region (Xu and Nicholson, 2009).

Quantifying the life traits of *Fusarium* spp. and their impact on the severity of FRC is essential for understanding the interactions between strains, symptoms and mycotoxin production. Although several studies have demonstrated a correlation between fungal biomass and ear symptoms, these relationships remain complex and dependent on environmental conditions, species and even the strains present (Reid et al., 1999; Burlakoti et al., 2007).

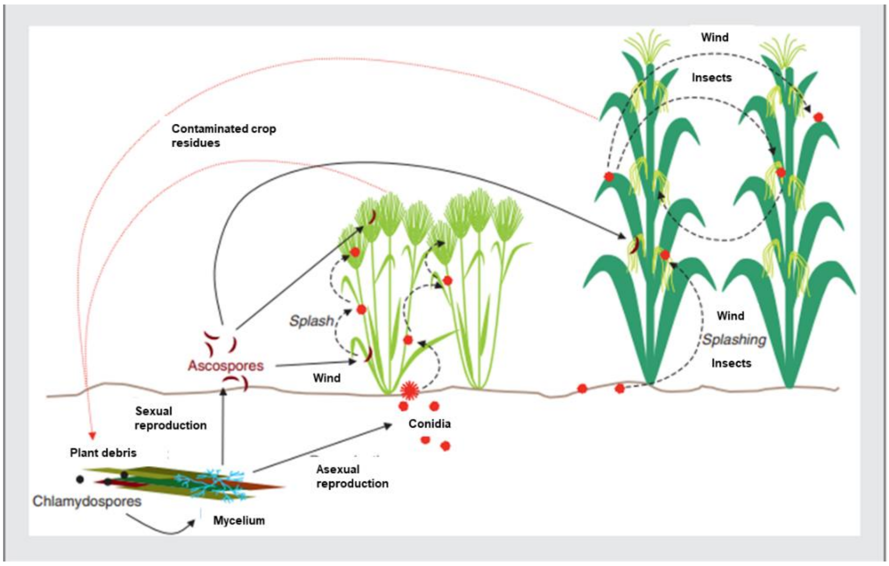


Figure 5: *Life cycle of phytopathogenic Fusarium species (Broydé 2013)*

II.3.3. Species identification methods

The identification of *Fusarium* species was initially based on morphological and biological criteria, such as sexual typing. However, the introduction of molecular techniques has considerably refined classifications.

- **Morphological criteria:**

Macroscopic approaches include growth rate, color and appearance of the mycelium, and pigmentation of the culture medium. Microscopic criteria mainly concern the shape, size and number of conidial septa. Macroconidia are sickle-shaped, with 1 to 9 septa, and their specific characteristics (apical and basal ends) enable certain species to be distinguished. Microconidia, on the other hand, are generally unicellular, adopting ellipsoidal, ovoid, pyriform or kidney-shaped forms. Chlamydospores can develop intercalarly or terminally.

- **Limitations of morphological approaches:**

Although macroconidial morphology has historically played a key role, phylogenetic studies have revealed that these criteria are not sufficient for accurate identification. Some species, such as F. *graminearum* and *F. pseudograminearum*, show phenotypic similarities, while other species possess atypical or absent morphological features.

- **Molecular approaches:**

Molecular methods, particularly DNA sequence analysis, have become essential for identifying complex species or validating morphology-based classifications. These tools make it possible to distinguish closely related species and revise genus taxonomy.

II.4. *Fusarium culmorum*, a major agent of FCR and FHB diseases in Tunisia

This fungus is frequently found in temperate regions, where it colonizes cereal crowns and grains, as well as plant debris in the soil. *Fusarium culmorum*, an ascomycetous fungus of the *Nectriaceae* family (Table 3), stands out for its morphological and biological characteristics.

On PDA (Potato Dextrose Agar) medium, *F. culmorum* develops rapidly, forming an initially white, flaky aerial mycelium that evolves to ochre to brownish-red hues. The underside of culture dishes is intensely colored, ranging from red to purple or dark brown (Figure 8). This fungus produces abundant sporodochia grouped in a central mass of spores, 1 to 2 cm in diameter, initially pale orange, but turning brown to dark brown with age. Some isolates can form rings of spores under alternating light and temperature conditions. Most strains secrete a diffuse red pigment into the agar, although some display olive-brown mycelium (Leslie and Summerell, 2006). Contrary to other *Fusarium* species, the sexual form of *F. culmorum* remains unknown, and it does not produce microconidia. Macroconidia, on the other hand, are abundant, short and robust, with a pointed apical end. Their dorsal surface is slightly curved, while their ventral surface remains straight. These conidia, produced by monophialids on branched conidiophores in orange sporodochia, generally have 3 to 4 septa and a uniform morphology (Leslie and Summerell, 2006).

Abundant, globose chlamydospores form rapidly, in 3 to 5 weeks, on PDA or CLA (Carnation Leaf-Piece Agar). They appear singly, in chains or in clusters, and persist longer than those formed on hyphae (Figures 7 and 9). These structures play a key role in the long-term survival of the fungus (Leslie and Summerell, 2006).

II.4.1. Taxonomic, ecological and pathological aspects

Fusarium culmorum can be easily morphologically confused with *F. sambucinum* and *F. crookwellense*, as these species can be isolated from the same hosts and in similar climatic regions. The apical cell of *F. culmorum* is generally blunt (rounded), although some isolates have a slightly papillary shape, which can be confused with that of *F. sambucinum*. The relatively rapid growth of *F. culmorum* distinguishes it from *F. sambucinum*, which grows more slowly. On the other hand, *F. culmorum* differs from *F. crookwellense* in the shape of its macroconidia. The macroconidia of *F. crookwellense* are longer, with a distinct foot-shaped base and tapering apical cell, while those of *F. culmorum* are short, stout and lack a distinct basal cell (Gagkaeva, 2010).

Phylogenetically, *F. culmorum* is closely related to *F. graminearum* and *F. crookwellense* (Leslie ans Summerell, 2006). Its potential sexual stage is heterothallic since this species carries a single locus with two opposite alleles (Kerényi et al., 2002) However, the sexual stage of these species has never been detected in natural conditions. In addition to genetic differences, these species can also be distinguished by their secondary metabolite profiles (Thrane, 1990).

Although a soil-borne fungus, *F. culmorum* can be splash-dispersed on the ear of certain cereals, such as wheat and barley, where it can cause ear blight symptoms and widely colonize the grains (Jenkinson and Parry, 1994; Jackowiak et al., 2005).

An ABC transport protein is required for the pathogenesis of *F. culmorum* on wheat, suggesting that wheat produces an antifungal compound that this protein is able to export from the cell (Skov et al., 2004).

III Mycotoxins: definition, origin and basic principles

Poisoning linked to the consumption of food contaminated with mycotoxins is a persistent worldwide problem. These metabolites, produced by micro-organisms, notably filamentous fungi, are low-molecular-weight secondary metabolites with proven toxic properties. The term “mycotoxin” comes from two roots: the Greek mukes, meaning “fungus”, and the Latin toxicum, meaning “poison”.

Several mycotoxins reported to date have a cosmopolitan distribution and pose serious risks to human health, such as carcinogenic effects and neurological disorders (Bhat et al., 2010). Mycotoxins are mainly produced by various species of fungal genera such as *Alternaria Aspergillus*, *Fusarium* and *Penicillium* (Murphy et al., 2006; Bhat et al., 2010; Reverberi et al., 2010). They infect crops in the field as well as during storage, under favorable conditions of temperature, humidity, water activity, etc. (Bhat et al., 2010).

The most common mycotoxins include aflatoxins, ochratoxin A, fumonisins, trichothecenes A and B, and zearalenone. A wide range of foodstuffs, such as corn, wheat, barley, rice, oats, nuts, milk, cheese, peanuts and cottonseed, are vulnerable to contamination. This can occur at various stages, notably between harvesting and drying (Didwania and Joshi, 2013).

III.1 Fusarium mycotoxins: typology and impact on food commodities

Fusarium species that colonize standing cereal ears can produce a variety of mycotoxins, some of which are of major importance to public health and the economy (Bottalico and Perrone, 2002). Mycotoxins produced by *Fusarium* species belong to various chemical families, such as trichothecenes, fumonisins, zearalenones, and enniatins (Figure 5). In addition, many *Fusarium* species can produce beauvericin, fusarin C, and moniliformin, which are often found in crops and food products (Nielsen et al., 2011; Richard-Forget and Oswald, 2012). Among these mycotoxins, trichothecenes stand out as a group of secondary metabolites synthesized by several species, especially *F. culmorum, F. graminearum, F. langsethiae, F. poae, F. sambucinum,* and *F. sporotrichioides.* Over 160 trichothecenes have been identified, the main ones being deoxynivalenol (DON), nivalenol (NIV), T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), and fusarenone X (FX), with DON being the most common contaminant (Munkvold et al., 2021). These toxins represent a major risk due to their toxicity to humans and animals, their economic impact, and their prevalence in cereal crops (Desjardins, 2006; Gratz et al., 2014; Charmet et al., 2016).

Recent advances in *Fusarium* taxonomy and phylogenetics, as discussed by Munkvold et al., (2021), have refined our understanding of the distribution of mycotoxin production among species and species complexes. The ability of *Fusarium* species to produce mycotoxins is determined not only by their genetic background but also by environmental conditions and interactions with plant hosts. The same species can produce several types of mycotoxins, and the same toxin can be synthesized by different species (Parent-Massin et al., 2013). For example, *F. graminearum* and *F. culmorum* are both major producers of trichothecenes, while fumonisins are more commonly associated with *F. verticillioides* and *F. proliferatum.* Additionally, genomic studies have revealed that mycotoxin biosynthesis pathways are regulated by complex gene clusters that can undergo horizontal gene transfer, potentially altering mycotoxin profiles among strains and species.

Moreover, mycotoxin production capacity varies between isolates and environmental conditions, both *in vitro* and *in vivo* (Yli-Mattila and Gagkaeva, 2010). The interaction between mycotoxin biosynthetic genes and environmental factors such as temperature, humidity, and substrate composition play a crucial role in determining contamination levels. As highlighted by Munkvold et al., these insights are critical for predicting and mitigating the risks associated with mycotoxin contamination in food and feed.

Trichothecenes, fumonisins, and zearalenone are attracting particular attention in the cereal industry due to their toxicity, their regulation in many countries, and their ability to contaminate cereals and by-products. Understanding the phylogenetic relationships and genetic mechanisms underlying mycotoxin biosynthesis is essential for developing targeted strategies to reduce contamination and improve food safety.

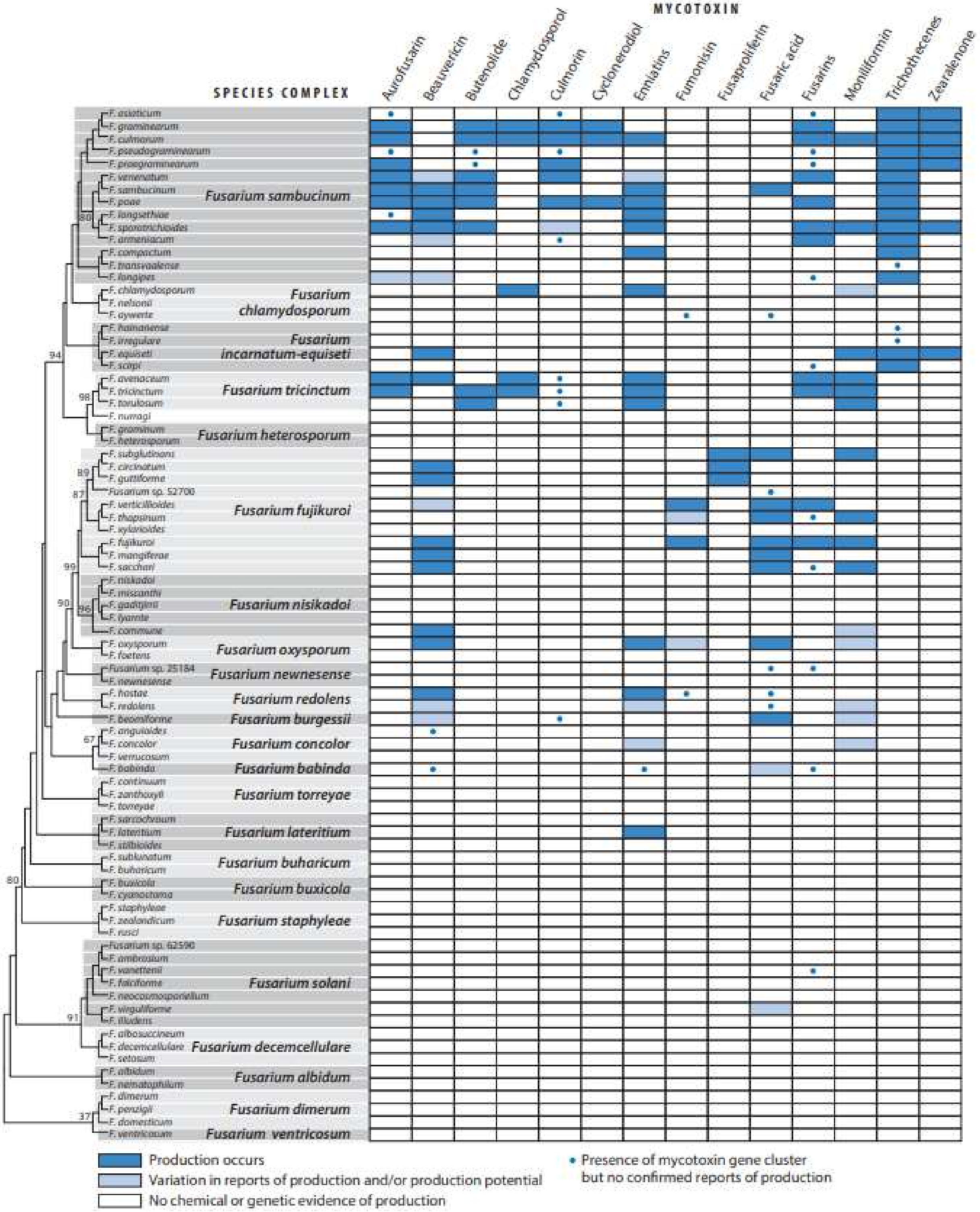


Figure 6: *Distribution of mycotoxin production among Fusarium species (Munkvold et al., 2021). The tree (cladogram) on the left shows the phylogenetic relationships of the selected species and the 23 species complexes. The grid on the right shows the mycotoxin production or potential of each species. In the tree, grey shading delimits the species complexes. The tree is derived from the phylogram reported by Geiser et al. (2013) and was inferred by maximum likelihood analysis from concatenated alignments of exon sequences from 19 genes. Branch support was determined by bootstrap analysis with 5,000 replicates. Most branches had bootstrap values of 100; therefore, only values below 100 are shown (numbers near the branches). When analyzed individually, some genes did not provide significant support for certain branches Geiser et al. (2013).*

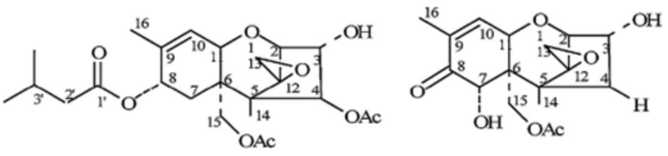
III.2 Structural properties and metabolic pathways of Trichothecenes

III.2.1. Chemical architecture of trichothecenes

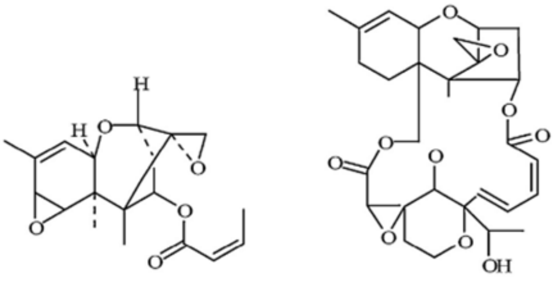
Trichothecenes are a class of sesquiterpenes characterized by a tricyclic ring composed of a cyclopentane, a cyclohexane, an oxygenated hexagonal ring and four methyl groups. This basic structure, known as trichothecene, is illustrated in Fig. 5. Depending on the functional groups present, trichothecenes are classified into four main categories (A to D). Type A trichothecenes have an oxygen function other than a carbonyl in position C-8, while type B trichothecenes have a carbonyl in the same position. Type C is distinguished by a second epoxy function at C-7,8 or C-9,10, and type D features a macrocycle formed between positions C-4 and C-15 by two ester bonds (Krska et al., 2001).

These mycotoxins are among the most important produced by *Fusarium* species on cereals and are grouped into two subcategories: trichothecenes type A (TCTA) and type B (TCTB) (Moss and Thrane, 2004). TCTBs include DON and its derivatives, NIV and their acetylated forms, such as 15-acetyl-deoxynivalenol (15-ADON), 3-acetyl-deoxynivalenol (3-ADON) and fusarenone X (FX) (Gauthier et al., 2015).

Type A trichothecenes, including T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS, and neosolaniol NEO (Juan et al., 2012), are considered more toxic to humans and animals than type B trichothecenes (Krska et al., 2001). These mycotoxins are primarily associated with *F. poae* (DAS and NEO) and *F. langsethiae* and *F. sporotrichioides* (T-2 and HT-2), while *F. acuminatum, F. avenaceum* and *F. tricinctum* produce other toxic metabolites, such as moniliformin, beauvericin, and enniatins (Munkvold et al., 2021).



Group A: T-2 toxin Group B: Deoxynivalenol



Group C: Crotocin Group D: Satratoxin G

Figure 7: *Trichothecenes chemical structure groups A-D (Zhang et al., 2014)*

III.2.2. Metabolic pathways of trichothecenes

Trichothecenes are oxygenated sesquiterpenes derived from the isoprenoid biosynthetic pathway (Alexander et al., 1997). Their production begins with trichodiene (Fig. 6), a natural metabolite initially isolated from *Trichothecium roseum* (Machida and Nozoe, 1972). The process begins with sesquiterpene cyclization catalyzed by the enzyme trichodiene synthase, followed by a series of oxygenation reactions (up to eight steps) and four successive esterification.

Trichothecene biosynthesis also involves transport protein and a set of regulatory genes (Desjardins and Proctor, 2007). Fifteen genes, collectively known as Tri genes, have been identified (Fig. 6). Of these, twelve are grouped together in a cluster designated the Tri cluster, while the other three are distributed across two distinct loci in the genome (Richard Forget and Oswald, 2012).

III.2.4. Impact and regulation of trichothecenes

Trichothecenes are highly toxic compounds, affecting a wide range of living organisms, although their toxicity varies according to species and trichothecene type (Eriksen, 2003). Group A trichothecenes, such as T-2 toxin, are of particular concern due to their higher toxicity than type B trichothecenes (Bhat et al., 2010).

In mammals, ingestion of trichothecenes causes digestive and hematological disorders, such as ulcers, hemorrhages, gastrointestinal necrosis and, in some cases, fatal outcomes (Eudes et al., 2000). Side effects also include immunodepression, reduced fertility, reduced growth, vomiting and feed refusal (Lattanzio et al., 2009; Rocha et al., 2005).

In plants, notably wheat, DON affects germination and inhibits grain and coleoptile development (Rocha et al., 2005; Snijders and Perkowski, 1990). The toxicity of trichothecenes to plants is generally measured using coleoptile elongation tests (Eudes, 1998; Eudes et al., 2000).

Trichothecenes also exert inhibitory effects at various cellular levels, such as protein, DNA and RNA synthesis, cell division and mitochondrial function. They also disrupt cell membranes, contributing to their phytotoxicity and overall toxicity (Rocha et al., 2005).

In response to these risks, contamination thresholds for trichothecenes in food are strictly regulated in many countries to limit their impact on human and animal health.

III.3 Mycotoxin Safety Standards

In Tunisia, there are currently no established national standards or regulations specifying maximum allowable levels of mycotoxins in food products or animal feed. However, regulations addressing mycotoxin contamination have been implemented in over 100 countries (FAO, 2003; Van Egmond et al., 2007), with significant variations in permissible limits across nations (Pinotti et al., 2016). Notably, some European Union member states began setting maximum levels for aflatoxins as early as 1974, and these regulations have since evolved to cover a broader range of mycotoxins, following updates based on scientific risk assessments (Zinedine & Idrissi, 2007).

The European framework was historically based on Regulation (EC) No. 1881/2006 (later amended by EC No. 1126/2007), which established maximum permissible levels for specific mycotoxins in cereals and cereal-based products intended for human consumption (Atanasova-Penichon & Richard-Forget, 2014; Savoie et al., 2015).

More recently, this framework has been repealed and replaced by Regulation (EU) 2023/915, which entered into force on May 25, 2023. This regulation consolidates and updates the maximum levels permitted for a broad range of contaminants, including key mycotoxins such as aflatoxins (AF), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON), and fumonisins (FB). Furthermore, Regulation (EU) 2024/1038 of April 8, 2024, amended Annex

I of Regulation (EU) 2023/915 to revise certain maximum levels, particularly for DON and related trichothecenes, to reflect the latest scientific evaluations (European Commission, 2023; European Commission, 2024).

Economically, only a limited number of mycotoxins are considered of major importance due to their frequency and toxicity: AF, OTA, ZEA, DON, and FB. The updated maximum levels for these mycotoxins within EU member states are detailed in Annex 2 (Cheli et al., 2013; Cheli et al., 2014).

IV *Trichoderma* as a biocontrol agent

IV.1 Characteristics and applications of *Trichoderma*: a cosmopolitan fungus with multiple benefits

Trichoderma is a cosmopolitan, saprotrophic fungal genus found in diverse environments such as forest soils, roots and leaves, and in habitats ranging from salt marshes to desert soils. Its rapid growth has earned it the status of “soil fungus”, and its ability to degrade complex substrates makes it a key player in the soil ecosystem. What's more, it shows remarkable resistance to toxic chemicals, which contribute to their presence in great abundance, particularly on decaying wood, due to heterotrophic interactions and its role as an opportunistic endophyte. Long used as a biocontrol agent against phytopathogens, *Trichoderma* is recognized for its ability to produce specific enzymes, giving it great economic importance in agriculture and biotechnology.

Recent taxonomic and phylogenomic studies have significantly expanded our understanding of this genus, revealing over 400 recognized species to date (Ding et al., 2020). These advances have also highlighted its exceptional metabolic diversity, which underpins its biotechnological importance in agriculture, as a biocontrol agent and growth promoter, and in industry, for enzyme and secondary metabolite production.

While *Trichoderma* species are mainly known as epiphytes associated with plant surfaces sometimes beneficial, sometimes opportunistically harmful their role as true endophytes colonizing internal plant tissues remains less well documented and is an active area of research (Cai & Druzhinina, 2021). Genomic resources such as the Mycocosm portal continue to support this research by providing high-quality genome sequences and comparative genomics tools, thereby deepening our understanding of the ecological versatility and functional potential of *Trichoderma* (Mycocosm, 2025).

IV.2 The *Trichoderma* Genus: Taxonomic history, evolution and classification

The genus *Trichoderma*, first described by Persoon in 1794 (Samuels, 1996), has a complex taxonomic history marked by initial ambiguities. A major breakthrough came in 1969, when Rifai proposed a structured classification, grouping the species into nine distinct categories (Harman et al., 2004a). Originally classified in the phylum *Deuteromycotina*, class *Hyphomycetes*, order *Phialosporae*, and family *Dematiaceae*, it was later associated with the genus *Hypocrea Fr.* within the *Hypocreales*, reflecting its teleomorphic form (Dennis & Webster, 1971).

Significant morphological studies, such as those by Bissett (1991), and the introduction of molecular methods by Samuels (1996) have advanced the classification of *Trichoderma*, clarifying interspecies relationships (Klyce, 1984). Molecular techniques for species identification were further refined by Hermosa et al., (2000), complementing earlier morphological approaches (Howell, 2003).

These advancements have considerably expanded the recognized diversity of *Trichoderma*. In 2000, Kiffer and Morelet identified 36 species (Migahed, 2003), while subsequent research by Samuel et al., (2006) and Bisset et al., (2003) introduced additional methods to define new species, particularly in Asia. Combining morphological, molecular, and biochemical approaches, Harman et al., (2004) increased the number of identified *Trichoderma* species to 104, illustrating the genus's remarkable biodiversity and the growing sophistication of taxonomic techniques.

IV.3. Characteristics of *Trichoderma* species

Fungi of the genus *Trichoderma* were historically classified among the so-called “imperfect fungi” (*Deuteromycetes*), as only their asexual (anamorphic) stage was initially known (Gupta et al., 2014). However, it is now well established that many *Trichoderma* species also possess a sexual (teleomorphic) stage, classified within the genus *Hypocrea* (Woo et al., 2023; Jaklitsch, 2009).

These fungi are characterized by the abundant production of conidia, typically green but sometimes of other hues, and by rapid growth on a variety of culture media (Klyce, 1984). The undersides of colonies in Petri dishes often appear buff, colorless, yellow, green-yellow, or amber. Additionally, several species can produce large quantities of thick-walled chlamydospores within their submerged mycelium (Vinale et al., 2008).

Trichoderma spp. are further distinguished by the brightly colored and characteristic fruiting structures associated with their sexual stage (*Hypocrea*), which typically manifest as small, orange to reddish-brown stromata on wood or plant debris (Harman et al., 2012). This dual reproductive capacity sexual and asexual—contributes significantly to their ecological success and genetic diversification.

These fungi thrive mainly on herbaceous and woody plants, where their teleomorphic forms can sometimes be observed. Nevertheless, many biocontrol and plant-associated strains of *Trichoderma* typically lack an observed sexual stage and reproduce exclusively asexually, forming clonal, sometimes heterokaryotic populations (Gupta et al., 2014). As highlighted by Woo et al., 2023, this predominantly asexual reproduction strategy has favored rapid adaptation and diversification, enabling these fungi to colonize a wide range of ecological niches and to evolve independently.

Importantly, *Trichoderma* species exhibit remarkable genetic diversity, which underlies their ability to synthesize a wide array of biologically active compounds and enzymes of ecological and commercial importance (Kubicek et al., 2011; Woo et al., 2006). This versatility explains why *Trichoderma* has emerged not only as an effective biocontrol agent against plant pathogens but also as a promising microorganism for eco-sustainable agriculture, contributing to enhanced plant growth, soil health, and stress tolerance (Woo et al., 2023).

IV.4 Biodiversity and distribution of *Trichoderma* species

Extensive studies on the taxonomy and biodiversity of *Trichoderma* species have been carried out in North America, Europe and other parts of the world, providing a better understanding of their geographical distribution (Bissett et al., 2003; Lieckfeldt et al., 2001). Kullning et al., (2000) reported the identification of 76 isolates from northern India, Nepal and Russia, including seven main species, *T. asperellum, T. ghanense, T. atroviride, T. hamatum, T. oblongisporum, T. virens,* and *T. harzianum*, and five new taxa. Their work revealed that *T. harzianum* exhibits great genetic diversity.

Kubicek et al (2003) isolated and identified 96 *Trichoderma* strains using internal transcribed spacer sequence analysis (ITS 1 and 2), of which the most frequent species were *T. harzianum, T. spirale, T. virens, T. koningii, T. asperellum, T. atroviride, T. reesei, T. hamatum, T. viride* and *T. ghanense*. In Southeast Asia, Kubicek et al (2003) also reported the existence of nine new species, including *T. asperellum, T. ghanense, T. atroviride, T. hamatum, T. koningii, T. harzianum, T. spirale, T. viride,* and *T. virens*. In China, Zhang et al (2005) evaluated the biogeography and biodiversity of *Trichoderma* species in four distinct regions: Hebei province (north), Zhejiang province (southeast), Tibet (west) and Yunnan province (south-west). They identified several species, including *T. koningii, T. asperellum, T. atroviride, T. velutinum, T. viride, T. cerinum, T. harzianum, T. virens, T. sinensis, T. longibrachiatum* and *T. citrinoviride*, in addition to two new species recently accepted.

In tropical areas, Hoyos-Carvajal et al (2009) observed that *T. asperellum* was more widespread than *T. harzianum*. The latter was found mainly in poorly decomposed soils, such as crop residues or dead leaves, in cold regions of Peru, Colombia and Brazil.

Studies in Sardinia by Migheli et al (2009) examined the impact of abiotic factors on the distribution of *Trichoderma* species and found high diversity in disturbed and undisturbed environments. Similarly, research in Japan, Indonesia, Vietnam and Mongolia isolated 332 strains, including *T. harzianum, T. virens, T. hamatum* and *T. crissum* (Jaklitsch and Voglmayr, 2015).

In Tunisia, early surveys by Sadfi-Zouaoui et al. (2009) documented the presence of several *Trichoderma* species distributed across different soil types and agroecological zones, including *T. longibrachiatum*, *T. harzianum*, *T. atroviride*, *T. hamatum*, and *T. saturnisporum*. These findings provided the first comprehensive picture of *Trichoderma* biodiversity in Tunisian agricultural and forest soils.

More recently, biodiversity studies have revealed an even richer and more complex *Trichoderma* community structure. For example, Saadaoui et al. (2023) applied multilocus phylogenetic analyses (ITS, tef1, and rpb2) to isolates from wheat rhizosphere soils and identified not only the dominant *T. harzianum* but also *T. afroharzianum*, *T. atrobrunneum*, *T. lixii*, and *T. lentinulae*, reported for the first time in Tunisia.

Additionally, studies such as Yangui et al. (2018) have isolated *T. harzianum* as an endophyte from cork oak (*Quercus suber*) forests in northwestern Tunisia, demonstrating the ecological plasticity of these fungi beyond cultivated soils.

IV.5 *Trichoderma* as a potential antagonist

Trichoderma, present in a wide variety of soils throughout the world, is widely recognized as an opportunistic non-pathogenic symbiont of plants (Samuels, 2004). A key feature of several *Trichoderma* species is their mutualistic interaction with plants as endophytes, enabling them to play a crucial role as antagonistic agents (AA) against various fungal plant diseases (Bongiorno et al., 2016). These fungi are able to establish themselves easily in the rhizosphere of plants, where they can survive for several months (Ellis, 2013).

The presence of *Trichoderma* in the soil plays a key role in suppressing the population of other competitive microorganisms, consolidating its role as the dominant biocontrol agent at local level (Rasool Azarmi, 2011). In the rhizosphere, a wide variety of microorganisms coexist, including *Trichoderma* and root exudates, which favor the attraction and colonization of this fungus (Vinale et al., 2008; Table 2).

The activation of plant defenses by *Trichoderma* is enhanced by the production of various MAMPs (Microbial-Associated Molecular Patterns), such as xylanases, swollenins, peptaibols and cerato-platanins, which play a key role in pathogen resistance (Maaløe, 2002; Mathys et al., 2012).

IV.6 Efficacy and benefits of *Trichoderma* in crop protection

As an antagonist, *Trichoderma* offers several advantages over other biocontrol agents.

IV.6.1 Effects on root colonization rate

Trichoderma colonization refers to its ability to adhere to and penetrate plant roots, resisting the toxic metabolites they secrete in response to attack from foreign organisms. *Trichoderma* species are particularly effective at rapidly colonizing roots and stimulate plant defense mechanisms such as the production of chitinases, β-1,3 glucanases, phenylalanine, peroxidases and hydroperoxidase lyase. They also activate signaling pathways involved in the biosynthesis of phytoalexins, low-molecular-weight antimicrobial compounds. *Trichoderma*'s resistance to toxic plant metabolites by several species of the genus is linked to the presence of ABC transport systems in its strains, which enable it to tolerate these compounds better than other fungi. This ability to colonize roots is enhanced by *Trichoderma* strains possessing repellents, hydrophobins and overexpression of ABC transporters. For example, electron microscope studies have shown that *T. harzianum* penetrates to the outer cortex of cucumber roots, resulting in increased production of chitinase and peroxidase. This interaction appears to be symbiotic:

Trichoderma appropriates nutrients while protecting the plant from disease. The *T. harzianum* strain, for example, stimulates the growth of plants such as tomatoes, cotton and tobacco, while protecting them against various fungal pathogens.

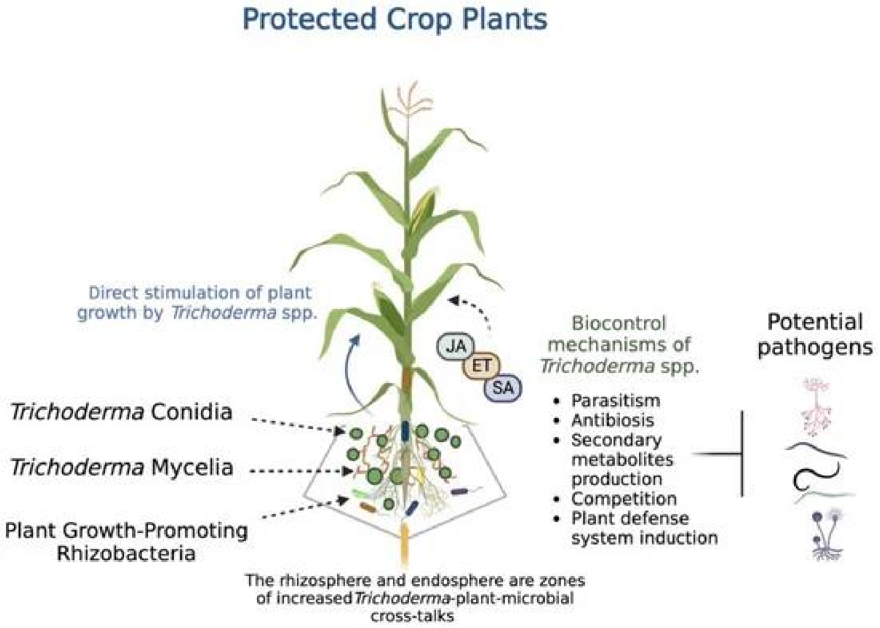


Figure 8: *Direct biostimulation and biocontrol properties of Trichoderma species. ((Guzmán-Guzmán* et al.*, 2023))*

IV.6.2 Effect of *Trichoderma* on Plant Tolerance to Abiotic Stresses

Trichoderma also shows remarkable resilience to various abiotic stresses, improving plant tolerance to difficult environmental conditions. Seeds treated with this fungus grow faster than untreated ones, even under conditions of heat, salt or osmotic pressure stress, suggesting that the fungus-plant association improves tolerance to abiotic stresses (Ahmad et al., 2015). Studies have shown that *Trichoderma* promotes root growth, nutrient uptake, particularly potassium, and water retention, factors that contribute to better plant adaptation to stressful conditions (Rasool Azarmi, 2011; Bimenya et al., 2002). In addition, some *Trichoderma* species, such as *T. harzianum* and *T. asperellum*, have demonstrated significant resistance to high salt concentrations, making them particularly effective in saline soils (Poosapati et al., 2014).

In addition, *Trichoderma* can grow even in competitive soils and resist toxic chemicals such as insecticides and fungicides, thanks to its ABC transport system, which confers some resistance to chemical compounds (Chaparro et al., 2011; Harman, 2006). Regarding thermal tolerance, some *Trichoderma* isolates can withstand temperatures as high as 52°C, demonstrating their ability to survive in extreme conditions while maintaining vigorous hyphal growth (Poosapati et al., 2014). Finally, *Trichoderma'* s production of siderophores under conditions of low nutritional availability enables it to limit the growth of pathogens, reinforcing its effectiveness as a biocontrol agent (Vinale et al., 2013).

IV.6.3 Effect of *Trichoderma* in Plant Protection against Biotic Pathogens

Trichoderma is renowned for its ability to enhance plant resistance to pathogens, an interaction that plays an important role in biological control. In agricultural fields, *Trichoderma spp.* is found mainly in the rhizosphere soils (directly connected to plant roots) and non-rhizosphere soils (away from roots), where it is one of the dominant fungi (Yadav et al., 2000). It has been observed that inoculating plants with *Trichoderma* increases their resistance to attack by pathogens, whether fungi or other harmful microorganisms. This fungus acts by colonizing roots and inhibiting pathogen growth, not only at the time of inoculation, but also when applied at different times or sites in relation to the pathogen (Yadav et al., 2000). *Trichoderma* species also show strong resistance to a variety of ecological conditions, and the biocontrol effect is particularly effective under biotic stress, such as infestation by soil-borne pathogens. Competition for nutrient resources, particularly iron, and the production of antifungal compounds, enable *Trichoderma* to limit the growth of other pathogenic fungi, such as *Fusarium* and *Botrytis* (Vinale et al., 2013; Thangavelu et al., 2004).

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Chapter II: Geographical distribution of *Fusarium* species involved in Fusarium Head Blight and Fusarium Crown Rot and their related mycotoxin accumulation

Geographical distribution of Fusarium species involved in Fusarium head blight and Fusarium crown rot of wheat in Tunisia and their mycotoxin accumulation

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Abstract

Fusarium Crown Rot (FCR) and Fusarium Head Blight (FHB) are among the most destructive diseases of wheat worldwide. A broad range of *Fusarium* species is associated with both diseases and can be isolated from stems and kernels of wheat plants. *Fusarium culmorum* and *F. graminearum* are the most frequent species involved, and both can produce the harmful mycotoxin deoxynivalenol (DON) that inhibits protein synthesis. This study aimed to assess the distribution of both Fusarium species and DON accumulation in crowns and kernels in geographic areas where wheat is cultivated in Tunisia. Sixty- four fields from four bioclimatic zones were sampled during two crop seasons (2020/2021, 2021/2022). Based on morphological traits, a subset of 250 representative Fusarium strains was identified by elongation factor 1α gene sequencing. DON contamination in wheat samples was determined by HPLC/DAD. Chemical analyses showed that DON contamination in crowns ranged from around 0.1 to 17 mg kg^{-1}, while in kernels it was detected at a very low level (up to 2.4 mg kg^{-1}). *F. culmorum* was the species frequently isolated from both crown and kernel samples. However, several

Fusarium species, belonging to eight different phylogenetic species com plexes, were isolated, showing a greater diversity in the crown than in the kernels. We report here for the first time the occurrence of *F. anthophilum*, *F. nygamai*, *F. algeriense*, *F. redolens* and *F. brachygibbosum* in Tunisian wheat. Moreover, this study provides important information on the toxicological risk related to DON contamination of wheat in Tunisia.

1 | Introduction

Durum wheat, extensively cultivated in the Mediterranean basin, represents one of the most important staple food crops in the human diet worldwide, providing an essential source of car bohydrates, fibre, proteins and vitamins. Among Mediterranean countries, in Tunisia, wheat is cultivated on a surface area of about 0.6 million hectares with an estimated annual production of 1–1.5 million tonnes (FAOSTAT 2022). The production and quality of this crop are largely influenced by several environmental factors and biotic stresses due to the broad range of microorganisms that can colonise the wheat plants during the whole crop season (Leslie et al., 2021). Among the fungi involved in wheat diseases, mycotoxigenic genera, especially Fusarium, are of great concern as they represent both a phytopathological issue and a toxicological risk for the consumers (Leslie et al., 2021). Indeed, many species of *Fusarium* can produce an array of mycotoxins in planta that are harmful products of their secondary metabolism (Leslie et al., 2021). The two most important wheat diseases, FCRand FHB, are caused by attacks of multiple *Fusarium* species (Leslie et al., 2021; Ma et al., 2024). FCR is one of the dominant soilborne diseases in semi- arid environments such as the Pacific Northwest, Australia and the Mediterranean regions. In Tunisia, Fusarium species have been widely documented as significant contributors to wheat diseases (Gargouri et al., 2003; Khemir et al., 2020). The potential yield loss is considerable, with studies reporting losses of up to 44% in bread wheat and up to 56% in durum wheat (Chekali et al., 2024). The characteristic symptoms of FCR include the formation of 'white heads' containing shrunken or missing grains. The pathogen hinders water transport at the base of the stems, leading to the formation of white heads, and infected plants show browning at the base of the stems (Ma et al., 2024). The most commonly reported causal agents of FCR worldwide are *F. culmorum* and, to a lesser extent, *F. pseudograminearum*, the latter considered by the European Food Safety Agency (EFSA) as a potential quarantine microorganism in European Union territory (EFSA 2022) but often misidentified in the past as *F. graminearum* Group 1 (EFSA 2022). In addition, together with these fungal species, some other mycotoxigenic Fusarium species can be isolated from FCR- diseased plants (Ma et al., 2024). On the other hand, although FHB has been reported in all cereal cultivation areas, it is detected more in regions with temperate and subtropical climates, characterised by high humidity, moderate temperatures and frequent rainfall during the flowering stage of wheat. *F. graminearum* and *F. culmorum*, members of the *F. sambucinum* species complex (FSamSC) are reported as the main species causing FHB worldwide, although several other Fusarium species are frequently associated with this disease, such as *F. avenaceum*, a member of the *F. tricinctum* species complex (FTSC), F. cerealis, *F. langhsethiae*, *F. poae*, members of the FSamSC and members of the *Fusarium incarnatum-equiseti* species complex (FIESC; Haidukowski et al., 2022). In addition to direct yield losses, the Fusarium species causing FCR and FHB can produce a range of mycotoxins that have toxic effects on humans and domestic animals. These toxins include trichothecenes, potent inhibitors of protein synthesis, causing symptoms such as skin irritation, haemorrhagic syndrome, feed rejection and vomiting (Desjardins, 2006). In addition, Fusarium species can produce zearalenone (ZEA), related to oestrogenic disorders in humans and animals, the possible carcinogenic fumonisins (FUMs) and other less toxic mycotoxins such as moniliformin (MON), enniatins (ENNs) and beauvericin (BEA; Munkvold et al., 2021). Trichothecenes are classified into two main groups: type A, that includes T-2 toxin (T-2), HT-2 toxin (HT-2) and diacetoxyscirpenol (DAS), and type B, that includes deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives 3- and 15- acetyl- deoxynivalenol (3- AcDON and 15- AcDON) and 4-acetyl-nivalenol (Desjardins, 2006). Several national and international authorities have adopted regulatory limits and guidelines to monitor mycotoxin levels in various food and feed products. In particular, the limit of 1750 µg kg^{-1} has been fixed for DON in unprocessed wheat, whereas the limit of 100 µg kg^{-1} has been fixed for ZEA and, re cently, for T-2 and HT-2 (Commission Regulation EU 2024/1038, https://eur-lex.europa.eu/eli/reg/2024/1038/oj/eng) in unprocessed durum wheat. Among these mycotoxins, DON has been proven to act as a pathogenicity/virulence factor for infection of wheat heads, thus playing a role in the pathogenesis of FHB (Proctor et al., 1995). While the role of DON in FHB has been largely studied, the production and role of DON in FCR during the colonisation of stem tissues have been rarely reported. Moretti et al., (2014) reported the induction of DON synthesis by *F. graminearum* during the infection of the stem base, having a role in stem colonisation by the fungus. Beccari et al., (2011) and Covarelli et al., (2012) have reported that *F. culmorum* caused disease symptoms in the whole plant before actual fungal colonisation, suggesting a role of DON in inducing a host response far from the colonisation points. Moreover, all three studies mentioned above have shown that, although both *F. graminearum* and *F. culmorum* cannot grow systemically beyond the third node, DON synthesized by the pathogens in the stems of wheat plants has been detected in the last internode and even in the heads. The translocation of DON from stem base to the head indicates that FCR infection may be considered an additional source of mycotoxin contamination of wheat kernels (Moretti et al., 2014). On the other hand, especially in semi- arid regions, less attention has been given to stems, despite their use in animal feed and the potential risk they pose to animal health (Häggblom and Nordkvist 2015). Climate plays a crucial role in the development of both FCR and FHB and the production of DON in the wheat plants. However, the reports on both diseases in Tunisia are often related to a single location and limited sampling and periods. A wide survey would provide a significant overview of the occurrence of FCR and FHB in the main durum wheat-growing areas of the country as well as mycotoxin contamination in wheat tissues. Therefore, this study aimed to (1) evaluate the occurrence of FCR and FHB causal agents in different geographical areas of northern Tunisia where the wheat crops are mostly cultivated; (2) identify the Fusarium species associated with FCR and FHB diseases; and (3) assess the DON contamination in wheat crowns and kernels.

2 | Materials and Methods

2.1 | Assessment of Fusarium Presence in Wheat Fields

During the 2020/2021 and 2021/2022 crop seasons, a total of 64 wheat fields were surveyed to evaluate the occurrence of FCR and FHB, identify and characterise the species associated with these diseases and assess DON contamination in both wheat crowns and kernels. The fields were selected from various climatic zones within Tunisia's cereal- growing regions in collaboration with the National Institute of Field Crops, ensuring a representative sample. Twenty- five fields were surveyed during both 2021 and 2022, while an additional 14 fields were included in 2022. The locations were as follows: 16 fields in the humid bioclimatic zone (Nefza region), 18 fields in the Eastern sub- humid zone (Bizerte region), 16 fields in the Western sub- humid zone (Bousalem and Beja regions) and 14 fields in the semi- arid bioclimatic zone (Zaghouan region; Figure 1, Table 1).

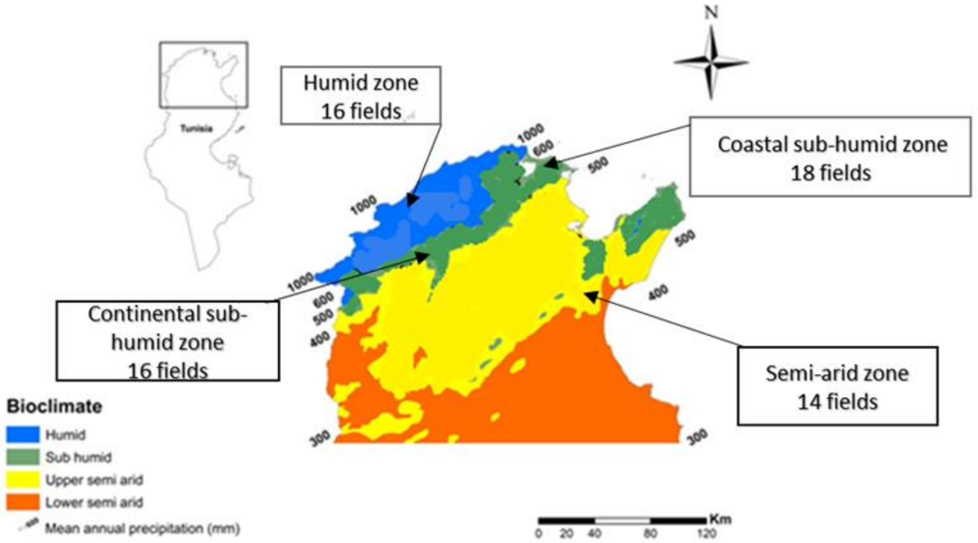

Figure 1. *Fusarium species distribution of surveyed fields in the different bioclimatic zones in northern Tunisia during 2020-2022 crop seasons.*

TABLE 1 | Range and mean values of Fusarium frequency detected on wheat crowns and kernels collected from fields located in different bioclimatic zones during two surveyed years.

		***Fusarium* frequency (%)**							
		Crowns				Kernels			
		2021		2022		2021		2022	
Bioclimatic zone	**No. of inspected fields**	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Humid	16	60-96	77	60-100	80	0-3	1	0-3	1,4
Eastern sub-humid	18	48-80	59	40-90	64	02-06	4,1	0-2	0,4
Western sub-humid	16	40-44	42	14-60	37	0-2	1	0-2	1
Semi-arid	14	08-18	12	14-62	28	0-3	0,8	0	0

2.2 | Sampling and Fungal Isolation

For each field surveyed in 2020/2021 and 2021/2022, 50 plants were randomly collected and used for detecting Fusarium presence and for fungal isolation. These plants were also used to evaluate the DON accumulation both in crowns and kernels. For each field, one piece collected from the crown of each plant (50 plants) and subsamples of 100 kernels randomly selected from the whole harvested grains were surface- disinfected with 2% sodium hypochlorite solution for 2 min, followed by two rinses with sterile distilled water (SDW) for 1 min, and transferred on potato dextrose agar (PDA) amended with 100 mg L−1 streptomycin sulphate and 50 mg L−1 neomycin sulphate. Petri dishes were incubated at 25°C ± 1°C for 5–7 days under a 12 h light/dark cycle. After incubation, the developed fungal colonies were observed under a stereoscope in order to select all fungal species morphologically identified as Fusarium species. For each field, the number of kernels/crowns infected by Fusarium species out of the total plated kernels or crowns was used to calculate the Fusarium incidence, expressed as percentage. Two hundred and fifty isolates, selected to represent all morpho logically identifiable types (Leslie and Summerell 2006), were used for identification by gene sequencing (Table S1).

2.3 | Molecular Identification of Fusarium Species

Two hundred and fifty isolates, 134 from crowns and 116 from kernels, were grown for 3 days at 25°C on cellophane disks over laid on PDA plates. Mycelium of each isolate was collected, frozen and lyophilised. Genomic DNA was extracted and purified from 10 to 15 mg of lyophilised mycelia using the Wizard Magnetic DNA kit (Promega) according to the manufacturer's instructions. Quantity and integrity of DNA were checked by a spectrophotometer (Thermo- Scientific NanoDrop) and electrophoresis on a 0.8% agarose gel by comparison with a standard DNA (1 kb DNA ladder; Fermentas). Elongation factor gene (EF-1α), the most informative marker used for Fusarium identification at species level (Geiser et al., 2004), was amplified and sequenced using the primer pair EF1/EF2 (O'Donnell et al., 1998). In detail, the PCR mixture (15 µL) containing 15 ng DNA template, 1.5 µL (10×) Hot Start buffer, 0.45 µL of each primer (10 mM), 1.2 µL of dNTPs (2.5 mM each) and 0.125 µL of Hot Start Taq DNA polymerase (5 U/µL; Fisher Molecular Biology) was amplified using the fol lowing reaction conditions: an initial 2 min at 95°C; followed by 35 cycles of 50 s at 95°C; 50 s at 59°C; 60 s at 72°C; and a final 7 min at 72°C. The PCR products were verified by electrophoresis on a 1.5% agarose gel and visualised on a UV transilluminator. PCR products were purified with the enzymatic mixture EXO/ FastAP (ExonucleaseI, FastAP thermosensitive alkaline phosphatase; Thermo Scientific). Sequence reactions were performed for both strands using a BigDye Terminator v. 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacturer's recommendations. Labelled products were purified by filtration through Sephadex G- 50 (Sigma- Aldrich) and analysed in ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Partial FASTA sequences were analysed and assembled using the BioNumerics v. 5.1 software (Applied Maths) and aligned using the ClustalW algorithm (Thompson et al., 1994). Phylogenetic relationships among and within Fusarium species were studied using the maximum- likelihood method with MEGA 7 software (Kumar et al., 2016). The bootstrap analyses (Felsenstein 1985) were conducted to determine the confidence of internal nodes using a heuristic search with 1000 replicates. Sequences derived in this study were deposited in the GenBank database (Table S1).

2.4 | Deoxynivalenol Analysis

Kernels and crowns from each field were respectively bulked, and subsamples were finely ground using an MLI 204 laboratory mill (Bühler S.p.A.). Five grams of crowns was extracted with 25 mL acetonitrile/ water (84:16, vol/vol) by orbital shaking for 60 min at 250 rpm. The extract was filtered through filter paper (Whatman no. 4), and 8 mL was cleaned up through a Mycosep #227 column (Romer Labs). The purified extract (5 mL equivalent to 1 g) was dried down under an air stream at 50°C and reconstituted in 500 mL of HPLC mobile phase. Five grams of kernels were extracted with 25 mL of water by blending. Extracts were filtered through filter paper (Whatman no. 4) and glass microfibre filter (Whatman GF/A). Then, 2 mL of extract was cleaned up by DONTest immunoaffinity column (VICAM). The eluate was dried and reconstituted in 250 mL of HPLC mobile phase. An aliquot of 100 µL of extract was injected into an HPLC appa ratus (Agilent 1100 Series), with the column heater set up to 40°C and a diode array (DAD) detector that was set to 220 nm. The analytical column was a Synergi Hydro-RP 80A (150 × 3 mm, 4 µm, Phenomenex). The mobile phase was acetonitrile/water (10:90, vol/vol). The flow rate of the mobile phase was 0.5 mL/ min. In these analytical conditions, the retention time of DON was 5.1 min. The detection limits (LOD) of DON, based on a signal- to-noise ratio of 3:1, were 0.05 mg kg−1 in crowns and 0.005 mg kg−1 in kernels. The mycotoxin was quantified by comparing peak areas with a calibration curve obtained with standard solutions.

2.5 | Statistical Analysis

Data were analysed using Student's t test, analysis of variance (ANOVA) with Graphpad Prism 8 software. Significance of mean differences was determined using the HSD test of Tukey, and responses were judged significant at the 5% level ($\alpha = 0.05$) with a 95% confidence interval. The correlations between the Fusarium contamination and DON amount were determined by Pearson correlation using the same software.

3 | Results

3.1 | Incidence of *Fusarium* from wheat crowns and kernels

The survey conducted in the 64 selected fields showed that Fusarium species were detected in all fields at crown level, while 48% of the fields showed kernel infection. Therefore, 48% of the fields exhibited simultaneous infection in both plant tissues. Overall, there was a significant difference in the incidence of *Fusarium* species isolation between wheat crowns and kernels ($p < 0.001$). The contamination ranged from 8% to 100% on wheat crowns and from 0% to 6% on wheat kernels during the two cropping seasons studied (Table 1). Additionally, the growing season did not significantly affect *Fusarium* incidence in either crowns or grains, with p values of 0.71 and 0.33, respectively (Figure 2, Table 2)

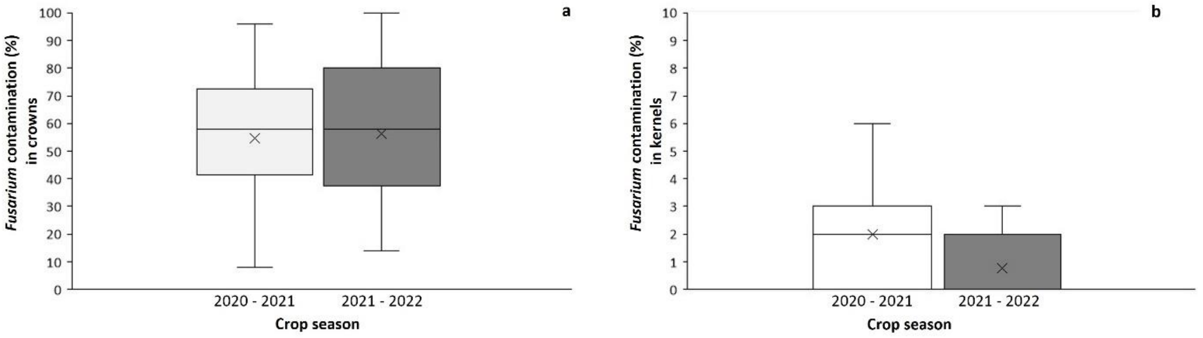

Figure 2. *Fusarium presence in wheat crowns (a) and kernels (b) during the cropping seasons 2020-2021 and 2021-2022.*

TABLE 2 | Statistical analysis of the Fusarium frequency in crowns and kernels between the two crop seasons (2020-2022) using Student's t test.

	Kernels	**Crowns**
P value	0,3335	0,707
Difference between means± SEM	-0,7400 ± 0,7190	1,886 ± 6,233
R^2 (η^2)	0,1169	0,001475

The frequency of isolation of *Fusarium* species from crowns varied significantly among the considered bioclimatic zones, ranging from 60% to 100% in the humid zone, from 40% to 90% in the Eastern sub- humid zone, from 14% to 60% in the Western sub- humid zone, and, with the exception of a single field (62% contamination), from 8% to 22% in the semi- arid zone (Table 1, Figure 3). In contrast, the *Fusarium* infection of kernels showed no significant difference among the bioclimatic zones with a p-value of 0.24 (Table 3).

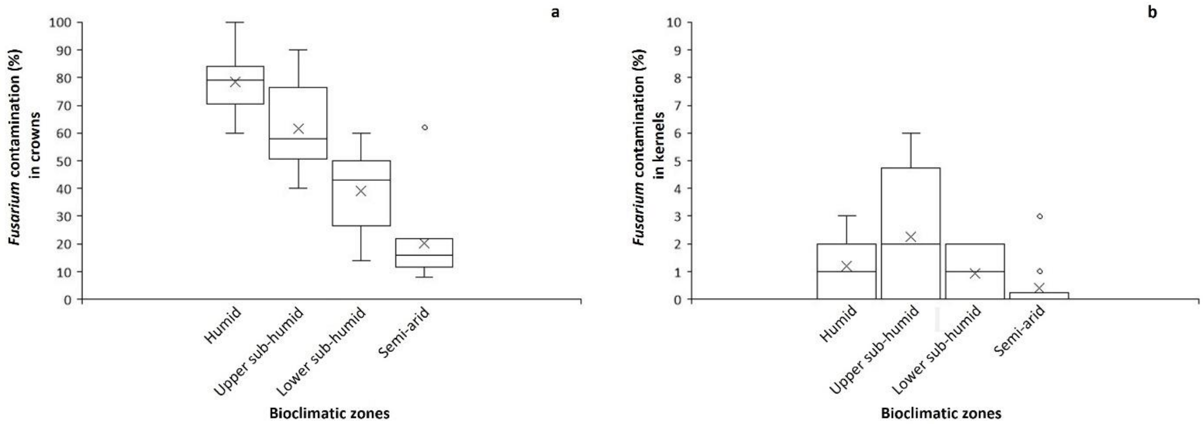

Figure 3. *Fusarium presence in wheat crowns (a) and kernels (b) collected from four different bioclimatic zones of Tunisia. × represents the mean value and the line inside the boxes represents the median.*

TABLE 3 | ANOVA analysis of Fusarium incidence in crowns and kernels across the different studied bioclimatic zones.

		Sum of Squares	P value
Kernels	Zone	3.522	0,2372
	Residual	9.348	
Crowns	Zone	38.911	<0.0001
	Residual	22.034	

3.2 | Morphological and molecular dentification of *Fusarium* strains

During the two crop seasons, 618 *Fusarium* strains were ob tained from both crowns and kernels. Based on morphological traits, 12 different species were detected from crowns and nine species from kernels (Table 4). For *Fusarium* species distribution, some species, including *F. algeriense* and members of FIESC, *Fusarium solani* (FSSC), *F. oxysporum* (FOSC) except for a single isolate from kernels, and *F. fujikuroi* (FFSC) species complexes were isolated only from crowns, while *F. graminearum*, *F. sambucinum*, both belonging to FSamSC and *F. avenaceum* (except for a single isolate from crown), were obtained only from kernels (Table 4). *Fusarium culmorum* was the predominant species occurring on both crown and kernel samples.

It was detected in all monitored fields except for fields located in the semi-arid zone, with frequency ranging between 48.4 and 84.3% on crowns and between 67.5 and 90.4% on kernels (Table 4). *F. culmorum* was not detected in the semi- arid zone, where the dominant spe cies (41.3%) occurring on crowns was identified as members of FIESC (Table 4). A set of 250 *Fusarium* strains (Table S1), selected as representative of the species detected on crowns and kernels according to morphological traits and source of isolation, was genetically identified at species level by sequencing an EF-1α gene fragment. In particular, for each strain, a specific PCR amplicon of about 650 nucleotides was obtained and sequenced. For phylogenetic analysis, all sequences were aligned and cut at the ends in order to consider a common fragment of around 530 positions. The phylogenetic tree (Figure 4) was resolved into nine well- separated clades, supported by high significance (bootstrap val ues > 70). The clades corresponded to FSamSC (Clade A), FIESC (Clade B), *F. brachygibbosum* (Clade C), FSSC (Clade D), FTSC (Clade E), FOSC (Clade F), FFSC (Clade G), *F. redolens* species complex (Clade H) and *F. burgessii* species complex (Clade I). Most of the strains (69%) clustered in Clade A. In particular, 166 isolates, 72 from crowns and 94 from kernels, were identified as *F. culmorum*, grouping together with *F. culmorum* reference strain NRRL 52792. In the same clade, three isolates from ker nels showing 100% homology grouped with *F. graminearum* reference strain NRRL 52929. In particular, BLAST analysis al lowed better identification of these three isolates as *F. brasilicum* (100% identity with NRRL 31238). In this clade, a single isolate was identified as *F. sambucinum*, and three isolates, one from crown and two from kernels, were identified as *F. pseudograminearum*. In Clade B, all 14 isolates were obtained from crowns. In detail, 11 out of 14 showed 100% homology among them and with *F. clavum* reference strain CBS 131015, and a single isolate showed 100% homology with *F. equiseti* ITEM 7633. Only five isolates, four from crowns and one from kernels, grouped with *F. brachygibbosum* reference strain NRRL 25093, although a great variability was observed. In the FTSC (Clade E), two well-separated groups were detected: 10 isolates were identified as *F. avenaceum* and six isolates as *F. acuminatum*. In Clades D and F, corresponding to FSSC and FOSC, respectively, all isolates were obtained from crowns, with the exception of a single isolate from kernels. In particular, in Clade F, we observed three differ ent groups: a group including seven isolates and two including three isolates each (Figure 4). BLAST analysis showed that each group showed 100% of identity with *F. oxysporum* NRRL 52763, *F. vasinfectum* ATCC 7808 and *F. curvatum* CBS 247.61 strains, respectively. In Clade G, corresponding to FFSC, all isolates were obtained from crowns, three identified as *F. anthophilum* and three as *F. nygamai*. Seven isolates (six from crown and one from kernels) were identified as *F. redolens* (Clade H) and 13 isolates, also from crowns, showed high similarity with *F. algeriense* NRRL 666533 reference strain (Clade I).

TABLE 4 | Distribution of Fusarium species, expressed as a percentage, detected in kernels and crowns of wheat plants collected in 2020–2022, in four bioclimatic zones of northern Tunisia.

	Crowns				**Kernels**			
	Humid	Eastern sub-humid	Western sub-humid	Semi-arid	Humid	Eastern sub-humid	Western sub-humid	Semi-arid
Fusarium contamination (%)	78	62	39	20	1.2	2.2	0.9	0.4
***Fusarium* species**								
F. culmorum	84.3	48.4	66.7	n.d.	90.4	88.2	67.5	n.d.
F. pseudograminearum	n.d.	1.6	n.d.	n.d.	n.d.	n.d.	4.6	n.d.
F. sambucinum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.3	n.d.
F. graminearum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.0	n.d.
FOSC	3.2	15.6	4.7	n.d.	n.d.	n.d.	2.3	n.d.
FIESC	n.d.	6.3	14.3	41.3	n.d.	n.d.	n.d.	n.d.
F. acuminatum	6.3	n.d.	n.d.	n.d.	3.2	n.d.	7.0	50.0
F. avenaceum	n.d.	1.6	n.d.	n.d.	6.4	9.5	7.0	50.0
FSSC	n.d.	3.1	n.d.	5.8	n.d.	n.d.	n.d.	n.d.
F. brachygibbosum	n.d.	1.6	14.3	n.d.	n.d.	n.d.	2.3	n.d.
F. algeriense	n.d.	14.0	n.d.	23.5	n.d.	n.d.	n.d.	n.d.
F. redolens	n.d.	6.3	n.d.	11.8	n.d.	2.3	n.d.	n.d.
F. anthophilum	6.2	n.d.	n.d.	5.8	n.d.	n.d.	n.d.	n.d.
F. nygamai	n.d.	1.5	n.d.	11.8	n.d.	n.d.	n.d.	n.d.

Abbreviations: *FIESC*, *Fusarium incarnatum-equiseti* species complex; *FOSC*, *Fusarium oxysporum* species complex; *FSSC*, *Fusarium solani* species complex, n.d., not detected.

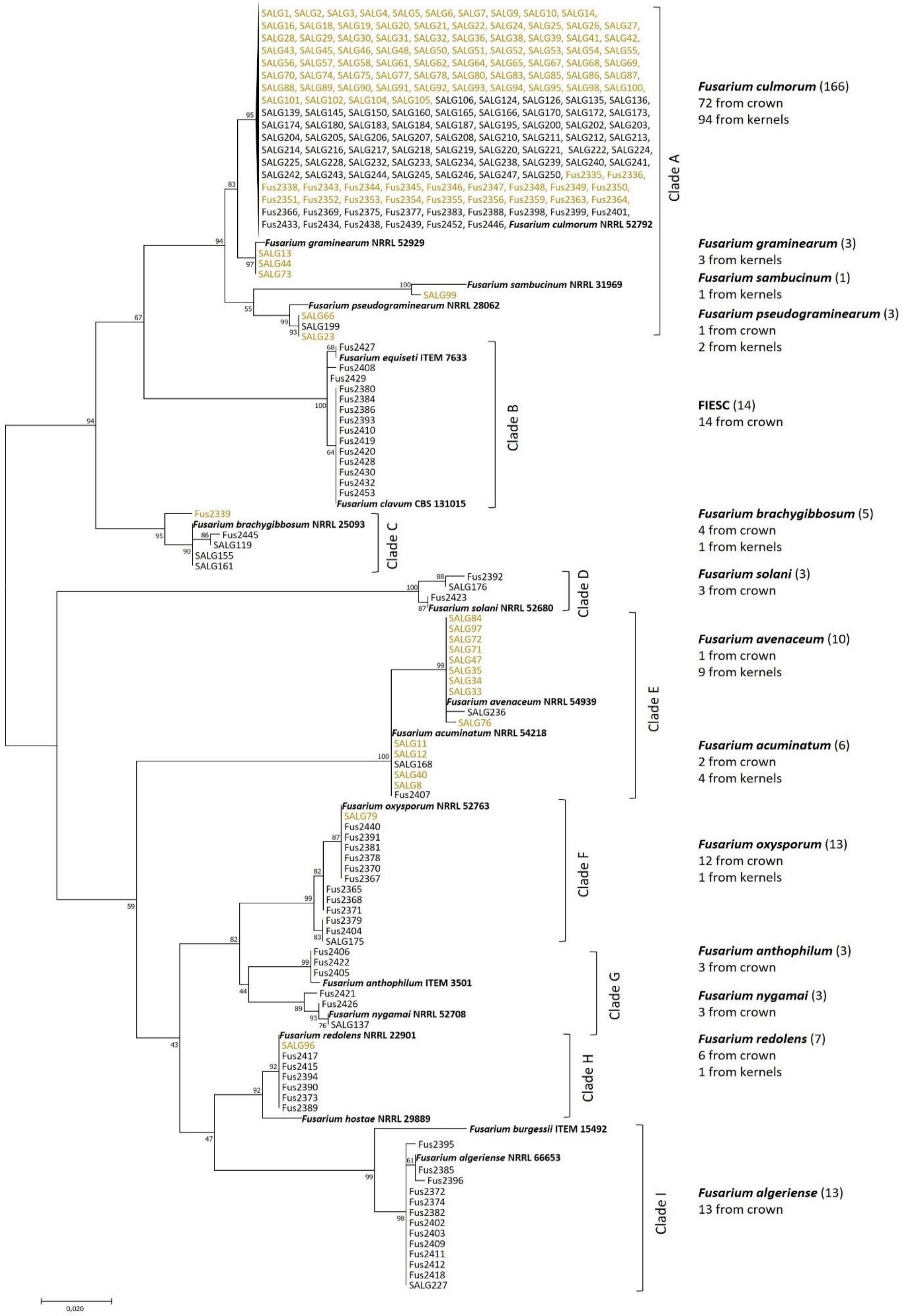

FIGURE 4 | Legend on next page.

Figure 4. *Phylogenetic tree based on EF-1α gene sequences of 250 Fusarium strains isolated from wheat plants in Tunisia and 17 reference sequences. The phylogenetic tree was generated by the Maximum Likelihood Method; bootstrap values >70 with 1000 replicates are shown near the branches. Fusarium strains from kernels are shown in orange, strains from crown in black. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.*

3.3 | Detection of DON in wheat crown and kernels

DON contamination was assessed on kernels and crown samples collected from 64 fields. During the two cropping seasons, from 29% (in semi- arid zone) to 75% (in humid zone) of crown samples were contaminated by DON, depending on the bioclimatic zone considered, with values ranging from 0.1 to 17 mg kg^{-1} (Table 5). In contrast, DON contamination was detected in more than half of the kernel samples (from 56% in the humid region to 75% in Western sub- humid zone), although at very low levels, ranging between 0.005 and 2.4 mg kg^{-1} (Table 5). However, a significant correlation between the DON amount in the crowns and in the kernels was not observed ($r = 0.35$). Furthermore, the growing season had no significant effect on the occurrence of DON in both crowns and kernels, with p-values of 0.2404 and 0.3687, respectively. Within each bioclimatic zone, the DON contamination in crowns varied significantly, ranging from 0.4 to 11.3 mg kg^{-1} in the humid zone, from 0.1 to 17 mg kg^{-1} in the Eastern sub-humid zone, from 0.4 to 2.6 mg kg^{-1} in the Western sub- humid zone, and from 0.2 to 10 mg kg^{-1} in the semi- arid zone (Table 5). However, within the kernel contamination, no significant differences were observed between the climatic regions, because almost all values never exceeded 0.8 mg kg^{-1}, except for two samples collected in the humid and in the Eastern sub-humid regions (2.4 and 1.15 mg kg^{-1}, respectively). The Pearson correlation test revealed a slight positive correlation between the incidence of *Fusarium* infection and DON level in the crown tissue (Figure 5a), with a coefficient of 0.28. This correlation is statistically significant ($p < 0.05$), suggesting that higher levels of *Fusarium* infection are associated with increased DON levels. In contrast, at kernel level, there was no significant correlation between

Fusarium incidence and DON level (r = 0.11, p = 0.90; Figure 5b). This is probably due to the high incidence of zeroes in the dataset.

TABLE 5 | Deoxynivalenol (DON) amount detected on wheat kernels and crowns collected from different bioclimatic zones during 2020–2022 crop seasons.

Bioclimatic zone	Inspected fields (n.)	Crowns			Kernels		
		Positive samples (%)	DON (mg kg^{-1})		Positive samples (%)	DON (mg kg^{-1})	
			Range	Mean value		Range	Mean value
Humid	16	75	0.4-11.3	1.8	56	0.01-2.4	0.22
Coastal sub-humid	18	72	0.1-17.0	3.7	72	0.005-1.15	0.08
Continental sub-humid	16	44	0.4-2.6	0.7	75	0.007-0.12	0.02
Semi-arid	14	29	0.2-10.0	1.1	64	0.002-0.03	0.006

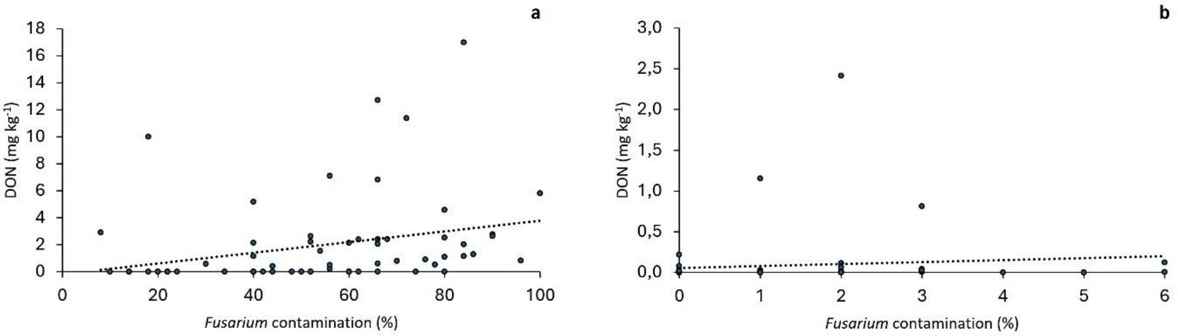

Figure 5. *Correlation between Fusarium presence and the occurrence of DON in crown (a) and in kernel (b) samples.*

4 | Discussion

The results of this study highlight significant differences in the incidence of *Fusarium* species causing FCR and FHB across northern Tunisia's bioclimatic zones. As previously reported in studies carried out in Algeria, Morocco and southern Italy (Abdallah-Nekache et al., 2019; El Yacoubi et al., 2012; Laraba et al., 2017; Touati- Hattab et al., 2016), in this study, *F. culmorum* is also considered the main FCR causal agent. FCR is more abundant in areas with high temperatures and drought (Balmas et al., 2015; Obanor and Chakraborty 2014), conditions that char acterise many countries of the southern Mediterranean area. In our study, all fields showed contamination at the crown level (up to 100%), while only 48% of fields had grains infected by Fusarium species (up to 6%). These results corroborate those of Kammoun et al., (2009), who found that FHB rarely occurs in Tunisia and is mainly confined to the humid and Eastern sub-humid regions, where irregular rainfall and predominance of the rain- fed cropping system could lead, under particular conditions, to the disease. These findings also align with observations made in other southern Mediterranean areas by Abdallah- Nekache et al., (2019) in Algeria and Balmas et al., (2015) in Sardinia, southern Italy, where FCR has proven to be endemic, while FHB appears sporadically. In another geographic area, Australia, characterised by similar environmental conditions as northern Africa, Obanor and Chakraborty (2014) reported that FCR is widespread unlike FHB, which is restricted to more specific environmental conditions. Our results highlight a significant effect of bioclimatic zone on Fusarium contamination for FCR. The infection of crowns in the humid zone ranged from 60% to 100%, whereas in the semi- arid zone it was notably lower, as reported in a previous Tunisian study (Chekali et al., 2016). In contrast, no significant differences were observed in grain infection across the zones. This can be attributed to the low overall frequencies of infection detected in the grains. However, up to 48% of wheat fields had Fusarium- infected grains, showing that the occurrence of Fusarium species exhibits a significant variability, although lower than that reported for the species isolated from the crowns. Interestingly, different Fusarium species were identified within the same field in the comparisons between crowns and grains. These data could be related to results shown by Covarelli et al., (2012) and Moretti et al., (2014) who proved, respectively, that *F. culmorum* and *F. graminearum* infecting the crown could not grow systemically beyond the third node of the wheat plants and therefore the infections causing FHB, as a consequence of the colonisation of spikes by both species, are only due to infections occurring at flowering. The results obtained over the 2 years of sampling revealed that 12 Fusarium species were isolated from crowns and nine species from kernels. In Canada, Hafez et al., (2022) documented a higher variability in the diversity and abundance of *Fusarium* strains isolated in grains compared to those that occurred in crown and stem samples. Such variation could be attributed to several factors such as the different hydration of the colonised tissues, the environmental conditions at the different stages of plant growth, or the specific biological cycle of the different species of *Fusarium*. A great variability of *Fusarium* species was also reported in studies on the fungal diversity occurring in wheat tissues, such as stems (Łukanowski 2009) and spikes (Rojas et al., 2020). In addition, all the above-mentioned reports suggest that different plant tissues usually host a wide range of *Fusarium* species, both pathogenic and nonpathogenic, that compete in the same niche and influence plant health (Xu and Nicholson 2009). Although significant diversity was observed, *F. culmorum* was the predominant species in both crowns (54%) and grains (81%). These results are in line with those of Gargouri et al., (2003), who showed that *F. culmorum* is the main species isolated from crowns in all wheat-growing areas of Tunisia, and Kammoun et al., (2009), who demonstrated that *F. culmorum* is the predominant species on wheat kernels in Tunisia. This finding also aligns with other studies conducted worldwide, such as in Iraq (Matny et al., 2017), Algeria (Abdallah- Nekache et al., 2019) and Turkey (Tunali et al., 2008), where *F. culmorum* was consistently identified as the dominant agent of FCR and FHB. Interestingly, *F. culmorum* was not detected in semi-arid areas, which may be explained by the drought conditions during the years of sampling. As *F. culmorum* requires adequate humidity for infection, particularly during wheat plant emergence, the lack of moisture in semi-arid regions could limit its presence. Our findings are consistent with previous studies that have shown *F. culmorum* to be prevalent in high rainfall areas (Smiley et al., 2005; Strausbaugh et al., 2004; Xu et al., 2018), where sufficient moisture supports its infection cycle. Moreover, *F. culmorum* has been consistently reported as less frequent in semi-arid regions, as noted by Backhouse et al., (2004), Nelson Memorial Symposium (2001) and Özer et al., (2020). The wide occurrence of *F. culmorum* in the crown and its dominance in the grains trigger further studies on its genetic diversity and population structure. All 166 *F. culmorum* strains identified here were placed in a single clade through EF- 1α sequencing. Laraba et al., (2017) studied the genetic struc ture of a wide population of this species isolated from crowns and grains in Algeria by using microsatellite markers (SSRs) and identified two sympatric populations. Further studies on our set of strains of *F. culmorum* are in progress to assess its genetics in order to gain information aimed at better management of the disease in the field. In the semi- arid areas, mostly species members of FIESC and *F. algeriense*, and, to a lesser extent, *F. anthophilum*, *F. nygamai*, *F. redolens* and members of FSSC, were isolated from the crowns. This may be explained by the high adaptability of these species to drier climatic conditions. Previous studies, including those by Minati and Mohammed- Ameen (2019), have already reported the presence of FIESC and FSSC members as frequent patho gens in semi- arid environments. The high occurrence of FIESC members is of particular concern, because among these species, many strains can produce a wide range of mycotoxins, including type A and B trichothecenes (Villani et al., 2016), that could accumulate in planta. The discovery of species never reported before in Tunisia, such as *F. algeriense*, *F. anthophilum*, *F. nygamai*, *F. brachygibbosum* and *F. redolens*, occurring in crowns and rarely in kernels, indicates that the diversity of *Fusarium* species in this country is underestimated and highlights the need for regular monitoring of *Fusarium* species diversity, as each *Fusarium* species could have specific pathogenic potential in given ecological contexts. In addition, each *Fusarium* species has its own specific mycotoxin profile (Munkvold et al., 2021), and thus a correct species identification is important for a better mycotoxin risk assessment. The detection of members of the FOSC reveals a distinct distribution according to bioclimatic conditions, with increased frequency in the Eastern sub-humid zone, and absence in the semi-arid one. Although FOSC members are generally considered weak pathogens for FHB and FCR, their occurrence suggests that they could play a complementary role in the development of these diseases. On the other hand, within FOSC, many strains are nonpathogenic and are known to protect crops against FOSC pathogenic members, mainly due to their extensive ability to colonise the root surface and outcompete the pathogenic strains for nutrients. The pathogenicity of the FOSC strains isolated in this study is also under analysis through pathogenicity tests, to evaluate the possible use of nonpathogenic strains in wheat fields to control *Fusarium* pathogenic species causing FCR. Among species colonising the crowns, *F. brachygibbosum* has also been isolated with a significant percentage in the Western sub-humid climatic zone. Interestingly, this species rarely occurred in the grains at harvest, only within the same climatic zone. This is reason for concern, due to its ability to produce type A trichothecenes such as DAS and type B trichothecenes such as NIV (Laraba et al., 2021), that are all highly harmful mycotoxins. *Fusarium graminearum* was infrequently isolated exclusively from grains, specifically in the Western sub- humid zone. *F. graminearum* has rarely been reported in the southern Mediterranean countries, including Tunisia (Khemir et al., 2020), although this is one of the most important fungal species associated with wheat crops worldwide. Its rare occurrence is probably due to the climatic conditions, because *F. graminearum* requires high humidity at flowering. However, in particular climatic seasons, *F. graminearum* was the main species associated with FHB in sub- humid regions of Tunisia (Abdallah- Nekache et al., 2019). This suggests constant monitoring is required for the possible occurrence of *F. graminearum* in wheat kernels in Tunisia because of its ability to produce DON in planta, representing a severe risk for the final products at harvest. Indeed, DON occurred in crowns and kernels in 55% and 67% of the fields, respectively, with 16% of the fields showing contamination in both parts of the plant. The contamination in crowns was significantly higher than in grains, findings in agreement with the report of Minati and Mohammed- Ameen (2019) in Iraq. Our analyses revealed significant differences in DON contamination at the crown level between bioclimatic zones, highlighting a strong influence of humidity on fungal infection and mycotoxin production, consistent with findings by Abdallah- Nekache et al., (2019). The high contamination of crowns was also confirmed by Xu and Nicholson (2009) who suggested that crowns, being in closer contact with Fusarium-infested soil, tend to be contaminated by high DON levels when FCR occurs. In contrast, no significant variation in grain contamination was observed between bioclimatic zones. Similar findings have been reported in southern Italy, where DON contamination of kernels remained consistently low across various climatic conditions (Balmas et al., 2015; Haidukowski et al., 2022). The findings of this study highlight the critical need for rigorous mycotoxin monitoring, particularly in the basal parts of plants where DON contamination is more pronounced. This poses significant risks when using crop residues, such as straw, for animal feed. The results of the correlation between the incidence of Fusarium species and the quantification of DON also reveal distinct dynamics depending on the plant parts examined. A significant positive correlation was observed for the contamination at crown level, indicating that higher levels of Fusarium species incidence are associated with increased DON accumulation in this part of the plant, as reported by Minati and Mohammed-Ameen (2019) and Hafez et al., (2022). This correlation at crown level suggests that infections at basal level not only promote disease progression but also DON synthesis, thus contributing to greater contamination of basal tissues. Because *F. culmorum* is the dominant species in the crowns, and no other DON-producing species have been detected in the crown, except extremely rarely some strains of *F. pseudograminearum*, the occurrence of DON in stems and crowns can be associated with *F. culmorum*. The high level of DON occurrence in the crown is also a reason for concern for the final contamination of the kernels, because a proportion of the DON can be trans located from the stems to the spikes, as previously reported by Moretti et al., (2014). The high level of DON accumulation in the stems is worrisome not only for the possibility of translocation in the ears, but also because DON, which is an inhibitor of protein synthesis, is accumulated in the plant tissues from the early stages of growth, affecting some of the physiological functions of the plant and reducing its fitness and productivity. In contrast, no significant correlation was observed between *Fusarium* incidence and DON levels at grain level. This is mainly due to the low level of DON detected in the grains, making it difficult to establish a correlation, but also because of the possible translocation of DON from stems of FCR-affected plants. Therefore, a correct management strategy aimed at reducing DON accumulation should include seed coating with effective fungicides and eventually crop rotation and tillage. In conclusion, this study highlights that (a) FCR is widely spread in Tunisia in all bioclimatic zones, while FHB occurs rarely and in seasons characterised by high humidity from flowering to harvest; (b) the variability of Fusarium species is high in both crowns and spikes, with *F. culmorum* the predominant species in both parts of the plant; (c) DON occurs at high levels in crowns but shows poor contamination in kernels; and (d) the possible translocation of DON produced in crowns to the kernels must be better monitored. Therefore, our data stress the importance of accurate and continuous monitoring of Fusarium species and their related mycotoxins occurring in wheat plants in Tunisia and the need for effective management practices to protect both human and animal health, as well as the economic viability of affected crops.

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Chapter III: Investigation of the relationship between Fusarium Crown Rot, grain infection and DON mycotoxin contamination in nine wheat varieties in Tunisia

Background

Fusarium culmorum is a widespread and significant pathogen in Tunisia, primarily responsible for Fusarium crown rot (FCR) in wheat. This pathogen leads to substantial yield losses globally. For example, yield estimates from the Pacific Northwest of the United States indicate that FCR can reduce wheat grain yield by up to 35% under natural inoculum levels (Smiley *et al.*, 2005). Similarly, in Australia, FCR caused by *F. pseudograminearum* routinely results in 10% yield loss in cereals (Murray & Brennan), while in China, the loss can be as high as 51.6% (Xu et al., 2016). In Tunisia, recent reports from the semi-arid regions have documented yield losses as high as 56% (Chekali *et al.*, 2024). Thus, *F. culmorum* poses a dual threat to wheat production not only reducing grain yield, but also contributing to increased fungal contamination and facilitating the accumulation within plant tissues of harmful mycotoxins, such as DON, a potent inhibitor of protein synthesis, produced by some *Fusarium* species, including *F. culmorum*, toxic to both humans and livestock, posing significant risks to food safety and agricultural sustainability (Moretti *et al.*, 2014; Knight et al., 2017). Infected wheat plants by *F. culmorum* typically exhibit characteristic symptoms, including brown discoloration along the stem, which can extend to multiple internodes as the plant matures. These lesions are most concentrated in the lower stem, although severe cases may result in the infection spreading to the crown and root tissues (Chakraborty *et al.*, 2006; Cook, 2010). *Fusarium culmorum* is notable for producing several trichothecenes during infection, including nivalenol (NIV) and DON, along with their acetylated derivatives. FCR mainly affects the stem base, crown, and root tissues, but it also contributes to mycotoxin contamination in wheat straw, a vital raw material for livestock feed. This highlights the importance of understanding the relationship between FCR severity and trichothecene distribution, as contamination in wheat straw could threaten feed safety (Covarelli *et al.*, 2012).

Data on the extent of *Fusarium* colonization in wheat plants are variable across studies. Mudge *et al.*, (2006) reported that *Fusarium* infection can extend up to the head tissues, while other studies found fungal colonization restricted to internodes of the wheat culms, without reaching the heads (Covarelli *et al.*, 2012; Winter *et al.*, 2013; Moretti *et al.*, 2014). In field inoculation studies with *F. pseudograminearum*, plants exhibiting "whitehead" symptoms showed dense colonization in at least the first three internodes (Knight & Sutherland, 2016). Along with the pathogen's colonization, trichothecenes, including DON, are translocated upwards within the plant. Research has shown that DON is present in all plant tissues, with concentrations decreasing from the crown to the head following stem base inoculation with *F.*

pseudograminearum, *F. culmorum*, or *F. graminearum* (Covarelli et al., 2012; Moretti et al., 2014; Mudge et al., 2006; Winter et al., 2013).

Given the widespread presence of *F. culmorum* in Tunisia, understanding its impact on both yield loss and mycotoxin accumulation is crucial. Addressing these issues is not only vital for mitigating economic losses from reduced crop productivity but also for safeguarding public and animal health.

The aim of this study is to investigate the impact of *Fusarium culmorum* infection on wheat yield, fungal contamination, and mycotoxin accumulation in wheat tissues. A trial was conducted using nine of the most cultivated commercial varieties of durum wheat, and agronomic parameters were assessed in inoculated and no inoculated plots. Fungal contamination and mycotoxin accumulation were measured in both stem and kernel tissues. The correlation between disease incidence, severity, and the above-mentioned parameters was established to gain a comprehensive understanding of the pathogen's effects on wheat production and quality.

Materials and Methods

Field trial

The trial was set up at the EL-Marja experimental station of the National Institute of Field Crops (INGC) (36°31’50.2” N 8°57’14.9”E) in northwest Tunisia, during two cropping season 2020-2021 and 2021-2022. The station is located in the sub-humid area characterized by a mean annual rainfall about 540 mm and a temperate climate with cold moist winters and hot dry summers. The monthly rainfall during the four cropping seasons is summarized in Figure 1. The dominant field trial soil type is Limon-clay.

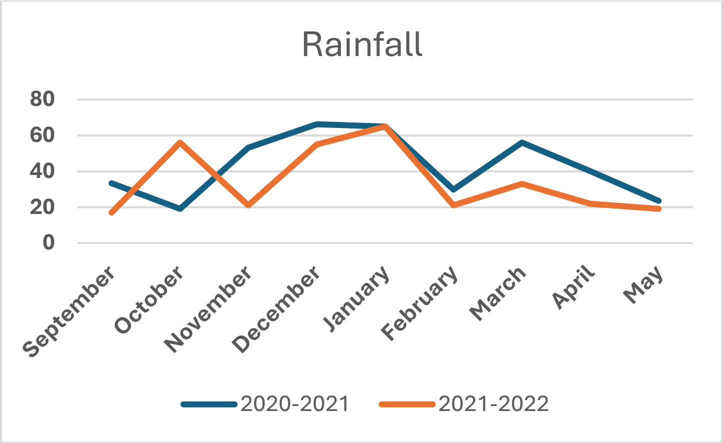

Figure 1*: Rainfall, expressed in mm, during the two crop seasons (2020-2022)*

The trial was conducted on a 450 m^2 plot previously planted with rapeseed and followed a split-plot design to assess the response of nine wheat varieties (Khiar, Karim, Maali, Utique, Caroica, Dhahbi, INRAT100, Monastir and Razzek) to inoculation with *F. culmorum*. Wheat varieties constituted the primary factor (main plots), while treatment with or without inoculation (respectively marked "In" for inoculated and "Or" for control) represented the secondary factor (sub-plots). The experimental design, organized in randomized complete blocks with four replications, comprised 120 m^2 plots spaced 1 meter apart, with strips of triticale at the edges as an isolating crop (Figure 2). Sowing was carried out at a density of 190 seeds/m^2 (six rows of 10 m × 1.2 m) and optimized by the application of Plantstart soil activator (1.5 L/ha). Nitrogen inputs were split into three applications of ammonium nitrate (140 kg/ha on January 19, 2021, 150 kg/ha on March 2, 2021 and 150 kg/ha on March 26, 2021), and weed control provided by Nikos (0.6 L/ha) and Amilcar (1.25 L/ha) on February 9, 2021.

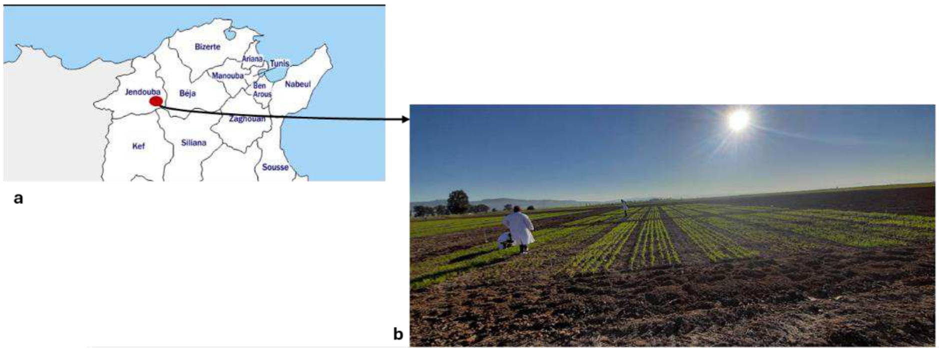

Figure 2: *Field trial design during two crop seasons (2020-2022) a) Location of the trial site on the geographical map b) Real picture of the implemented field trial.*

Fusarium culmorum **inoculum preparation and inoculation procedures**

Four strains of *F. culmorum* were used. They were grown on a PDA medium prepared in 90 mm Petri dishes. Two types of inoculum were prepared: a spore suspension and an inoculum on oats substrate. For the first inoculum a mixture of oats and wheat (1:1) was soaked in water for 24 hours, drained and autoclaved three times. Then, the grains were inoculated with the four *F. culmorum* strains and incubated at 25°C under a regime alternating 12 hours of near-UV light and 12 hours of darkness. After 2-3 weeks, the spores developed on the grains were recovered with sterile distilled water, filtered, and the resulting spore suspension adjusted to a concentration of 10^5 spores/mL.

For the second type, grain invaded by mycelium as described above were dried on sterile filter paper for one week, ground, then stored in paper bags for later use.

Inoculations were carried out at two key times: first, by soaking the seeds in the spore suspension enriched with 0.01% Tween 20, followed by drying before sowing, and then at tillering stage, by incorporating the second inoculum (0.53g/m^2) directly into the soil. Each variety was tested on four lines inoculated at the tillering stage to assess their response.

Evaluation of agronomic parameters

Agronomic parameters, except for grain yield, were evaluated in 2021, while grain yield was assessed over two years

Number of plants per square meter (NP/m^2)

Plants per square meter were counted at the end of the emergence stage. This assessment was used to estimate plant population density after emergence. For this purpose, the two middle rows of seedlings were selected in each experimental plot. The number of plants present on one linear meter of these two lines was counted manually. The data obtained for each line were then averaged to provide a representative estimate of stand density for each plot. This rigorous method guarantees accurate measurement of seedling establishment, an essential parameter for assessing seedling homogeneity and the potential impact of treatments or experimental conditions on emergence.

Number of tillers per square meter (NT/m^2)

The evaluation of the number of tillers per square meter was carried at the tillering stage. To carry out this measurement, the two median rows of seedlings were chosen in each plot to best represent the population. The number of tillers present on one linear meter of each of these two lines was accurately counted. The data obtained for each line were then averaged to provide a reliable estimate of the average number of tillers per square meter in each plot.

Number of ears per square meter (NE/m^2)

An assessment of the number of ears per square meter was carried out, at the maturity stage. The aim of this assessment was to determine crop density at an early stage of wheat development. For this, the number of plants per linear meter was estimated by counting the number of plants on two median rows of seedbed. The plants present on these two lines were counted, and then the average of the two counts was calculated to obtain an accurate estimate of planting density.

Number of kernels per ear (NK/E)

At the end of the maturity stage and to evaluate and calculate the number of kernels per ear, mature ears were selected randomly from each plot. The kernels from each ear are carefully removed and counted. To calculate the average number of kernels per ear, the total count is divided by the number of ears sampled.

Yield Assessment (T/ha)

At the end of the maturity stage, the mature ears were carefully harvested, and the yield assessed by weighing the grains obtained for the whole plot. The yield was then calculated by dividing the total weight of the harvested grains by the area of the plot (in square meters), then multiplying the result by ten to obtain a value expressed in tons per hectare. Then, to assess the impact of inoculation on the agronomic parameters measured, the rates of reduction in these parameters were calculated using the following formula:

$$\text{Reduction rate (\%)} = \frac{\text{Values observed in ordinary plants } - \text{Values observed in inoculated plants}}{\text{Values observed in ordinary plants}} * 100$$

Disease parameters

Disease incidence

During the two cropping seasons, once the plants reached maturity, 30 plants were randomly collected from each plot of the nine varieties, both inoculated and non-inoculated and from the four replicates. A subsample of these plants was stored for DON contamination and DNA quantification analysis.

Incidence was assessed by the frequency of isolation of *F. culmorum* species from the crown and kernels of the collected plants. Isolation was proceeded as follows, for crowns: fragments of 0.5 cm of each plant were cut. The fragments were then disinfected with 70° alcohol for 30s, rinsed twice with sterile distilled water and dried on sterile filter paper. The fragments thus disinfected were placed on PDA medium diluted 4-fold at a rate of five plants per dish. The plates were incubated at 25°C. After 4 to 7 days, the colonies corresponding to the *F. culmorum* species that had developed from the fragments were counted and related to the total number of plants analyzed. The same isolation process was used to assess the incidence on kernels.

Disease Severity

Disease severity was assessed for all varieties, by determining the brownish discoloration on the stem bases. The brown color that appeared between the first and the second internodes of stems was evaluated using a 0-4 scale (0 = completely healthy, 1 = less than 25% necrosis, 2 = 25–50% necrosis, 3 = 50-75% necrosis and 4 = greater than 75% necrosis). Ratings were converted to severity indices using the following formula (Chen *et al.*, 2023);

$$DI= (\sum nX/4\ N) \times 100$$

With X scale value of each plant, n number of plants in the category, and N is the total number of plants assessed for each material.

Deoxynivalenol analyses

Deoxynivalenol analyses were conducted for both crown tissue and kernel. For crowns, a subsample from collected plants described above were milled and used for analysis. Kernels were collected at harvest and then milled.

The samples were first prepared by the method of (Weingaertner *et al.*, 1997) with minor modifications. Briefly, 5 gr of grains and crowns were extracted with 25 mL acetonitrile/water (84:16, v/v) by orbital shaking for 60 min at 250 rpm. The extract was filtrated through filter paper (Whatman no. 4, Maidstone, EE.UU.), and 8 mL were cleaned up through a Mycosep #227 column (Romer Labs, Union, MO, USA). The column was inserted into the mouth of the test tube containing the extract and pushed slowly into the tube until approximately 4 mL extract had passed through the column. Purified extract (3 mL equivalent to 0.6 gr) was dried down under air stream at 50 °C. Dried residue was reconstituted in 500 ml of HPLC mobile phase.

100 µL (0.12 gr of sample) of extract was injected into to HPLC apparatus (Agilent 1100 Series), a column heater set at 40°C and a diode array (DAD) detector that was set at 220 nm wavelength. The analytical column was a Synergi Hydro-RP 80A (150 x 3 mm, 4 µm, Phenomenex). The mobile phase was acetonitrile/water (10:90, v/v). The flow rate of the mobile phase was 0.5 mL/min. In these analytical conditions, the retention time of deoxynivalenol was 5.1 min.

The detection limits (LOD) based on signal-to-noise ratio of 3:1, was deoxynivalenol = 0.1µg/g. The mycotoxin was quantified by comparing peak areas with a calibration curve obtained with standard solutions.

Quantification of Fusarium DNA in plant tissue by quantitative PCR

Total genomic DNA was extracted from 50 mg of representative grounded kernels and crowns by using the “Wizard Magnetic DNA Purification System for Food” kit (Promega Corporation, Madison, WI), according to the manufacture's protocol. Quantity and integrity of DNA were checked at Thermo-Scientific Nanodrop (LabX, Midland, ON, Canada) and by comparison with a standard DNA (1 kb DNA Ladder, Fermentas GmbH, St. Leon-Rot, Germany) on 0.8% agarose gel after electrophoretic separation.

To detect F. culmorum, SYBR Green real-time PCR assay, based on the translation elongation factor 1-α (TEF-1α) housekeeping gene, was performed by using primers reported by Nicolaisen et al., (2009). Real time PCR were carried out in a final volume of 15 µl, containing 2 µl of total genomic DNA diluted 1:5, 170 nM of each primer, 0.5 µg/µl bovine serum albumin (BSA), and 1X RealMasterMix SYBR ROX (5 PRIME GmbH, Max-Volmer-Str. 8, 40724 Hilden, Germany).

The experiments were performed in the ViiA7 Real Time System, with a fast run mode, by using the following PCR conditions: 2 min at 95°C, 40 cycles of 1 sec at 95°C and 30 sec at 62°C, and a dissociation stage from 60°C to 95°C.

In each experiment of both assays, a series of 6 10-fold dilutions, from 10 ng to 0,1 pg, of genomic DNA obtained from pure culture of F. culmorum strains used in the field trial, a negative control (water as template) and a positive control (a wheat sample contaminated by a known quantity of fungal DNA) were included. The standard curves were used to quantify, by comparison, the *F. culmorum* DNA in each wheat sample; each DNA sample was analysed in triplicate.

Fusarium culmorum DNA amount was expressed as ng of fungal DNA onmg of wheat grounded kernels.

The amount of fungal DNA was determined from calibration curves obtained with fungal DNA ranging from 0.1 to 1,000 pg dissolved in the corresponding plant DNA matrix. Samples with cycle threshold values above that of the 0.1 pg DNA standard were considered free of fungal colonization in statistical analyses, and were accordingly assigned a value of ≤0.01 pg mg-

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) in GraphPad Prism version 8 to determine significant differences among treatment means. When ANOVA indicated significance, Tukey's Honest Significant Difference (HSD) test was applied for post hoc multiple comparisons. Relationships among the parameters studied were explored using Principal Component Analysis (PCA), conducted with RStudio.

Results

Disease assessment and pathogen detection

Incidence and severity of Fusarium Crown Rot

Fusarium Crown Rot was detected in 97.2% plots of the trials under inoculated and non-inoculated treatments during the two cropping seasons 2020-2021 and 2021-2022. Overall, the incidence and severity of FCR ranged respectively from 0% and 71.66% and 0% and 75%.

A significant difference was observed in the incidence and severity of FCR between the inoculated and non-inoculated plots, with p<0.001 (Figure 3). Thus, under inoculated conditions, the incidence and severity averaged respectively 51.38% and 38.93%. Whereas, under non-inoculated conditions, the incidence and severity averaged respectively 24.95%, and 13.55% (Figure 3).

Figure 3: *Incidence (%) and severity (%) of Fusarium Crown Rot in inoculated and non-inoculated plots*

Overall, there was no significant difference in the incidence and severity between the years of the study (p=0.1364, p=0.1480) respectively. Mean incidences were 40.7% et 35.6% in 2021 and 2022 respectively. The mean severities were 34.04 et 38.93 in 2021 and 2022 respectively (Figure 4).

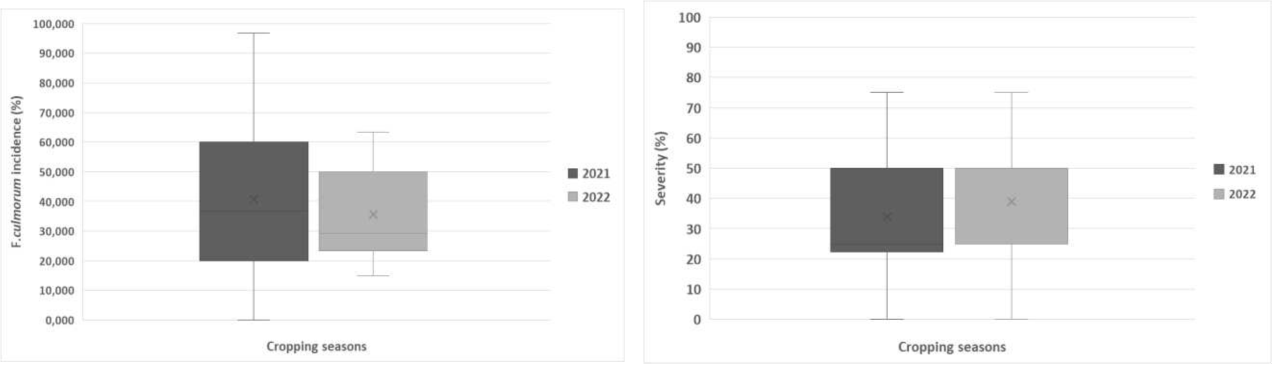

Figure 4*: Incidence (%) and severity (%) of Fusarium Crown Rot in the two crop seasons (2020-2022).*

Incidence of *F. culmorum* on wheat kernels

No significant difference in incidence was observed between 2021 and 2022. However, there was a significant increase in *F. culmorum* frequency between inoculated and non-inoculated samples (p = 0.0014; Figure 5)

In 2021, there was no significant difference between treatments, whereas in 2022, the inoculum increased significantly the contamination in kernels (p = 0.0002; Figure 6)

The incidence in inoculated samples ranged from 0% to 38% in 2021, while in non-inoculated samples, it was ranged from 0 to 6%.

In 2022 the incidence in inoculated samples ranged from 0 to 12%, whereas in non-inoculated samples, was ranged between 0 and 4%.

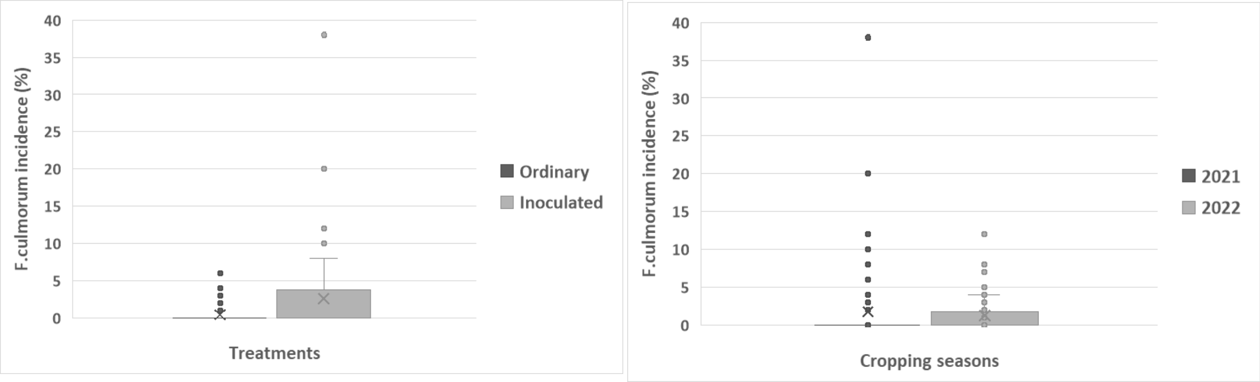

***Figure 5**: Fusarium culmorum incidence (%) in wheat kernel tissue in inoculated and no-inoculated plots and in the two crop seasons (2020-2022).*

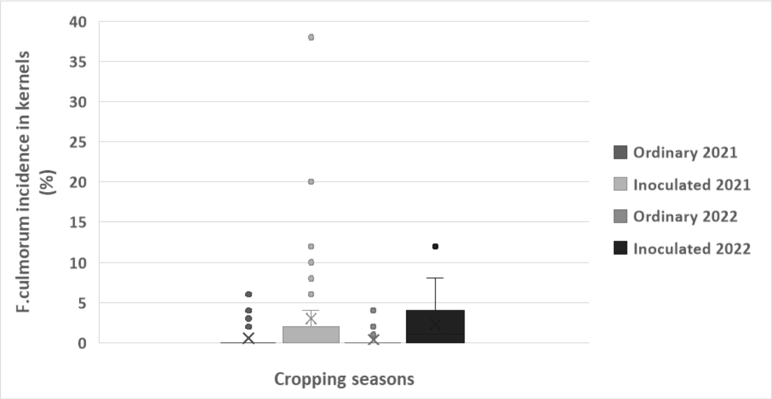

***Figure 6:** Fusarium culmorum incidence (%) in wheat kernel tissue in inoculated and no-inoculated plots during the two crop seasons (2020-2022).*

Quantification of F. culmorum DNA in crown tissue

Overall, there was no significant difference in DNA accumulation in the crown between 2021 and 2022 (p=0.210) and between inoculated and non-inoculated p=0.616 (Figure 7). However, in 2022, inoculation significantly increased fungal DNA concentration (p=0.0052), in contrast to in 2021, where no significant difference was observed (p=177).

In 2021, fungal DNA concentration under non-inoculated conditions had an average of 2.161 µg/g while under inoculated conditions 0.733 µg/g. In 2022, the average was 0.338 µg/g under non-inoculated conditions, and of 1.205 µg/g under inoculated conditions (Figure 8).

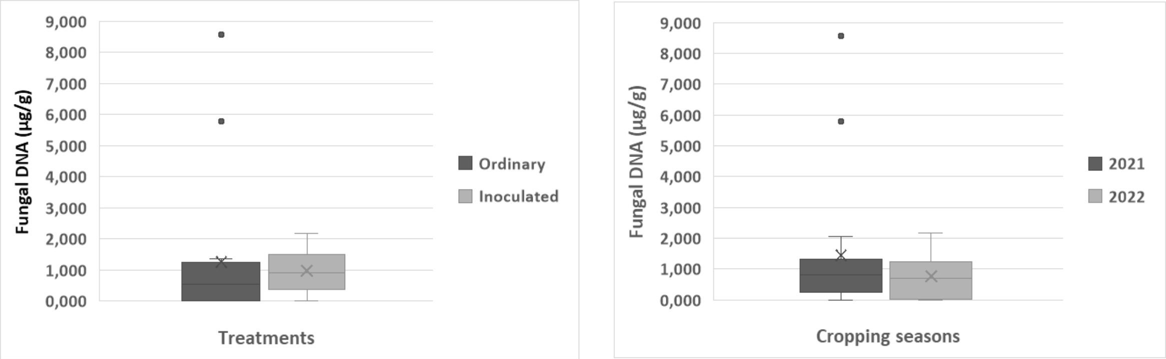

***Figure** 7: Fusarium culmorum DNA (µg/g) in wheat crown tissue in inoculated and no-inoculated plots and in the two crop seasons (2020-2022).*

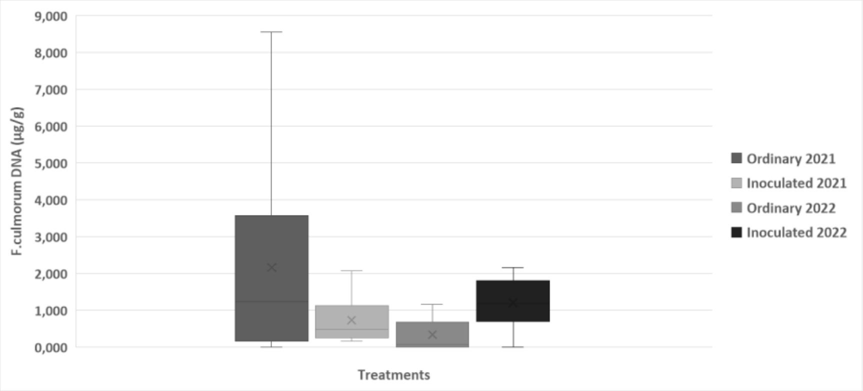

***Figure 8**: Fusarium culmorum DNA (µg/g) in wheat crown tissue in inoculated and no-inoculated plots for each of the two cropping seasons (2020-2022).*

Quantification of F. culmorum DNA in wheat kernels

Fusarium DNA concentration was significantly higher in 2021 than in 2022 (p = 0.0135). However, no significant differences were detected overall between inoculated and non-inoculated plants (Figure 9).

In 2021, fungal DNA quantities in inoculated samples ranged from 0 to 0.007 µg/g, while non-inoculated samples showed values between 0.0002 and 0.009 µg/g. In 2022, the fungal DNA levels in inoculated samples were between 0 and 0.0012 µg/g, whereas non-inoculated samples ranged from 0 to 0.001 µg/g (Figure 10)

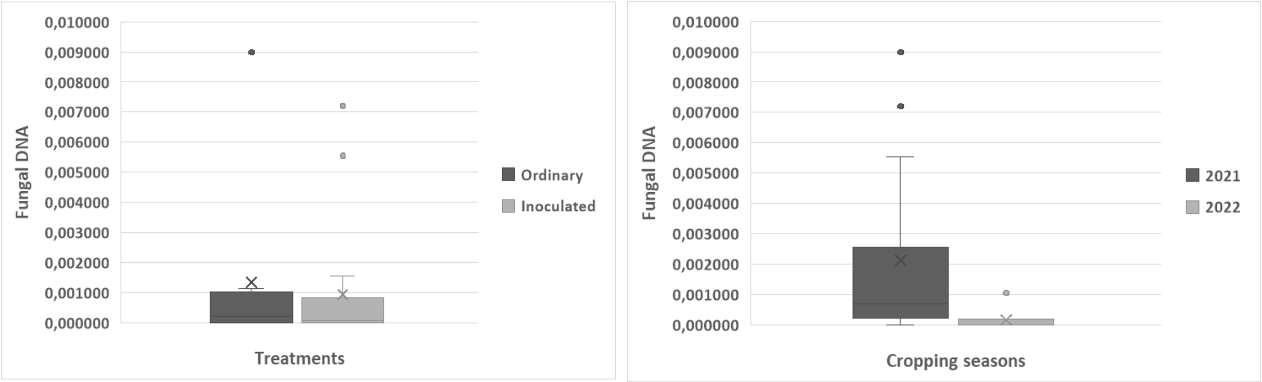

Figure 9: *F. culmorum DNA (µg/g) in wheat kernel tissue in inoculated and no-inoculated plots and in the two crop seasons (2020-2022).*

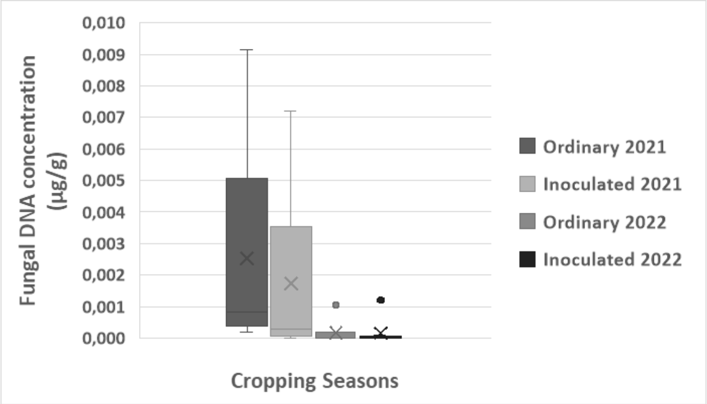

Figure 10*: Fusarium culmorum DNA (µg/g) in wheat kernel tissue in inoculated and no-inoculated plots and in the two crop seasons (2020-2022)*

Impact of F. culmorum inoculation on deoxynivalenol contamination in wheat crowns and kernels

Deoxynivalenol accumulation in crown Ttissue

Overall, there was a signification difference in accumulation of DON in the crown between 2021 et 2022 (p=0.0023). The DON level was significantly higher in 2021, reaching 38.656 mg Kg^{-1} (Figure 11). Overall, inoculation with *F. culmorum* increased significantly the DON levels in inoculated plants compared to non-inoculated conditions (p=0.0202; Figure 8). However, the trend differed between 2021 and 2022. In 2021, the average DON level was 8.43 mg Kg^{-1} under non-inoculated conditions and 10.83 mg Kg^{-1} under inoculated conditions. In contrast, in 2022, DON levels averaged 2.48 mg Kg^{-1} and 7.13 mg Kg^{-1} under non-inoculated and inoculated conditions, respectively (Figure 12).

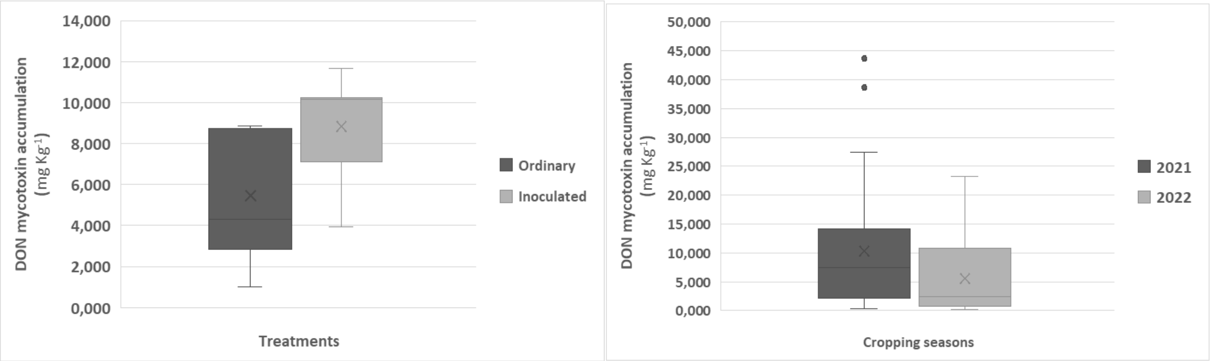

***Figure 11**: Deoxynivalenol accumulation (mg Kg^{-1}) in wheat crown tissue in inoculated and non-inoculated plots for each of the two crop seasons (2020-2022)*

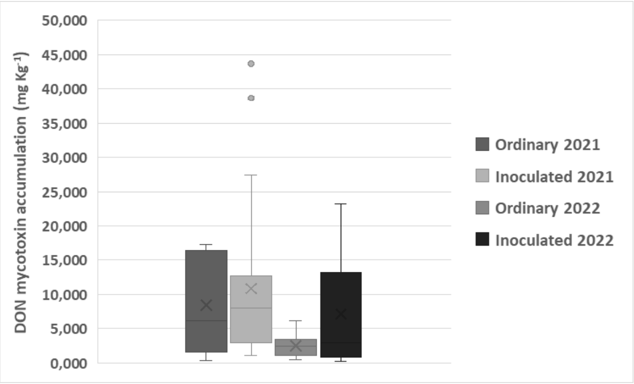

***Figure 12**: Deoxynivalenol accumulation (mg Kg^{-1}) in wheat crown tissue in inoculated and no-inoculated plots during the two crop seasons (2020-2022).*

Deoxynivalenol contamination of kernel tissue

DON mycotoxin concentration did not show a significant difference between 2021 and 2022. Similarly, no overall significant difference was detected between inoculated and non-inoculated samples. In both 2021 and 2022, the levels of DON did not significantly differ between these groups (Figure 13).

In 2021, DON concentrations in inoculated samples ranged from 0 to 0.160 mg Kg^{-1}, while non-inoculated samples showed values between 0.002 and 0.087 mg Kg^{-1}.

In 2022, DON levels in inoculated samples ranged from 0 to 0.066 mg Kg^{-1}, whereas non-inoculated samples exhibited levels between 0 and 0.028 mg Kg^{-1} (Figure 14).

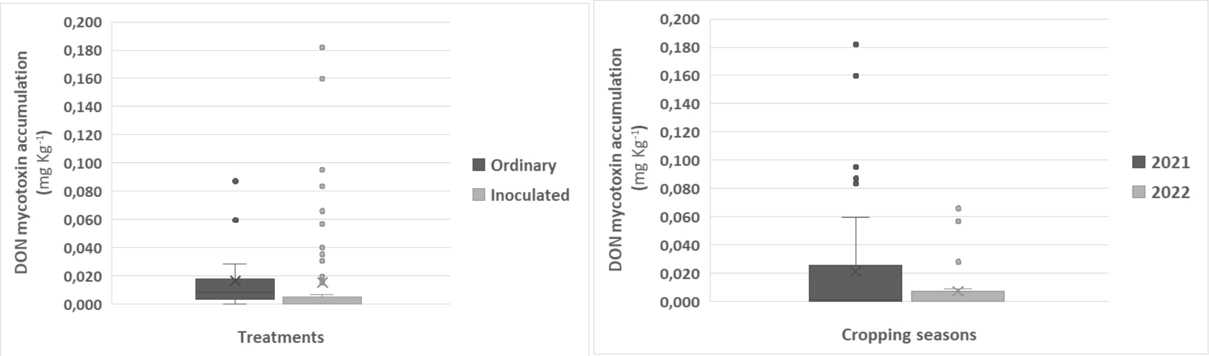

Figure 13: *Deoxynivalenol accumulation (mg Kg^{-1}) in wheat kernel tissue in inoculated and no-inoculated plots and in the two crop seasons (2020-2022).*

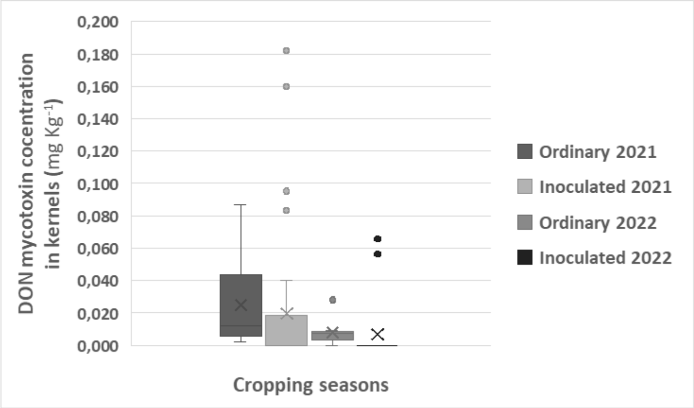

Figure 14: *Deoxynivalenol accumulation (mg Kg^{-1}) in wheat kernel tissue in inoculated and no-inoculated plots and in the two crop seasons (2020-2022).*

Impact of F. culmorum crown inoculation on agronomic parameters

Inoculation with *F. culmorum* decreased significantly tiller density (p= 0,0136). It ranged from 59 to 145, with an average of 120.23 tillers/m² under non-inoculated conditions, and from 55 to 120, with an average of 88.45 tillers/m under inoculated conditions (Figure 15). Similarly, inoculation with *F. culmorum* decreased significantly plant density (p<0,0001). It varied between 37 and 55, with an average of 47.83 plants/ m² under non-inoculated conditions, and between 25 and 49, with an average of 40.98 plants/ m² under inoculated conditions (Figure 4).

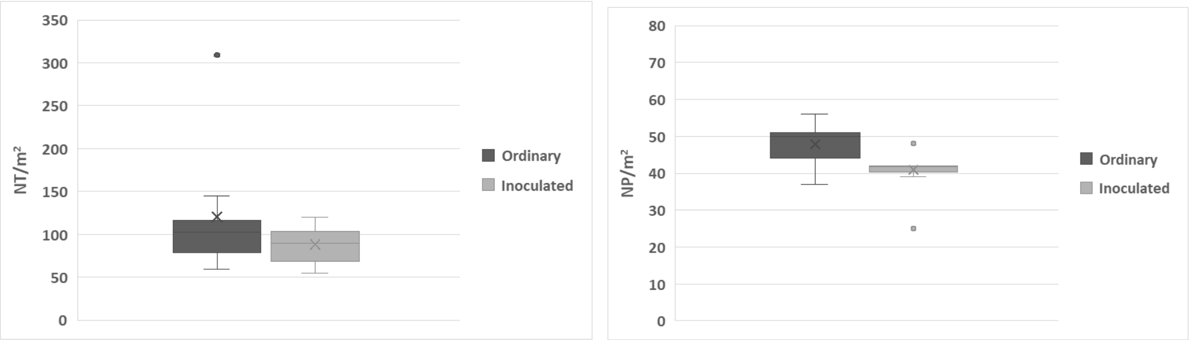

***Figure 15**: Tiller density (Number of tillers/m^2) and plant density (Number of plants/ m^2) in inoculated and no-inoculated plots during the two crop seasons (2020-2022).*

In contrast, inoculation with *F. culmorum* did not affect the number of ears per meter (p=0,1089), which ranged from 49 to 76, with an average of 60.54 ears/ m² under non-inoculated conditions, and from 28 to 72, with an average of 56.83 ears/ m² under inoculated conditions. Whereas inoculation decreased significantly the number of kernels per Ear (NK/E). The value ranged from 38 to 55, with an average of 44.70 NK/E under non-inoculated conditions, and from 27 to 55, with an average of 40.66 NK/E under inoculated conditions (Figure 16).

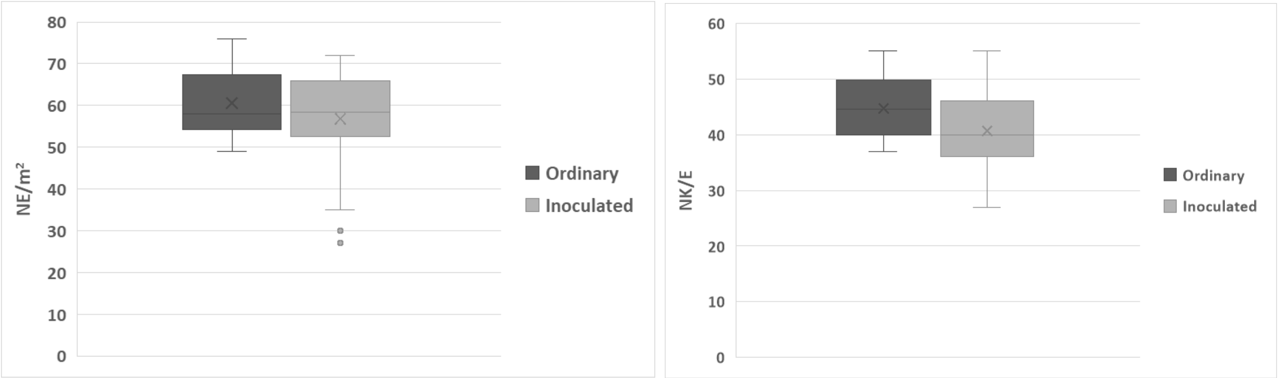

***Figure 16:** Ear density (NE/ m^2) and mean number of kernal/ear (NK/E) in inoculated and no-inoculated plots during the two crop seasons (2020-2022).*

Impact of F. culmorum crown inoculation on grain yield

In terms of yield estimated by tons per hectares, the statistical analysis did not reveal a difference between the two cropping seasons (p=0.540). However, *F. culmorum* inoculation decreased significantly the grain yield (p<0.0001). In 2021, it varied from 1.649 to 3.918 T/ha, with an average of 2.740 T/ha under non-inoculated conditions in 2021, and from 601 to 2.752 kg/ha, with an average of 2.161T/ha under inoculated conditions. In 2022, yield ranged from 2.201 to 3.160 T/ha, with an average of 2.668 T/ha under non-inoculated conditions, and from 1.798 to 3.081 T/ha, with an average of 2.338 T/ha under inoculated conditions (Figure 17).

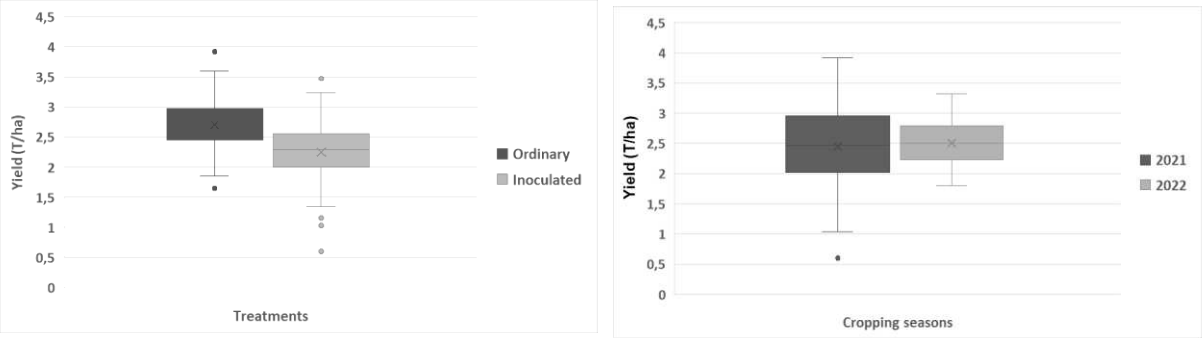

***Figure 17**: Yield (T/ha) in inoculated and no-inoculated plots during the two crop seasons (2020-2022).*

Impact of F. culmorum on the performance of the nine commercial durum wheat varieties

The performance of commercial durum wheat varieties was evaluated under inoculated conditions

Incidence and severity of Fusarium crown rot

Overall, in terms of FCR disease incidence in inoculated plants, only the *Maali*, *Karim* and *Carioca* varieties showed a significant decrease in 2021-2022 with p<0.0001, p=0.0435, p=0.007 respectively, the other varieties did not show a difference between years (Figure 18).

In 2020-2021, infestation rates varied significantly between varieties p<0.0001, with *Maali*, *Karim* and *Carioca* showing the highest rates (88.16%, 78.33%, and 75.84%, respectively) compared to *Dhahbi*, *Khiar*, (31.66% for both) *INRAT100*, *Utique* (39.16% for both) and *Monastir*, and *Razzek* (49.16 and 52.5%, respectively).

In 2021-2022, infestation rates between varieties were not statistically significant (p=0.760).

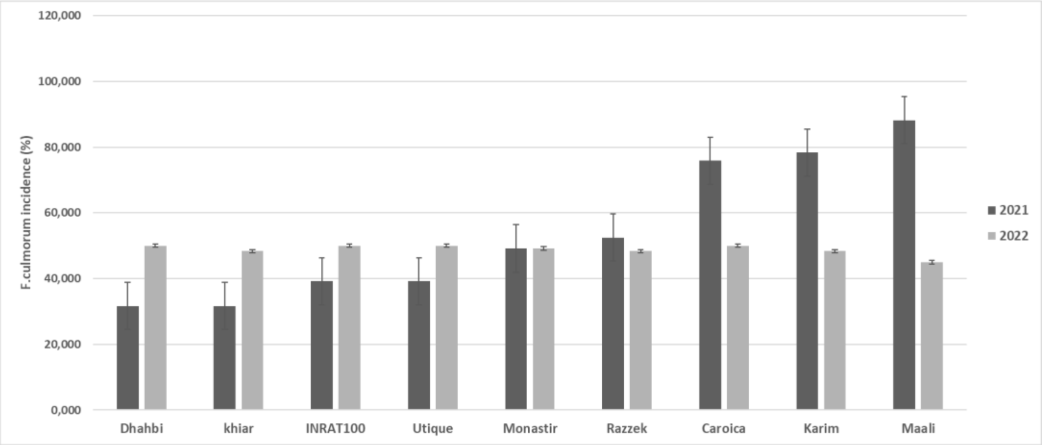

***Figure 18**: Fusarium culmorum incidence (%) for varieties under inoculated conditions during the two crop seasons (2020-2022)*

In the rates of severity, only the *Karim* variety showed a significant decrease in 2022 compared with 2021 (p=0.0281), with the other varieties showing no difference. However, statistical analysis did not show significant difference between varieties in the same year, p=0.0588 in 2020-2021 and p=0.2383 in 2021-2022 (Figure 19).

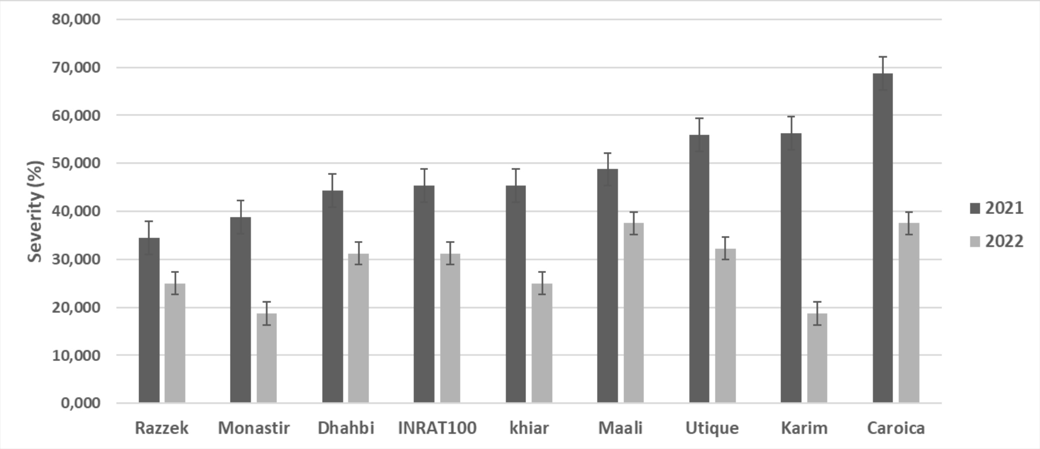

***Figure 19**: Disease severity (%) and incidence on wheat varieties under inoculated conditions for the two crop seasons (2020-2022)*

Incidence of Fusarium Head Blight

The *F. culmorum* infestation rate in the nine varieties under inoculation showed no significant difference between the years 2020-2021 and 2021-2022 (p=0.2726).

No significant difference was also found between varieties in the same year (p=0.1078; Figure 20).

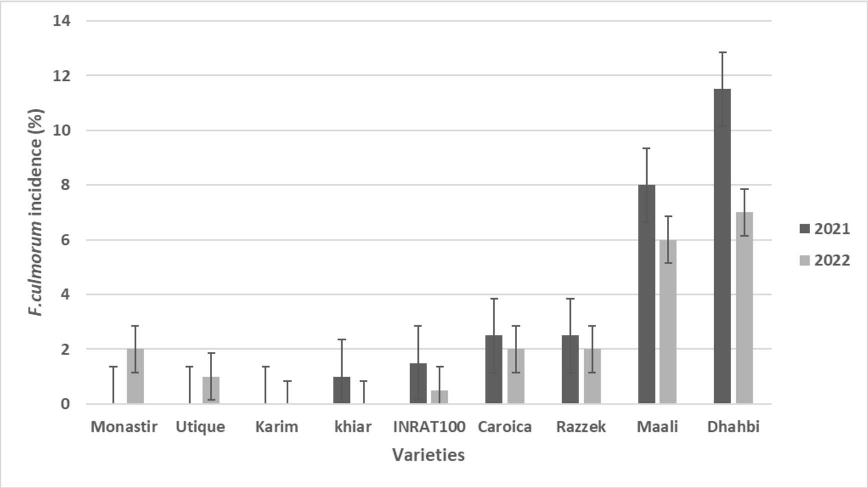

Figure 20: *Fusarium culmorum incidence (%) in the kernels of different wheat varieties during the two crop season (2020-2022)*

Quantification of *F. culmorum* DNA in crown tissue

The average concentration of *F. culmorum* DNA in the crowns did not show a significant difference between the two years p=0.1979, with a mean value of 0.733µg/g in 2021 and 1.204 /g in 2022. Also, between varieties the concentrations of the fungal DNA did not show significant differences p=0.1466 (Figure 21).

In 2021, concentrations ranged from 0.164 µg/g (*Karim*) to 2.070 µg/g (*Dhahbi)* recording the highest concentration.

In 2022, the lowest concentration was detected in the variety *Razzek* (0 µg/g), whereas the highest concentration was found in the variety *INRAT100* (2.167 µg/g). These differences in DNA concentration among the varieties and across the two studied years were not statistically significant.

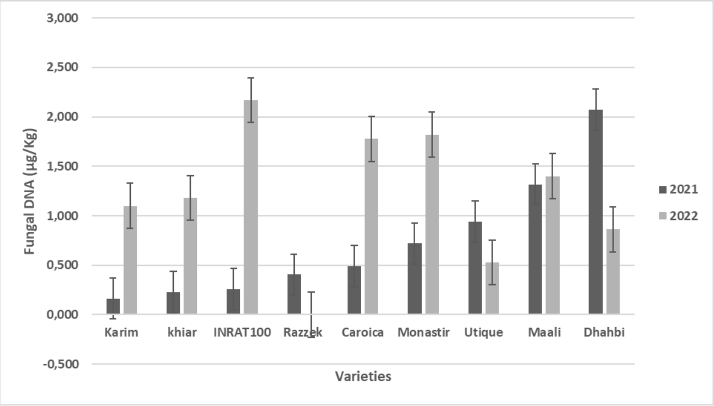

Figure 21*: Detection of Fusarim culmorum DNA (µg/g) in the crowns of the different varieties during the two crop season (2020-2022)*

Occurrence of F. culmorum DNA in Kernel Tissue

F. culmorum DNA concentrations were significantly higher in the 2020-2021 season compared with 2021-2022 for the Carioca, Karim, Razzek and Khiar varieties ($p < 0.0001$).

In 2021, DNA concentrations for the varieties *Carioca*, *Karim*, *Khiar* and *Razzek* were significantly higher than those of the other varieties ($p < 0.0001$).

In 2022, the *Dhahbi* variety showed the highest *Fusarium* DNA concentration of all the varieties studied ($p < 0.0001$), while no significant difference was observed between the other varieties (Figure 22).

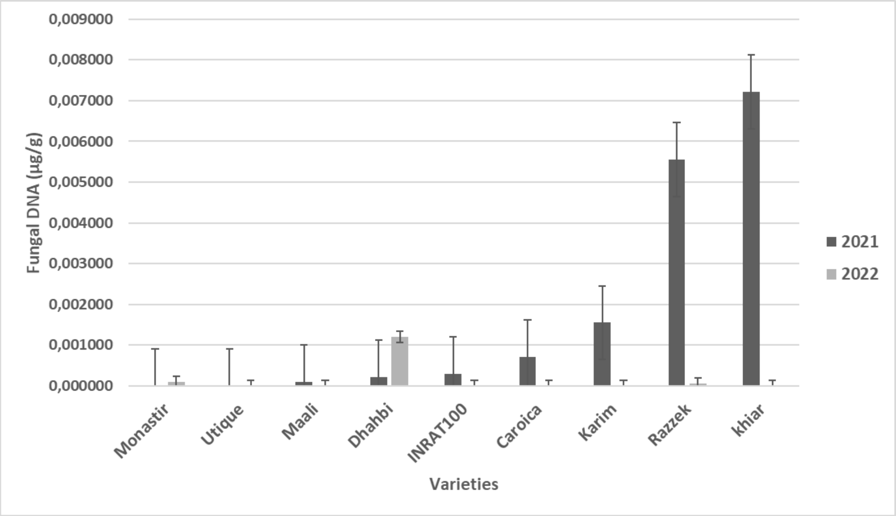

***Figure 22:** Detection of Fusarim culmorum DNA (µg/g) in the kernels of the different varieties during the two crop season (2020-2022)*

Dexynivalenol acuumulation in crown tissue

Between the two years, only the variety Utique showed a significant decrease in DON concentration ($p = 0.0067$), dropping from 23.04 mg·kg^{-1} in 2021 to 0.27 mg·kg^{-1} in 2022. Differences in DON levels between years for the remaining varieties were not significant ($p = 0.649$; Figure 23).

In 2021, the varieties *Maali* and *Utique* exhibited the highest and statistically significant DON concentrations, with 19.10 mg·kg^{-1} ($p = 0.0450$) and 23.06 mg·kg^{-1} ($p = 0.0027$), respectively. In 2022, the highest concentrations were recorded for *INRAT 100* and *Karim*, with significant levels of 18.08 mg·kg^{-1} ($p = 0.0044$) and 18.04 mg·kg^{-1} ($p = 0.0043$), respectively.

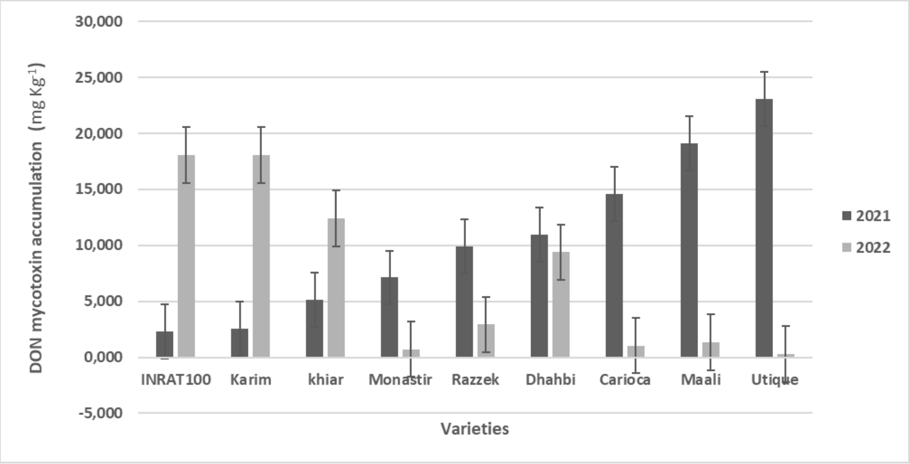

Figure 23: *DON mycotoxin accumulation (mg Kg^{-1}) in the different wheat varieties during the two crop season (2020-2022)*

Deoxynivalenol accumulation in kernel tissue

Overall, DON Mycotoxin levels were higher in 2021 p=0.0451.

In 2021, the *Dhahbi* variety recorded the highest DON mycotoxin concentration, with a significant difference from all other varieties ($p < 0.0001$), notably *Khiar* ($p = 0.0002$) and *Carioca* ($p = 0.0003$). On the other hand, there were no significant differences between the other varieties. In 2022, the *Dhahbi* variety also showed a significantly highest concentration compared to all the other varieties ($p = 0.002$; Figure 24).

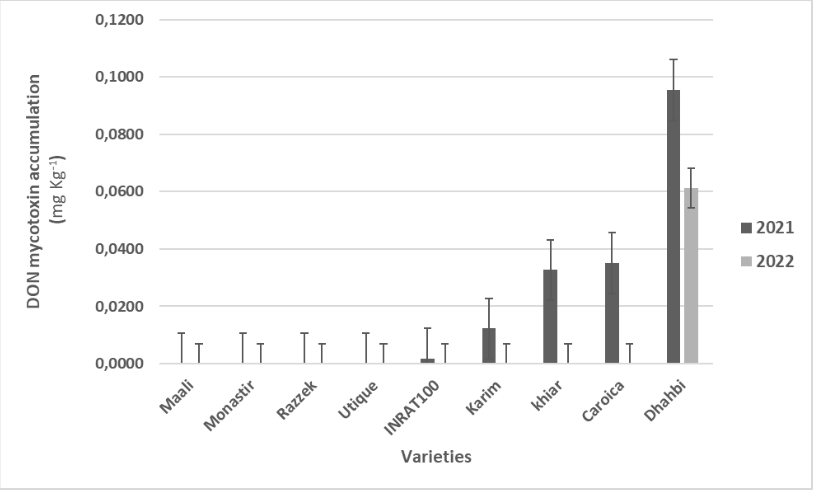

Figure 24: *DON mycotoxin accumulation in the kernels of the different wheat varieties during the two crop season (2020-2022)*

Impact on agronomic parameters

For agronomic parameters the decrease/increase was evaluated between inoculated and non-inoculated plot.

A significant difference was observed among varieties in the reduction rates of the number of tillers per square meter (NT/m^2) ($p < 0.0001$). The variety *INRAT100* exhibited the highest reduction rate (67.6%), while *Razzek* had the lowest reduction rate (0%). *Maali* did not show a reduction (Figure 25a)

Similarly, a significant difference between varieties was recorded in the reduction rates of the number of plants (NP/ m^2) ($p < 0.0001$). INRAT100 again showed the highest reduction rate (54.3%), whereas *Karim* displayed the lowest rate (0.97%). Interestingly, the variety *Utique* exhibited a negative reduction rate (-6.768%), suggesting a slight increase in plant density under inoculation conditions (Figure 25b).

For the reduction rates of the number of ears per square meter (NE/m^2), a significant difference was also observed ($p < 0.0001$). *Khiar* had the highest reduction rate (45.195%), while *Karim* showed the lowest reduction (1.576%). Notably, some varieties, including *Karim*, *Carioca*, *Dhahbi*, and *Monastir*, displayed negative reduction rates, indicating increased spike density in these cases (Figure 26a)

Regarding the reduction rates of kernels per Ear (NK/E), significant differences were identified ($p < 0.0001$). *Khiar* demonstrated the highest reduction rate (25.4%), while *Karim* recorded the lowest rate (1.2%) (Figure 26 b).

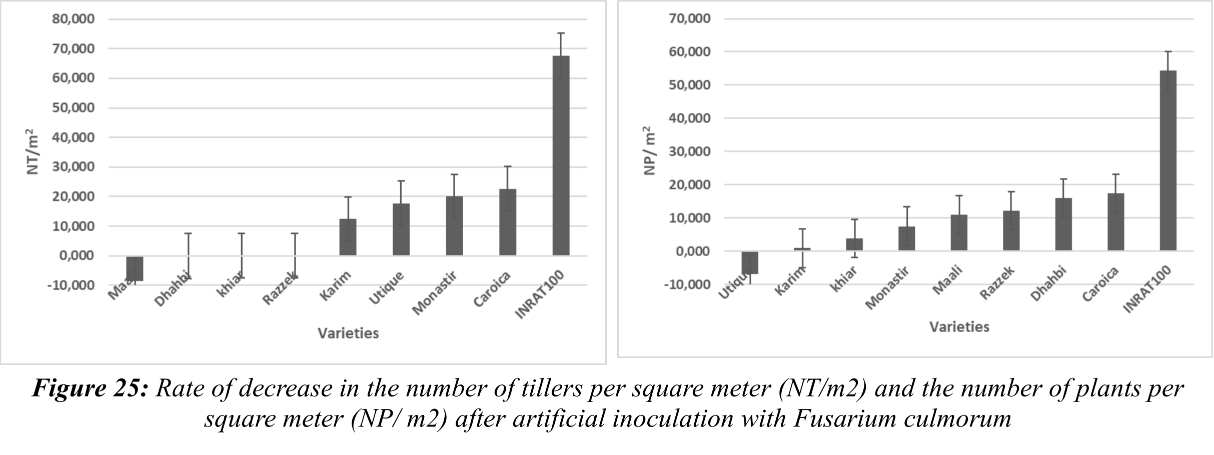

Figure 25: *Rate of decrease in the number of tillers per square meter (NT/m2) and the number of plants per square meter (NP/ m2) after artificial inoculation with Fusarium culmorum*

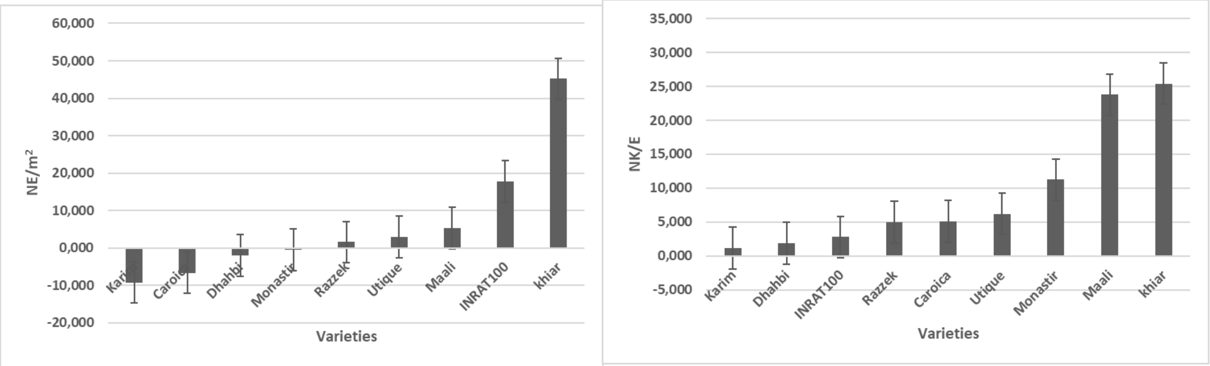

Figure 26*: Rate of decrease in the Ear density (NE/m2) kernel per Ear (NK/E) after artificial inoculation with Fusarium culmorum in the different wheat varieties*

Impact on grain yield

Yield losses were significantly higher in 2021, with an average reduction of 20.7% compared to 12% in 2022 with p=0.0192.

The varieties showed different relative yield losses in each of the studied years p=0.0032.

In 2021 overall yield loss, only the varieties *Dhahbi* and *Khiar* showed a significant difference p=0.0302 and p=0.0322, while no difference was observed in the other varieties. The variety *Dhahbi* experienced was signficatly the highest yield loss in both years, with 42.2% in 2021 and 21.7% in 2022. Conversely, *Khiar* in 2021 exhibited negative yield losses (Figure 27).

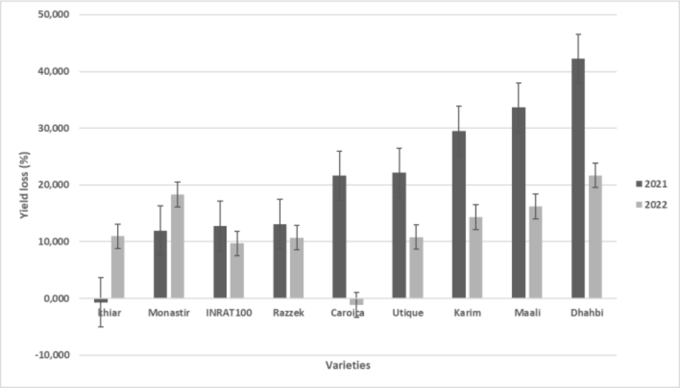

Figure 27: Rate yield loss in the different wheat varieties during the two crop seasons (2020-2022)

Correlation Analysis

Correlation between *F. culmorum* incidence, disease severity, yield, DON accumulation, and fungal DNA in wheat crown tissue

Principal component analysis (PCA) of five disease-related variables measured on wheat crown tissues following *F. culmorum* inoculation accounted for 66% of the total variance across the first two principal components (38.9% for Dim1 and 27.1% for Dim2; Figure 28). All variables showed positive associations, as indicated by their projection toward the right side of the correlation circle. PC1 was mainly shaped by yield loss (31.7%), symptom severity (26.3%) and DON concentration (20.2%), which clustered tightly and pointed in similar directions. This strong collinearity highlights that elevated disease severity was consistently associated with higher DON contamination and greater yield losses. PC2 was predominantly influenced by fungal DNA quantity measured by qPCR (54.%), with additional contribution from incidence (34.2%). Its proximity to DON concentration suggests a specific link between fungal biomass and mycotoxin production. Overall, this PCA identified two main clusters of positively correlated variables: one comprising yield loss, severity, and DON, which reflect visual and agronomic impacts; and a second, centered around fungal DNA and DON, highlighting the role of molecular markers in disease assessment. The alignment of all vectors in the same quadrant further emphasizes that more severe infections tend to be associated with greater pathogen pressure and higher agronomic losses.

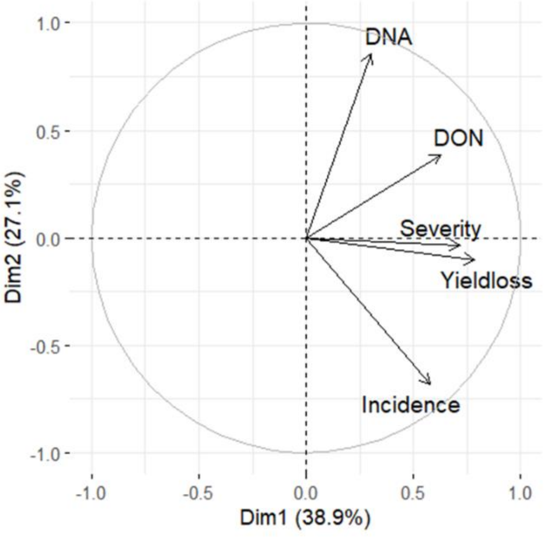

Figure 28: *Principal Component Analysis (PCA) of correlation between DON mycotoxin accumulation (mg Kg-1), Fusarium incidence (%), disease severity (%), yield loss (%) and fungal DNA (µg/g) detected on wheat crown level*

Correlations between F. culmorum incidence in kernel, DON accumulation and fungal contamination

The principal component analysis (PCA) performed on the wheat grain data showed that the first two dimensions explained 88.1% of the total variance, with 50.9% for the first (Dim1) and 37.2% for the second (Dim2) (Figure 29). The vectors representing the three variables - disease incidence, DON content and fungal DNA quantity - are all oriented to the right of the correlation circle, reflecting an overall positive correlation between these parameters. This suggests that high incidence situations are generally associated with higher DON contamination and fungal load. Dimension 1 (Dim1), which accounts for more than half the variance, is dominated by incidence (52.4%) and DON (45.8%), two highly correlated variables, as indicated by the very small angle between their vectors. Dimension 2 (Dim2), on the other hand, is mainly structured by fungal DNA, which contributes 93.5%, showing a moderate correlation with DON and less with incidence.

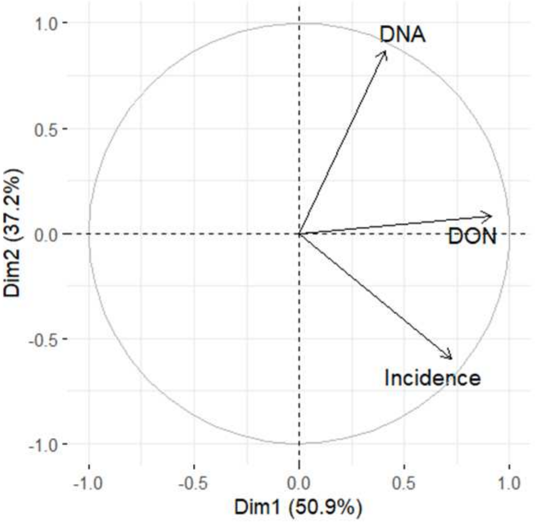

Figure 29: *Principal Component Analysis (PCA) of correlation between DON mycotoxin accumulation (mg Kg^{-1}), Fusarium incidence (%) and fungal DNA (µg/g) detected on wheat kernels*

Correlations between *F. culmorum* inoculation of soil and wheat stems and kernels parameters

Principal Component Analysis (PCA) was conducted to explore the relationships among disease severity, incidence, yield loss, fungal DNA content, and DON contamination, measured in both crown and kernel tissues (Figure 30). The first two principal components explained 50.6% of the total variance (Dim1: 30.6%, Dim2: 20.0%). Dim1 was mainly driven by Yield loss_c (27.7%), Severity_c (20.6%) while Dim2 was mostly influenced by DON_k (23.6%), DNA_c

(23.1%). The PCA biplot revealed a distinct separation between crown- and kernel-associated variables, indicating compartment-specific response profiles. The near orthogonality of the kernel and crown vectors—especially Incidence_k and Incidence_c—implies that infection and toxin accumulation in one tissue are not directly predictive of those in the other. These results hilights the tissue-specific nature of *F. culmorum* infection and its associated mycotoxin biosynthesis.

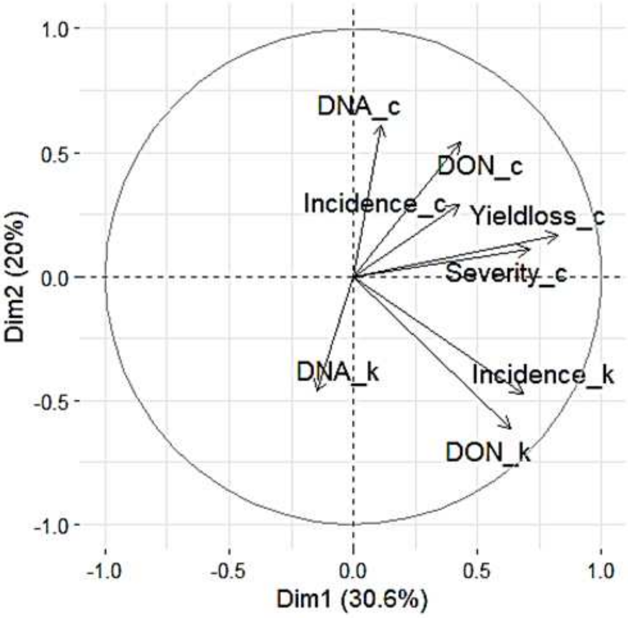

Figure 30: *Principal component analysis PCA of correlation between studied parameters in crown (c) and kernel (K): DON mycotoxin accumulation (mg Kg^{-1}) [DON c and DON K], Fusarium incidence (%) [Incidence c and Incidence K], yield loss (%) and fungal DNA (µg/g) [DNA c and DNA K].*

Discussion

Fusarium Crown Rot, caused mainly by *F. culmorum*, is a worrying disease in durum wheat crops, impacting both the health of the basal tissues of the plant and grain quality. While FHB directly affects the reproductive part of the plant, FCR develops from inoculation of the crown, and can have indirect repercussions on the grain, notably via the translocation of mycotoxins such asDON (Backhouse et al., 2004; Smiley et al., 2005). The complexity of interactions between fungal colonization in crown tissues, fungal contamination and mycotoxin synthesis in grains calls for an integrated approach combining phenotypic assessment, molecular quantification by qPCR and mycotoxin quantification.

This study aimed to investigate the impact of *F. culmorum*, inoculated at crown level, on fungal contamination, DON accumulation, and fungal biomass, assessed by qPCR, in both crown and grain tissues, as well as its effect on disease severity and yield losses. Multivariate analysis (PCA) revealed correlations between parameters measured in crown and grain tissues, highlighting the complex relationship between basal plant infection and final harvest quality. These results enrich our understanding of the pathogenic mechanisms specific to FCR and underline the importance of targeted monitoring and management strategies to limit the impact of this disease on cereal production.

The impact of *F. culmorum* crown inoculation varied markedly across seasons, affecting disease incidence and severity, fungal DNA accumulation, mycotoxin production, and agronomic performance. A consistent increase in FCR incidence and severity was observed in inoculated plots during both years, with no significant difference between years. This confirms the high pathogenic potential of F. *culmorum* under Mediterranean field conditions, in agreement with Chekali et al., (2013, 2019). These results align with Backhouse et al., (2004), who emphasized the critical role of environmental factors such as soil moisture and crown temperature over annual climatic variation in disease establishment.

Quantification of *F. culmorum* DNA in crown tissues revealed a significant increase following inoculation in 2022, while no significant difference was found in 2021. This suggests that fungal colonization depends not only on inoculum presence but also on host resistance traits or environmental stresses, as indicated by Lenc et al., (2015). Interestingly, kernel DNA concentrations were significantly higher in 2021 compared to 2022, regardless of inoculation. This uncoupling between crown and kernel colonization has been noted by Nicolaisen et al., (2009) and Waalwijk et al., (2004), emphasizing the importance of assessing multiple tissues to evaluate disease impact accurately.

The higher frequency of *F. culmorum* in kernels during 2022, especially under inoculation, supports systemic migration capacity of the pathogen, though kernel contamination was less marked in 2021 despite similar inoculum levels. This interannual variation reflects environmental modulation of fungal colonization and systemic movement, as described by Xu and Nicholson (2009) and Mudge et al., (2006).

DON accumulation followed similarly complex, tissue-specific patterns. Crown tissues from inoculated plants had significantly elevated DON levels in both years, with greater accumulation in 2022. However, kernel DON concentrations did not differ significantly between years or treatments, indicating limited systemic translocation of the toxin from crown to spike in some genotypes. This observation is consistent with Jestoi (2008) and Edwards et al., (2011), who reported that DON accumulation depends on both genotype and environment and does not always correlate with fungal biomass.

Agronomically, crown inoculation significantly reduced tiller and plant densities across years, corroborating previous findings that early infection impairs root function and resource allocation (Krnjaja et al., 2022; Bouatrous et al., 2023; Kazan & Gardiner, 2018). The number of ears per meter was unaffected, suggesting compensatory growth mechanisms in certain genotypes (Siham et al., 2014). Kernel number per ear was significantly reduced by inoculation, directly contributing to yield losses. Yield reductions were significant in both seasons, without year-to-year differences, confirming the persistent detrimental effect of crown infections on yield regardless of seasonal conditions (Bouarda et al., 2022; Tissaoui et al., 2022).

Considerable genotypic variability was observed in disease response. Maali, Karim, and Carioca showed high FCR incidence in 2020–2021 but significantly lower incidence in 2021–2022, indicating environmental influences rather than stable varietal resistance, consistent with Chekali et al., (2013). Despite these variations, severity scores were relatively uniform among varieties within the same year, supporting previous evidence that symptom severity does not always correlate with fungal biomass or yield loss (Mitter et al., 2006; Chakraborty et al., 2006).

Fungal DNA levels in crown tissues remained fairly stable across years and varieties, while kernel DNA concentrations varied notably. In 2021, Carioca, Karim, Razzek, and Khiar had higher kernel DNA levels, with Dhahbi surpassing others in 2022. This suggests differential systemic movement or resistance to kernel colonization, as reported by Nicolaisen et al., (2009).

DON accumulation mirrored DNA trends. Maali and Utique had higher DON levels in 2021, while INRAT100 and Karim were more affected in 2022. Notably, only Utique exhibited a significant decrease in DON accumulation across years, highlighting potential for improved mycotoxin resistance under variable Mediterranean conditions (Ferrigo et al., 2016).

Agronomic responses varied among varieties. INRAT100 suffered the greatest reductions in tiller number, plant density, and ear number per m², reflecting high susceptibility to early infections. Karim showed minimal reductions, suggesting resilience, as observed in other high-performing cultivars facing *Fusarium* stress (Tunali et al., 2006). Yield loss patterns were consistent: Dhahbi experienced the highest losses both years, whereas Khiar showed negative yield loss in 2021, possibly due to compensatory growth or tolerance mechanisms previously reported in partially resistant genotypes (Bai & Shaner, 2004).

Principal component analysis (PCA) of variables measured at crown level revealed a strong positive correlation between symptom severity, DON concentration and yield losses, as well as between fungal DNA quantity and DON level. These results highlight the complexity of interactions between *F. culmorum* infection intensity, its agronomic consequences and molecular biomarkers. They concur with those of Yang et al (2019), who found a significant relationship between symptom severity, DON accumulation and yield reduction in wheat trials. The aggregation of these variables on the first principal component (Dim1) reflects their interdependence and combined usefulness in characterizing the overall impact of Fusarium head blight. The strong contribution of fungal DNA to the second component (Dim2), close to the DON vector, suggests a close relationship between fungal biomass and mycotoxin production. This observation is consistent with the work of Deng et al., (2019) and Kazan & Gardiner (2018), who have shown that genetic regulation of trichothecene biosynthesis, particularly DON, is triggered by successful colonization of host tissues. Beccari et al (2011) and Winter et al (2013) have also demonstrated that the amount of fungal DNA is a reliable indicator of infection intensity and mycotoxic production. The strong correlation between yield losses and symptom severity is also supported by studies by Smiley et al., (2005), Moya-Elizondo (2011) and Khudhair et al., (2013), who have shown that severe infections by *F. culmorum* or *F. pseudograminearum* cause substantial losses in the field, linked to necrosis of vascular tissues impeding resource transport. Additionally, Hogg et al., (2007) demonstrated a strong correlation between visual disease symptoms and DON contamination, validating the usefulness of PCA for predicting mycotoxin levels based on severity. They also emphasized that wheat varieties exhibit considerable variation in DON accumulation, even when disease incidence is similar. Recent data from Buster et al (2022, 2023) reinforce our interpretation of the correlation circle: the joint localization of vectors in the same quadrant suggests systemic pathogenicity, where local infection of the crown can lead to the translocation of toxins and fungal DNA to other parts of the plant. This integrated approach thus confirms the value of combining classic indicators (incidence, severity) with molecular markers (qPCR) for a detailed assessment of genotype resistance, and highlights the relevance of simultaneously considering yield loss, mycotoxin load and fungal biomass as key criteria for estimating Fusarium risk.

PCA analysis of parameters measured in grains contaminated with F. culmorum also revealed a strong positive association between incidence, DON accumulation and fungal DNA quantity. The very high proportion of variance explained by the first two dimensions (88.1%) testifies to the robustness of this already well-documented tripartite relationship. The first component (Dim1), dominated by incidence and DON, reflects the close link between frequency of infected grains and toxic load. This corroborates the findings of Bai & Shaner (2004), who showed that the incidence of fusarium-damaged kernels is a good predictor of DON, particularly when infection occurs early in flowering. Similarly, Tunali et al., (2006) and Hellin et al., (2018) found a strong correlation between the frequency of infected grains and mycotoxin content, reinforcing the interpretation of Dim1 as an indicator of pathogen pressure and toxic production. The second component (Dim2), dominated by fungal DNA (93.5% contribution), reflects a complementary dimension. Although correlated with DON, fungal biomass does not systematically follow the evolution of incidence or toxin levels. Nicolaisen et al., (2009) have shown that qPCR can detect fungal biomass independently of visual symptoms, making it a valuable diagnostic tool. Beccari (2018, 2019) pointed out that the expression of DON biosynthesis genes is linked to the metabolic activity of the pathogen, but that divergences may exist depending on genotypes and environmental conditions. This would explain the moderate correlation between fungal DNA and DON, and its weak association with incidence. These results confirm the conclusions of Nielsen (2014) and Alisaac (2019), who recommended the combined use of these three variables for a more refined characterization of grain contamination. Today, this multifactorial approach is at the heart of sanitary monitoring strategies for wheat batches, particularly post-harvest, as Sohlberg (2022) and Brennan (2005) remind us. In short, PCA on grains distinguishes two complementary axes: one reflecting infectious and toxic pressure (incidence + DON), the other representing the actual fungal load (DNA). This structuring makes it possible to more accurately interpret the discrepancies sometimes observed between symptoms and mycotoxin contamination.

Finally, the PCA performed on all data measured in wheat collars and grains after soil inoculation with *F. culmorum* reveals a clear separation between the responses of these two tissue compartments. Although the first two dimensions explain around 50.6% of the total variance, the orthogonality of the vectors in the correlation circle reflects the compartmentalization of the infectious and mycotoxin biosynthesis processes. Dim1, dominated by crown severity and yield losses, reflects the direct agronomic impact of infection on the root system. These results concur with those of Minati et al., (2019) and Beccari et al., (2018), who showed that crown necrosis is linked to yield reductions through altered water and nutrient transport. Dim2, on the other hand, is shaped by DON accumulated in grains and fungal DNA measured at the crown. The co-influence of these two variables, oriented differently from those at the crown, suggests that translocation mechanisms to the ears are not systematic. This is confirmed by the quasi-orthogonality between the incidence variables in the kernels and those at the crown. Obanor et al (2014) have shown that Fusarium can preferentially colonize certain parts of the plant depending on infection stage and environmental conditions. This partial independence has also been described by Covarelli (2012) and Scherm et al., (2013), who observed distinct gene expression and DON biosynthesis profiles depending on the tissue. This would explain the poor ability to predict grain contamination from severe crown infection. The work of Fan et al (2021) also supports this compartmentalized view: according to their results, DON accumulation in grain is not always proportional to lesion severity or fungal biomass in the stem, due to independent genetic regulation according to fusariosis forms.

Conclusion

This study revealed the complexity of interactions between *F. culmorum*, infected tissues (crown and grain) and agronomic responses in durum wheat. Inoculation at the crown induced a significant increase in symptom severity, yield losses and mycotoxin accumulation. However, translocation of mycotoxins, particularly DON, from the crown to the grain remains partial and does not systematically follow fungal contamination at the crown. This dissociation underlines the importance of simultaneously assessing several plant compartments for a complete understanding of the impact of the disease. Principal component analysis highlighted strong correlations between severity, DON and yield losses, while distinguishing infectious processes specific to each tissue. These results confirm the need for an integrated approach, combining visual, molecular and chemical assessments, to improve sanitary monitoring and guide the selection of varieties more resistant to FCR.

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Chapter IV: *Trichoderma* spp. as biological control agent against *F. culmorum*

Trichoderma spp. as biological control agent against *F. culmorum*

Background

Fungal plant pathogens are among the most significant threats to global agriculture, causing severe damage to a wide range of crops. Fungal diseases account for approximately 70–80% of all microbial infections in agricultural systems, highlighting their impact on food production (Peng *et al.*, 2021). Among fungal pathogens, *Fusarium* species are particularly notorious for significantly reducing both the yield and quality of staple crops worldwide, with yield losses of up to 56% reported in Tunisia (Bentley *et al.*, 2006; Chekali *et al.*, 2024).

Common wheat (*Triticum aestivum* L.), as a strategic crop and vital cereal worldwide, plays a fundamental role in food security due to its high starch and protein content. However, its cultivation is often challenged by diseases caused by *Fusarium* species, which can be transmitted through seeds, reside in the soil, or persist in crop residues, leading to severe yield losses (Scherm *et al.*, 2013; Karlsson *et al.*, 2021). Among the most aggressive pathogens, *F. culmorum* is a soil-borne fungus known for causing FCR, seedling blight, and head blight in wheat and other small-grain cereals like barley. The symptoms of FCR vary with the timing of infection but generally manifest as brown discolorations on roots, coleoptiles, and pseudostems during the early stages, and later as lesions on leaf sheaths and sub-crown internodes, tiller abortion, whiteheads, shriveled grains, and lodging (Burgess *et al.*, 1975; Wildermuth *et al.*, 1992; Scherm *et al.*, 2013). On the other hand, FHB symptoms include prematurely bleached spikelets and shrunken, discolored kernels (Scherm *et al.*, 2013). Beyond its destructive impact on crop development and yield, *F. culmorum* contaminates grains (Pastuszak *et al.*, 2021) and stems (Mudge *et al.*, 2006) with DON, posing risks to food and feed safety.

To mitigate the harmful effects of fungal pathogens, bio-control agents (BCAs) offer a promising and sustainable approach. Among BCAs, *Trichoderma* species have gained attention due to their well-documented antagonistic activity against numerous plant pathogens, including *Fusarium* species (Reino *et al.*, 2007; Vinale *et al.*, 2008; Degenkolb *et al.*, 2015). *Trichoderma* species are abundant in agricultural soils, making them suitable for application without significant disruption to the soil microbiome (Papavizas, 1985). Their mechanisms of action include the production of antifungal metabolites, chitinases, and other active compounds; mycoparasitism through direct physical interaction with pathogenic hyphae; induction of systemic resistance in plants; and effective competition for nutrients and space in the rhizosphere (Howell, 2003; Klokočar-Šmit et al., 2008).

Given the widespread occurrence of FCR in most bioclimatic zones of Tunisia and the significant impact of *F. culmorum* on wheat yield and quality (Chekali et al., 2024), there is an urgent need for sustainable management practices. In this context, evaluating the biological control potential of the *Trichoderma* species offers a promising solution. As naturally occurring soil fungi, *Trichoderma* species can reduce the prevalence and severity of *Fusarium* infections through direct mechanisms such as mycoparasitism, competition, and the production of antifungal compounds, or indirectly through modulation of the host's innate immunity performance (Tyskiewicz et al., 2022). By integrating *Trichoderma* into management strategies, it may be possible to control the development of FCR, decrease DON accumulation in crowns, and ultimately protect both the productivity and safety of wheat crops in Tunisia.

This study aims to evaluate the biocontrol potential of *Trichoderma* strains isolated from cereal-growing regions of Tunisia against *F. culmorum*. Fungal isolates were obtained from agricultural soils in diverse regions and identified using molecular techniques. The antagonistic activity of forty-three *Trichoderma* isolates from five different species was assessed through dual culture bioassay against a *F. culmorum* strain isolated from infected wheat.

Materials and Methods

Trichoderma strain collection

Thirty-two soil samples were collected from cereal crops in five different regions of Tunisia: Bizerte, Béja, Nefza, Jendouba and Sidi Bouzid. These regions were chosen for their bioclimatic diversity, reflecting a broad spectrum of growing conditions in Tunisia. The soil samples were first air-dried, then lightly ground with a mortar and pestle to obtain a homogeneous texture. The samples were then mixed thoroughly. A 10 g sub-sample was taken and transferred to a beaker containing 100 mL sterile 0.01% agar water, to prepare a 1:10 dilution. From this solution, 10 mL was withdrawn and added to a second beaker containing 90 mL sterile 0.01% agar water, to obtain a 1:100 dilution. The solution was thoroughly mixed to ensure even distribution of the soil particles. This step was repeated to prepare successive 1:1000 and 1:10000 dilutions, the latter being generally suitable for isolating *Trichoderma* fungi from the soil. (Burgess, 2008). For each dilution, 1 mL of soil suspension was transferred to a Petri dish containing selective PCNB (Pentachloronitrobenzene) medium. The dishes were incubated under constant light for 5-7 days, until colonies development.

Molecular identification of *Trichoderma* species

Trichoderma strains were incubated for three days at 25°C on cellophane membranes placed on PDA medium. The mycelium from each strain was harvested, quickly frozen, and subsequently freeze-dried. Genomic DNA was extracted and purified from 10-15 mg of lyophilized mycelia using the Wizard Magnetic DNA kit (Promega, Germany) following the protocol provided by the manufacturer.

The DNA concentration and structural integrity were evaluated using a Thermo Scientific NanoDrop (LabX, Midland, ON, Canada). The results were then compared to a standard 1 kb DNA ladder (Fermentas GmbH, St. Leon-Rot, Germany) following electrophoretic separation on a 0.8% agarose gel. Species-level identification of *Trichoderma* was performed through PCR amplification and sequencing using the ITS5 forward primer (5′- GAAGTAAAAGTCGTA ACA AG -3′, 150 ng/µL) and the ITS4 reverse primer (5′- CCTCCGCTTATTGATATGC -3′, 130 ng/µL) (White et al., 1990). In detail, PCR mixture (15 µL) including 15 ng of DNA template, 1.5 µL of 10× PCR buffer, 1.2 µL of dNTPs (2.5 mM), 0.45 µL of each primer (10 µM), and 0.125 µL of Hot Start Taq DNA Polymerase (5 U/µL; Fisher Molecular Biology) was amplified using the following parameters: initial denaturation at 94°C for 2 min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min. A final extension at 72°C for 7 min followed. The PCR products were verified by electrophoresis on a 1.5% agarose gel and visualized on a UV transilluminator. The PCR products were cleaned using the enzymatic mixture EXO/FastAP (ExonucleaseI, FastAP thermosensitive alkaline phosphatase, Thermo Scientific, Waltham, MA, USA). Sequencing reactions for both strands were carried out using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's guidelines. The labeled products were then purified using Sephadex G-50 (5%) (Sigma-Aldrich, Saint Louis, MO, USA) filtration and analyzed with the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The partial FASTA sequences were processed and assembled with BioNumerics v.5.1 software (Applied Maths, Kortrijk, Belgium) and aligned using the ClustalW algorithm (Thompson *et al.*), 1994). Phylogenetic relationships among and within *Trichoderma* species were analyzed employing the Maximum Likelihood method with MEGA software version 7 (Kumar *et al.*, 2016). The confidence of the internal nodes was evaluated using bootstrap analysis (Felsenstein, 1985) with a heuristic search approach with 1000 replicates to evaluate the robustness of the tree.

It should be noted that although ITS sequencing is widely used as a first molecular approach for fungal identification, it does not always provide sufficient resolution for certain genera, and this limitation is particularly evident for *Trichoderma* (Cai & Druzhinina, 2020). Indeed, species belonging to the *Trichoderma harzianum* complex and closely related taxa often show highly similar or even identical ITS sequences, making it challenging to distinguish them reliably based solely on this marker.

In this study, a preliminary identification was based exclusively on ITS sequences. However, the sequencing of the other two informative genes (*tef1* and *rpb2*) would provide a more correct identification at species level and a robust taxonomic resolution.

Assessment of the antagonistic activity of *Trichoderma* strains against *Fusarium culmorum*

The antagonistic potential of *Trichoderma* strains against *F. culmorum* was evaluated using dual culture assays, following the protocol described by Bunbury-Blanchette and Walker, 2019). Small agar plugs (5 mm^2) were cut from the actively growing margins of seven-day-old cultures of both *F. culmorum* strain and *Trichoderma* strains. These plugs, one of *F. culmorum* and one of a *Trichoderma* strain, were used to inoculate Petri dishes (8.5 cm Ø) containing PDA medium. Each plug was placed 2 cm from opposite edges along the same diameter, maintaining a 4.5 cm gap between them.

For each combination (*F. culmorum* + *Trichoderma*), four replicates were conducted, with both fungi being inoculated simultaneously. Additionally, four control plates were prepared containing only *F. culmorum*. All plates were incubated in the darkness at room temperature for 7 days.

To assess the impact of *Trichoderma* on *F. culmorum* growth, the radial growth of *F. culmorum* colonies was measured seven days post-inoculation in the direction of the opposing colony, then, the inhibition percentage was calculated using the following formula:

$$\% \text{ inhibition} = (A–B)/A \times 100$$

where A = colony diameter of the phytopathogen in the control plate, B = colony diameter in the dual culture plate.

Testing of the production of antifungal inhibiting volatile compounds

Antagonistic activity assay for volatiles

The antagonistic activity of the volatiles produced by *Trichoderma* spp. against *F. culmorum* was tested using the “Sandwiched Petri plate method” described by (Li *et al.*, 2018). After inoculating the different *Trichoderma* strains onto PDA plates, *F. culmorum* inoculated plates were placed on top of each *Trichoderma* plate, sealed with parafilm, and incubated at 25°C. A non-inoculated PDA plate was used as the control. Colony diameters were measured after 7 days. Each treatment included three replicates and was repeated twice.

The effect of volatile metabolites against the target pathogens was assessed according to the method reported in literature (Li *et al.*, 2018; Dennis & Webster). Petri plates containing PDA medium were inoculated with a 5 mm diameter disc from actively growing mycelia of antagonistic isolates. Another plate of the same diameter was inoculated with actively growing mycelia discs of test pathogens and inverted over the first plate containing the antagonist disc. The junction of both the Petri dishes was tightly sealed and incubated at room temperature in such a way that the antagonists lay under the lower disc. Sealed dishes with the pathogen inoculated in one plate and no antagonist inoculated in the other plate of the pair were used as a control. The experiment was performed in triplicate replication. Colony diameters were measured after 7 days. The percentage of inhibition was calculated by the formula mentioned above.

Fusarium culmorum inoculation and disease assessments on wheat seeds

A single *F. culmorum* strain obtained in previous studies from diseased wheat plants collected in 2021, was used for seeds inoculation. Protocols for inoculum preparation, inoculations and *Fusarium* disease assessments were described by Li et al., (2008). Briefly, wheat grains were first surface sterilized by immersion in a 70% ethanol solution for 2 minutes, followed by three rinses with sterile distilled water. The sterilized grains were then inoculated by soaking in a spore suspension of *F. culmorum* supplemented with Tween 20 at a concentration of 1.5×10^5 spores/mL for 10 minutes under gentle agitation to ensure uniform coverage. After inoculation, the grains were air-dried under sterile conditions before sowing.

Trichoderma inoculum was prepared using PDA plates at half-concentration. Inoculated plates were incubated for 7 days under a combination of cold white and black, fluorescent lamps with a 12-hour photo period. The spores were then harvested, and the concentration of the spore suspension adjusted to 1×10^6 spores/ml. Seeds coated with the various treatments were germinated in Petri dishes on ten layers of water-saturated filter paper.

Seeds germination percentage was calculated after 5 days of incubation. Then, seedling growth parameters and FCR disease severity were evaluated 10 days after the incubation.

Disease severity was assessed by determining the brownish discoloration on the stem bases. The brown color was evaluated using a 0-4 scale (0 = completely healthy, 1 = less than 25% necrosis, 2 = 25–50% necrosis, 3 = 50-75% necrosis and 4 = greater than 75% necrosis). Ratings were converted to severity indices using the following formula (Chen *et al.*, 2023);

$$DI= (\sum nX/4\ N) \times 100$$

With X scale value of each plant, n number of plants in the category, and N is the total number of plants assessed for each material.

Results

Distribution and molecular identification of *Trichoderma* strains in Tunisian soils

In this study, thirty-two soil samples from various regions of Tunisia (Figure 1a) were used to isolate 43 *Trichoderma* strains (Table S1). *Trichoderma* contamination was detected in 65.6% of soil samples, with frequencies varying by region: 66.7% in Sidi Bouzid, 80% in Bousalem and Bizerte, 50% in Béja, and 62.5% in Nefza (Figure 1b).

Molecular analysis of the forty-three isolates revealed a predominance of *T. harzianum*, which made up 97.7% of the total strains. It was recorded with a frequency of 100% in all regions except Beja (90.9%). In addition to this dominant species, *T. citrinoviride* was also identified at a very low frequency of 2.3%.

In terms of geographical distribution, *T. harzianum* was found in all the studied regions. In contrast, *T. citrinoviride* was only detected in Beja.

Molecular identification of the 43 *Trichoderma* strains was based on amplification of the ITS region using primers ITS5 and ITS4. The sequences obtained were compared with the NCBI database using the BLAST tool, revealing high percentages of identity with different species of the *Trichoderma* genus. While most strains showed strong similarity with strains belonging to the *T. harzianum* complex, some were associated with other species. For example, isolate Tr1 showed 100% identity with *T. afroharzianum* (strain NY26), while Tr3, Tr7, Tr11 and Tr15 showed identities of 99.52%, 100%, 100% and 99.84% respectively with *Hypocrea lixii* (strain

NRCfBA-37). Similarly, Tr19 showed 100% identity with *Hypocrea intricata* (strain CM01) as well as with *T. inhamatum* (strain wb265).

However, it is important to highlight that partial amplification of the ITS region alone is insufficient for reliable species-level identification within the *Trichoderma* genus especially for closely related species within the *T. harzianum* complex as noted in recent guidelines (Cai and Druzhinina, 2020). For robust taxonomic resolution, other informative genomic markers, such as tef1-α, rpb2, will be included in future analyses.

Phylogenetic analysis showed that all strains grouped into three well-distinct clades (Figure 2), each supported by high bootstrap values (>70). The first clade, strongly supported (100% bootstrap value), included the majority if the strains analyzed (Tr1 - Tr46). This group also included several reference strains such as *T. harzianum* (strain T1), *T. afroharzianum* (strain CBS 124620) and *T. atrobrunneum* (strain CBS 548.92), belonging to the *T. harzianum sensu lato* complex. Within this clade, Tr1, Tr10 and Tr11 showed high similarity with the reference strains, indicating strong genetic proximity to them. The second clade, also well supported (98% bootstrap), included only Tr31 field strain and the reference strain *T. citrinoviride*. The third clade included species used as outgroups: *Ascochyta phacae* (strain CBS 184.55) and an T. *asperellum* (strain 12DEC). Overall, this tree reveals a moderate but well-structured diversity of the strains, dominated by representatives of the *T. harzianum* complex, accompanied by a few genetically distinct isolates such as Tr31. These results provide a valuable basis for assessing the biological potential of isolates and identifying those of particular interest for biocontrol.

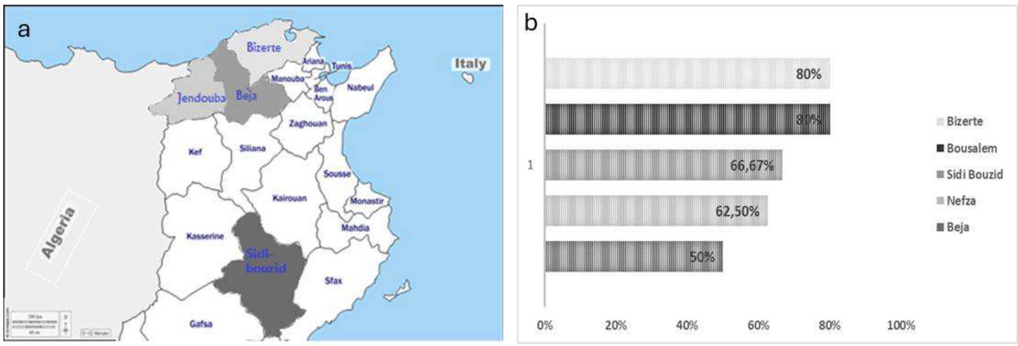

Figure 1: *Identification of Trichoderma spp. (a) Map of strains collection; (b) isolation frequency of Trichoderma spp.*

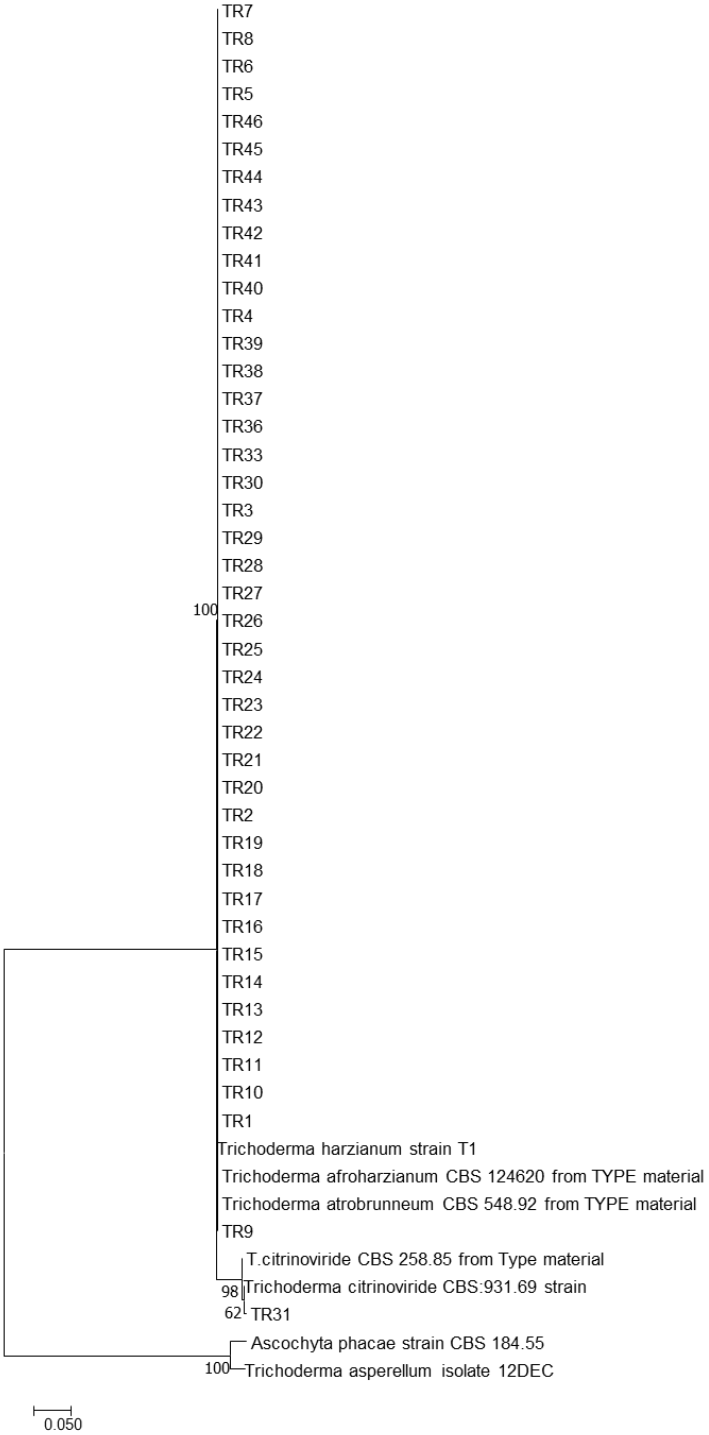

Figure 2: *Phylogenetic tree based on amplification of the ITS region sequences of 43 Trichoderma strains isolated from cereal crop soil in Tunisia and 7 reference sequences. The phylogenetic tree was generated using the Maximum Likelihood Method; bootstrap values > 70 with 1000 replicates are indicated near the branches..*

Antifungal activity of *Trichodema* strains

Radial growth inhibition of *F. culmorum*

The activity of *Trichoderma* strains against *F. culmorum* varied significantly, as shown in Figures 3 and 4.

Regarding radial growth and mycelial inhibition, isolates Tr13 and Tr15 exhibited the lowest growth rates (both measuring 1 cm) and also achieved the highest mycelial growth inhibition, with rates of 72% and 70%, respectively. In contrast, isolates Tr11, Tr33, Tr30, Tr26, and Tr23 showed no significant difference in radial growth compared to the control, with respective growth rates of 5, 5, 5, 4, and 4 cm. Among them, Tr11 strain exhibited no significant inhibition compared to the control (0.35%; $p = 0.932$), while Tr33 strain showed the lowest inhibition rate at 5% (Figure 3).

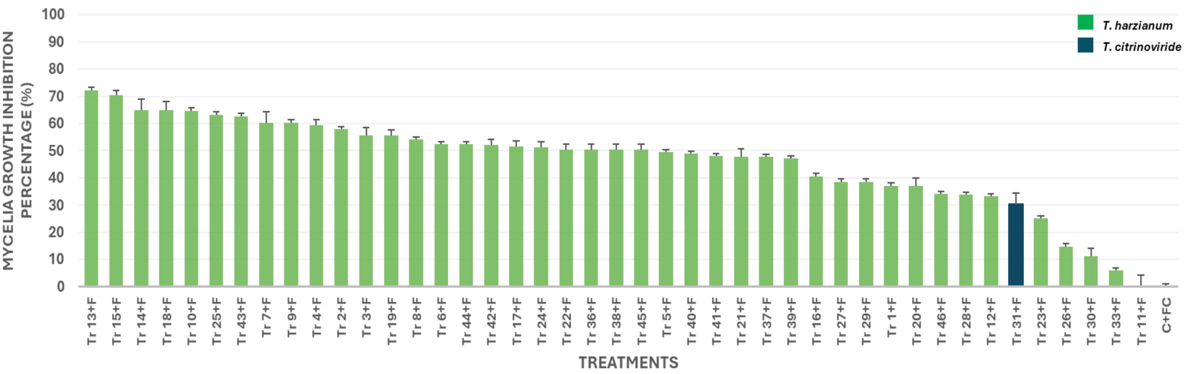

Figure 3: *Mycelia growth inhibition percentage (%) of F. culmorum by different Trichoderma spp strains*

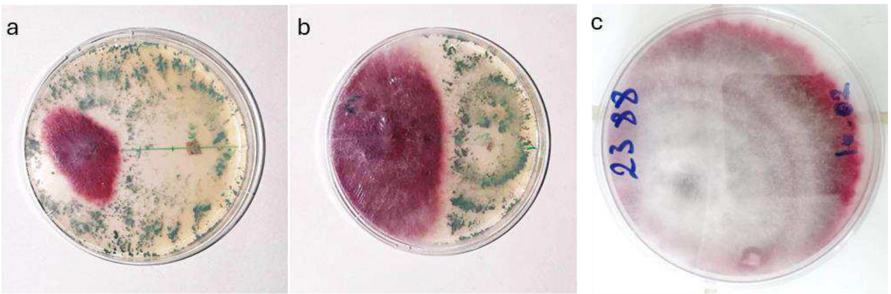

Figure 4: *Antagonistic effects of Trichoderma strains Tr 15 (a) and Tr 30 (b) against F. culmorum, control (c) on solid PDA media after seven days of incubation at 25°C.*

Effect of volatile compounds produced by *Trichoderma* strains on *F. culmorum*:

To further support the antifungal potential of *Trichoderma* strains, the effects of their volatile compounds on *F. culmorum* were evaluated *in vitro*. The results of pathogen growth inhibition by the strains are summarized in Figures 5 and 6.

The strains Tr42, Tr44 and Tr39 showed the highest radial growth, each reaching 4 cm, they also showed the lowest inhibition percentages, with values of 3, 5 and 6%, respectively.
The strains with the lowest radial growth, Tr24 (2 cm), Tr45 (3 cm) and Tr37 (3 cm), showed the highest inhibition percentages, reaching 37, 37 and 35%.

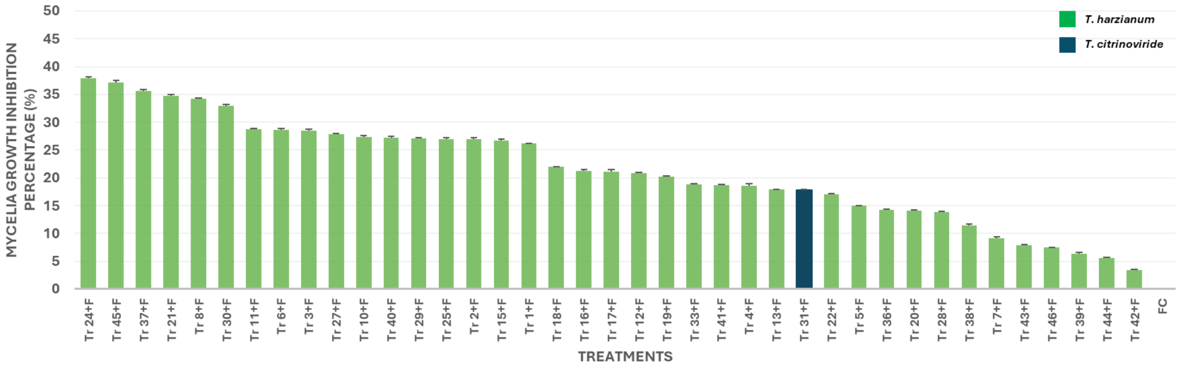

Figure 5: *Effect of Trichoderma volatiles compounds on mycelia colony growth inhibition (%) of F. culmorum*

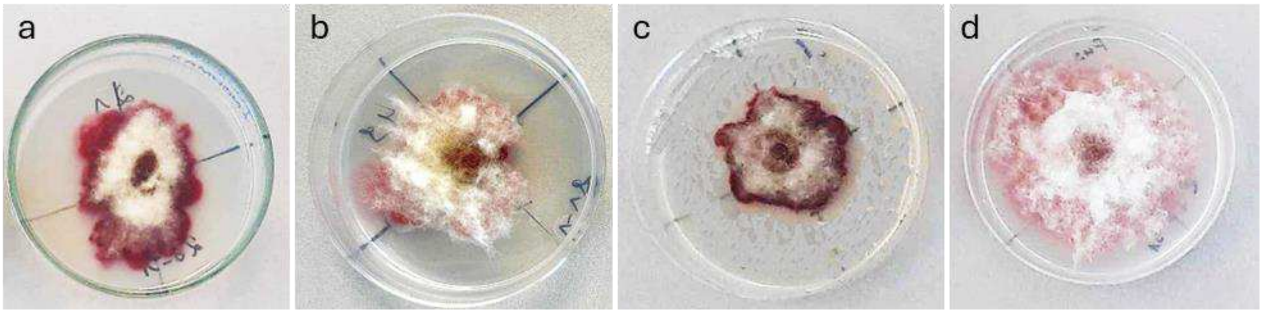

Figure 6: *Dual culture assay for bioactivity of volatile organic compounds of the Trichoderma strains on Fusarium culmorum: (a-c) mycelia growth under Trichoderma effect, (d) Control. Both the bioagent and pathogen were grown on PDA and exposed to each other for 7 days.*

Impact of *Trichoderma* spp treatment on wheat growth

Effect on germination

Overall, no significant difference was observed in the germination rate of seeds treated with *Trichoderma* compared to the non-treated control (p = 0.265). However, among the 17 tested isolates, five strains of *T. harzianum* Tr 1, Tr27, Tr44, Tr8, and Tr30 exhibited lower germination rates than the control (Figure 7).

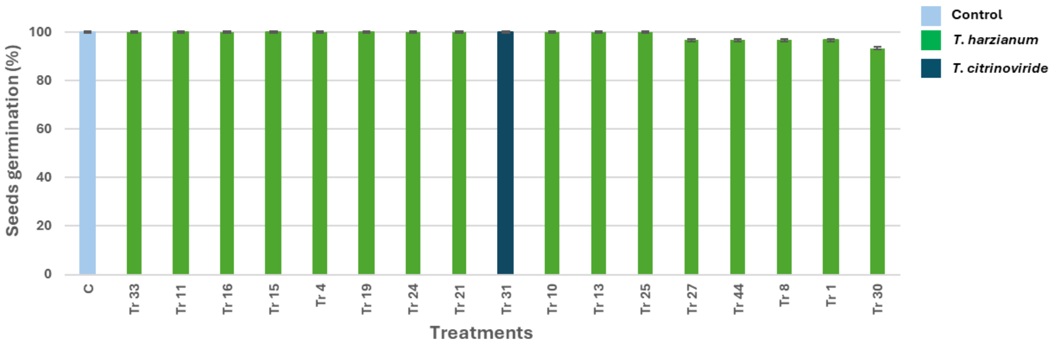

Figure 7: Effect of seed dressing with different *Trichoderma* spp. strains on germination (%) of wheat seeds.C: control with non-treated seeds

Effect on leaf growth

Regarding leaf development under *Trichoderma* treatment, no significant differences were observed for most of the isolates compared to the untreated control except for, *T. harzianum* (Tr 1, Tr 11, Tr21, Tr27, Tr44, and Tr16) and *T. citrinoviride* (Tr31) which significantly reduced leaf growth. Among all tested isolates, *T. harzianum* Tr44 resulted in the most pronounced reduction in leaf growth (p<0.0001; Figure 8).

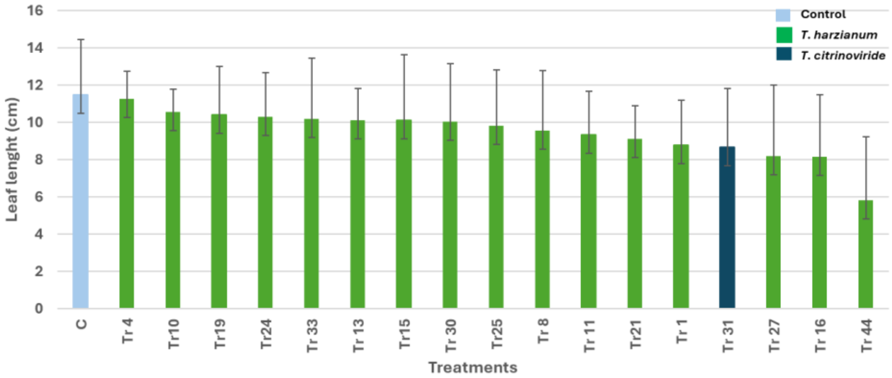

Figure 8: *Effect of Trichoderma seed dressing on leaf growth (cm) of wheat plants; C: control (non-treated seeds).*

Effect on root growth

For root development under *Trichoderma* treatment, seven strains of *T. harzianum* (Tr 4, Tr 10, Tr 13, Tr 21, Tr 24, Tr 25, and Tr 33), significantly enhanced root growth compared to the untreated control. In contrast, the strain Tr 44, also belonging to *T. harzianum* complex, exhibited the lowest root development among all treatments (p < 0.0001). For the remaining strains tested, no significant differences were observed. (Figure 9).

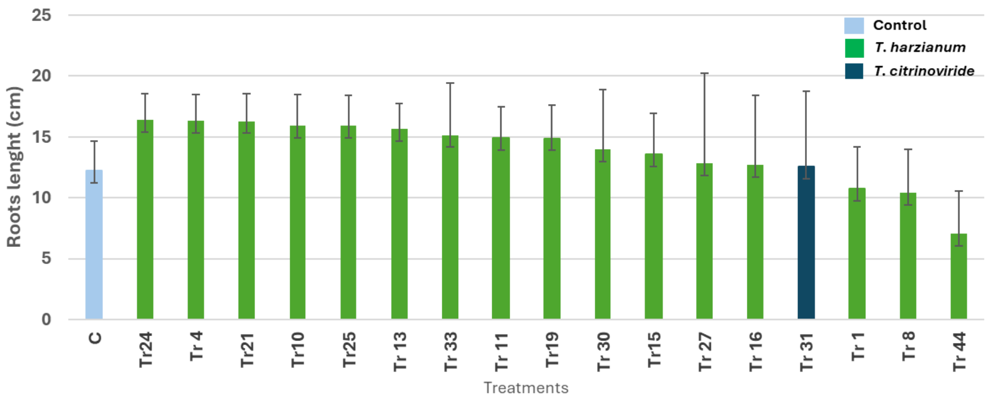

Figure 9: *Effect of Trichoderma strains on roots development (cm) of wheat plants C: control (non-treated seeds).*

Effect on leaf biomass

In terms of leaf biomass from seeds treated with *Trichoderma*, a significant increase in fresh leaf biomass was observed for *T. harzianum* (Tr33, Tr4, and Tr24) and *T. citrinoviride* (Tr31), compared to the untreated control (Figure 10). No significant differences were detected for the other isolates.

For dry leaf biomass, no statistically significant differences were found among isolates. However, in terms of average values, *T. citrinoviride* (Tr31) and *T. harzianum* (Tr8 and Tr13) recorded the highest dry biomass (0.10 g), while *T. harzianum* Tr44 exhibited the lowest value (0.06 g) (Figure 11).

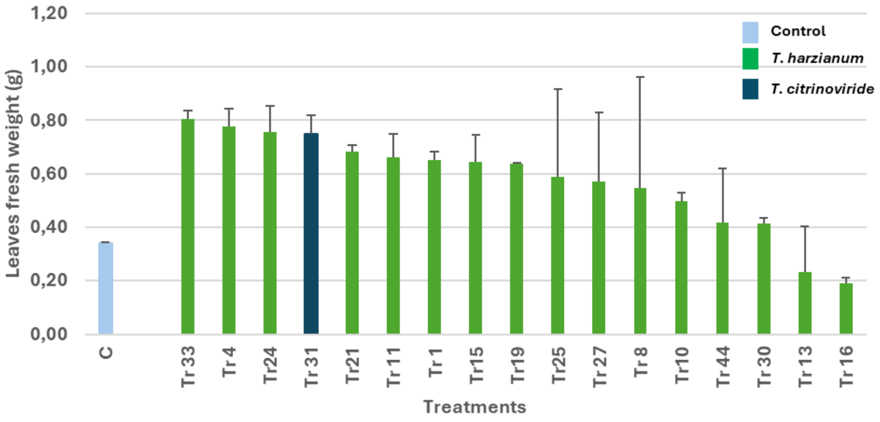

Figure 10: *Effect of Trichoderma strains on fresh weight of leaves biomass of wheat plants treated with Trichoderma; C: control (non-treated seeds)*

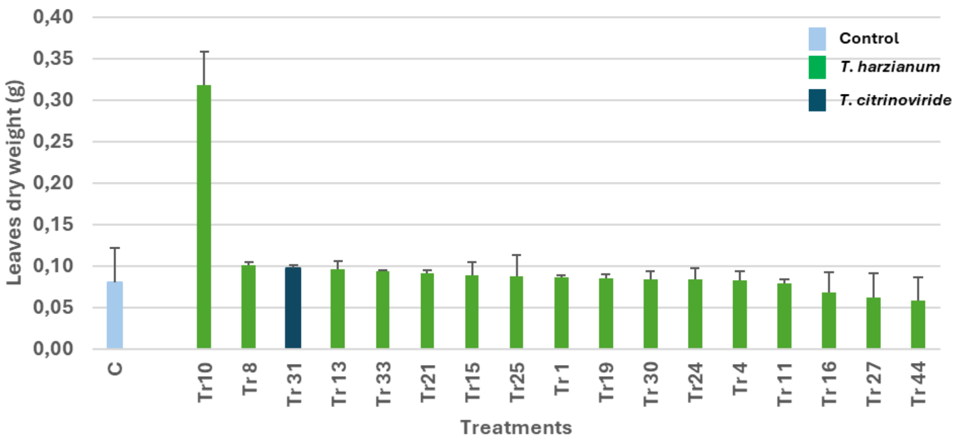

***Figure 11:** Effect of Trichoderma strains on dry weight of leaves biomass of wheat plants treated with Trichoderma; C: control (non-treated seeds)*

Effect on roots biomass

For root biomass of *Trichoderma* treated plants, the Tr4, Tr11, Tr21, and Tr33 *T. harzianum* strains significantly increased fresh root weight compared to the untreated control (Figure 11). No significant differences were observed for the remaining strains. Notably, *T. harzianum* Tr44 strain showed the lowest fresh root biomass (0.20 g), suggesting a possible inhibitory effect on root development (Figure 12).

As for dry root weight, no significant differences were detected between the treated and untreated plants for any of the strains (Figure 13).

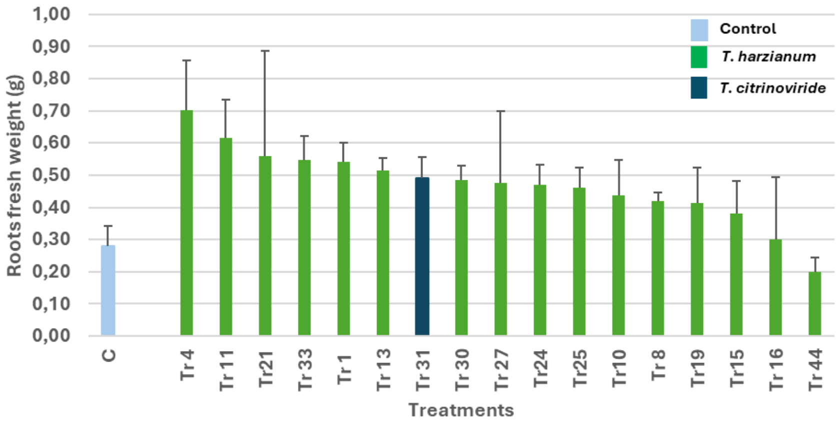

***Figure 12:** Effect of Trichoderma strains on fresh weight of roots biomass of wheat plants treated with Trichoderma; C: control with non-treated seeds*

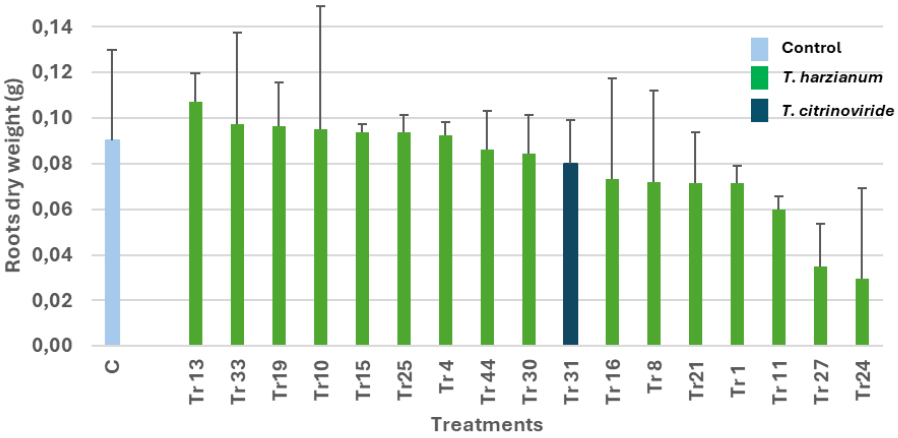

Figure 13: *Effect of Trichoderma strains on dry weight of roots biomass of wheat plants treated with Trichoderma; C: control with non-treated seeds*

Effect of *Trichoderma* spp on Fusarium Crown Rot

Effect on Disease Severity

Trichoderma strains exhibited a significantly variable impact on the severity of FCR. Notably, *T. harzianum* strains Tr10, Tr 11, Tr 19 and Tr21 significantly reduced disease severity, achieving the lowest severity values, 6% for Tr10 ($p = 0.0208$), 5% for Tr11 ($p = 0.0168$), and as low as 3% for both Tr19 and Tr21 strains ($p = 0.0087$).

In contrast to the other isolates, including Tr33 and *T. citrinoviride* Tr31, failed to mitigate disease symptoms and even exhibited exacerbated effects, with severity indices of 59% and 48%, respectively, both exceeding the level observed in the control C+F inoculated with *F. culmorum* (40%) (Figure 14).

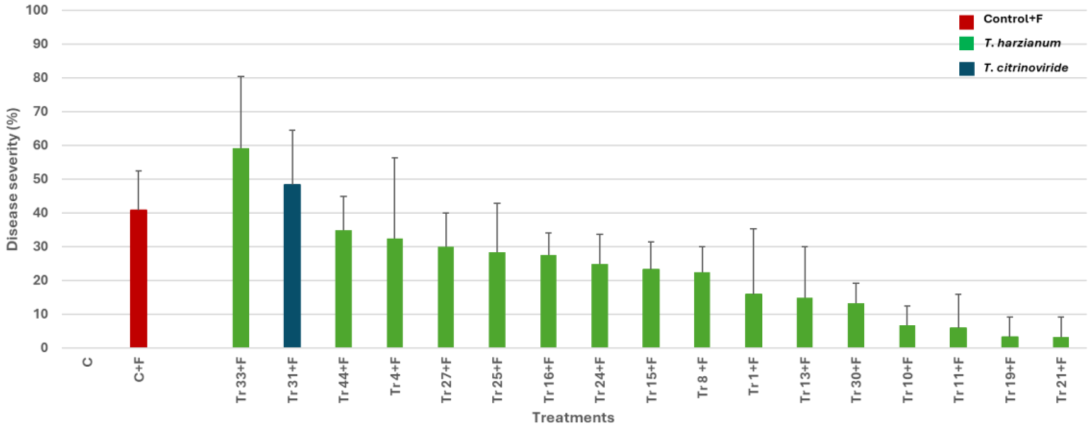

Figure 14: *Effect of Trichoderma strains on disease severity (%) of wheat plants contaminated with F. culmorum; C: control with non-treated seeds and C+F control with seeds inoculated with F. culmorum.*

Effect on Wheat growth

Wheat seeds germination

The impact of *Trichoderma* isolates on seed germination, compared with the *F. culmorum* inoculated control, showed that *T. harzianum* (Tr 4 and Tr 10 and Tr 11) significantly improved germination, reaching a rate of 98% each ($p = 0.0169$) (Figure 15 and 16).

Conversely, no significant differences were observed for the other isolates, whose germination rates ranged from 97% for *T. harzianum* Tr 19 and Tr 1, to 66% for *T. citrinoviride* (Tr 31), which recorded the lowest germination (Figure 15).

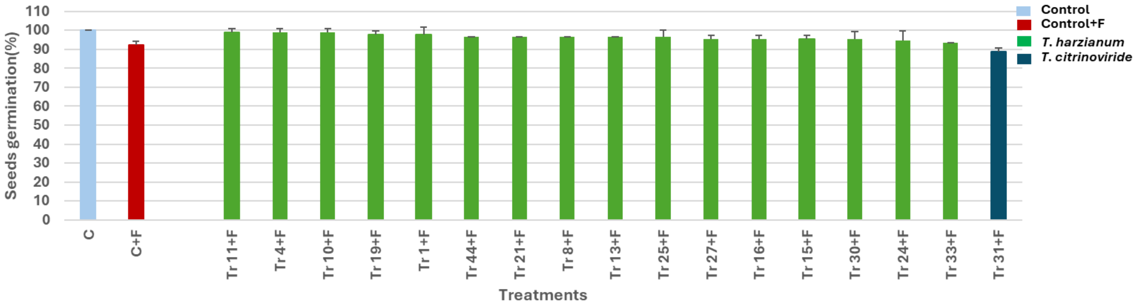

Figure 15: *Effect of Trichoderma strains on germination (%) of wheat seeds contaminated with F. culmorum; C: control with non-treated seeds and C+F control with seeds coated only with F. culmorum.*

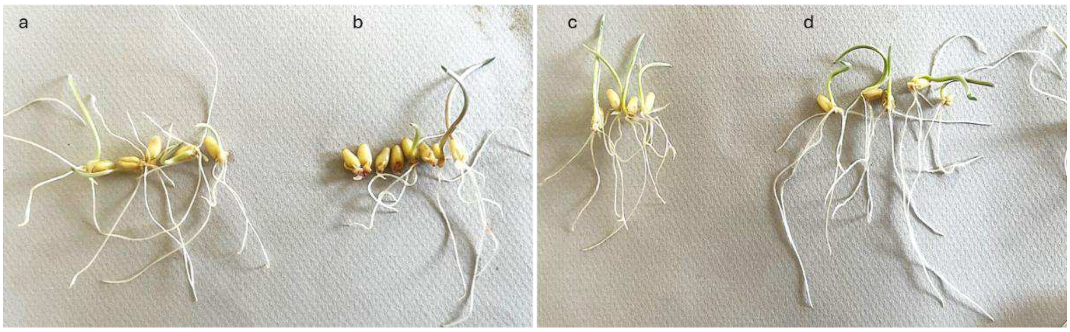

Figure 16: *Effect of Trichoderma strains on germination (%) of wheat seeds 5 days after inoculation: non-inoculated seeds (a) seeds inoculated with F. culmorum (b) seeds inoculated with Trichoderma (Tr 4 strain) and F. culmorum (c) and seeds inoculated with only Trichoderma (Tr 4) (d).*

Effect on leaf growth

The inoculation with *F. culmorum* resulted in a significant reduction in leaf development, with mean leaf height decreasing from 11 cm in the non-inoculated control to 4 cm ($p < 0.0001$).

In contrast, none of the *Trichoderma* strains induced a significant improvement in leaf growth compared with the *F. culmorum* inoculated control. Leaf growth rates varied between strains: *T. harzianum* Tr 19, Tr 21and Tr 44 strains showed the highest average heights, reaching 6, 5 and 5 cm, respectively thus exceeding the growth measured under the effect of *F. culmorum* (4 cm). Conversely, *T. citrinoviride* Tr 31 strain showed the lowest leaf growth, with an average height of 3 cm (Figure 17).

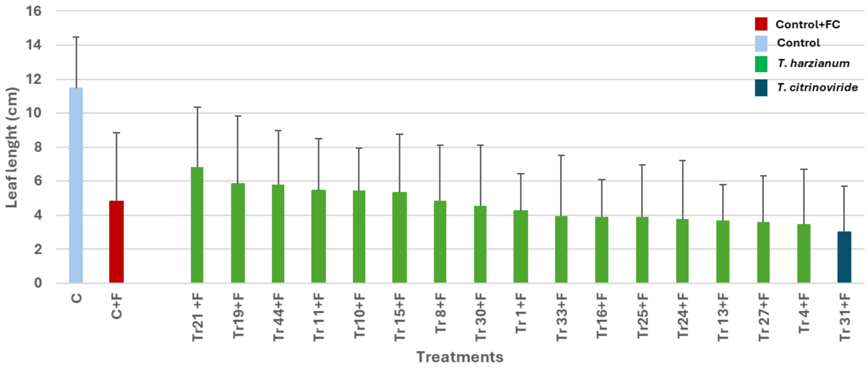

Figure 17: *Effect of Trichoderma strains on leaf development (cm) of wheat plants contaminated with F. culmorum; C: control with non-inoculated seeds and C+F control with seeds inoculated with F. culmorum.*

Effect on root growth

The inoculation with *F. culmorum* resulted in a significant decrease in root growth, reducing the average root length from 12 to 5 cm ($p < 0.0001$).

Among the *Trichoderma* tested strains, *T. harzianum* Tr 11 and Tr 44 strains favored the best root growth, reaching 8 cm ($p = 0.0187$) and 7.9 cm ($p = 0.0318$) respectively, thus significantly exceeding the growth recorded under the effect of *F. culmorum* (5 cm).

In contrast, none of the other isolates showed any significant difference from the *F. culmorum* infected control. Isolate *T. citrinoviride* (Tr 31) recorded the lowest root growth, with an average length of 3 cm. (Figure 18).

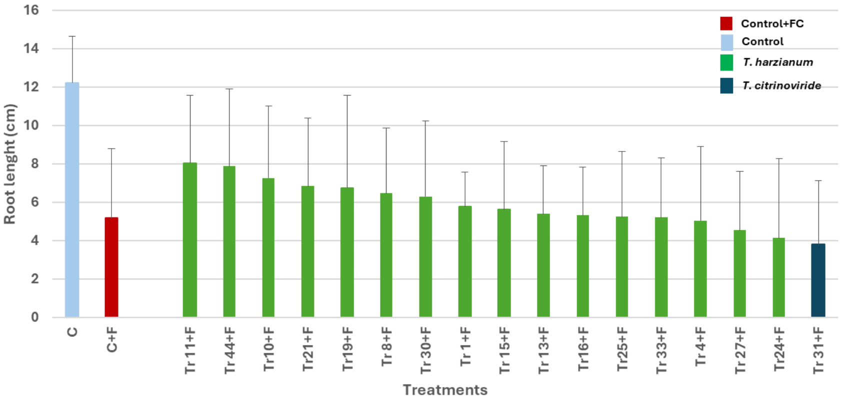

Figure 18: *Effect of Trichoderma strains on roots development (cm) of wheat plants contaminated with F. culmorum; C: control with non-inoculated seed and C+F control with inoculated seeds with F. culmorum.*

Effect on leaf Biomass

For seeds inoculated with *F. culmorum* and treated with *Trichoderma* strains no significant difference was observed for leaf fresh weight compared to the inoculated control (p=0.157, Figure 19).

Regarding dry weight (Figure 19), *T. harzianum* Tr1 strain significantly increased leaf biomass (0.08 g, p = 0.0024), whereas no significant differences were observed for the other strains (Figure 20).

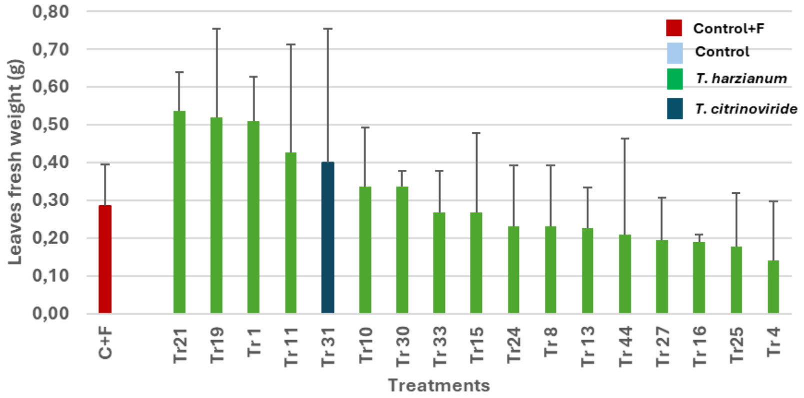

Figure 19: *Effect of Trichoderma strains on fresh weight of leaf biomass of wheat plants inoculated with F. culmorum; C+F control inoculated with F. culmorum*

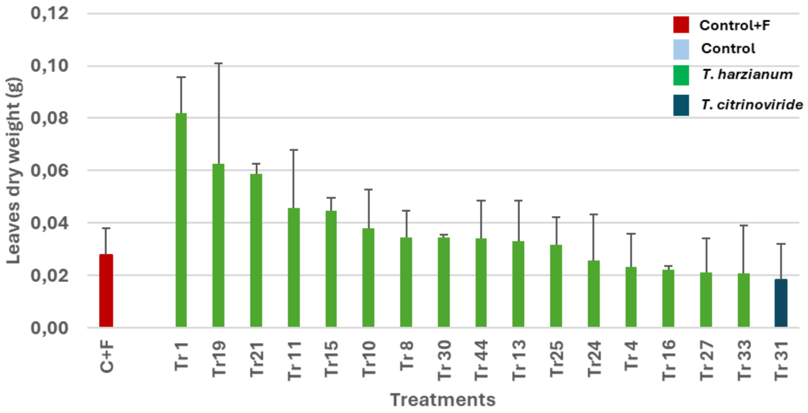

Figure 20: *Effect of Trichoderma strains on dry weight of leaves biomass of wheat plants inoculated with F. culmorum; C+F control inoculated with F. culmorum*

Effect on root Biomass

At the root level, no significant differences were observed between the treated seeds with the tested isolates and the inoculated control (p=0.062, Figure 21).

Regarding dry weight, *T. harzianum* Tr16 strain showed a significantly higher value (0.08 g, *p* = 0.015), while no significant differences were detected for the other strains (Figure 22).

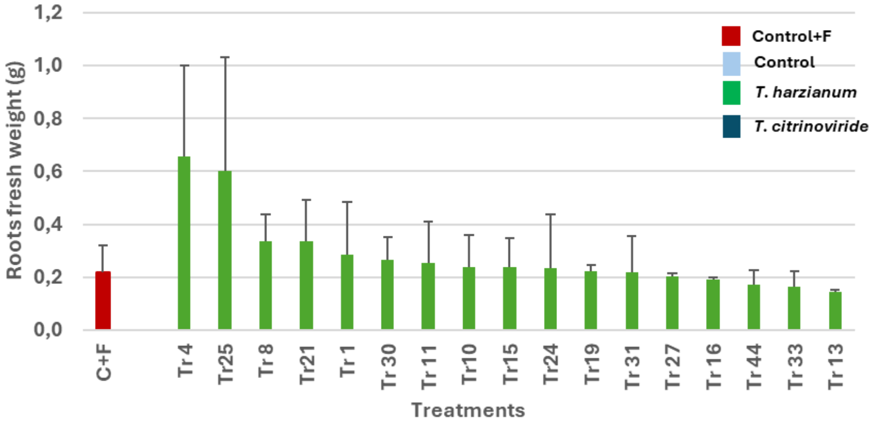

Figure 21: *Effect of Trichoderma strains on fresh weight of roots biomass of wheat plants inoculated with F. culmorum with C+F control inoculated with F. culmorum*

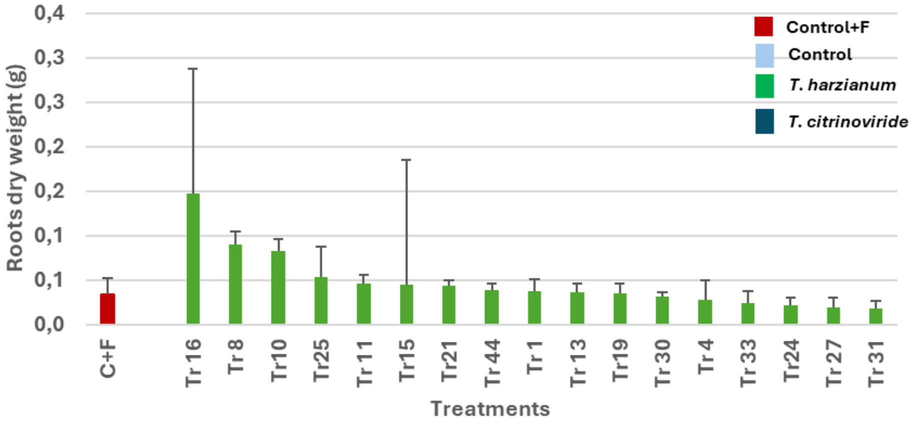

Figure 22: *Effect of Trichoderma strains on dry weight of roots biomass of wheat plants inoculated with F. culmorum; C+F control inoculated with F. culmorum*

Clustering of *Trichoderma* strains

As a bio-stimulant for wheat plant growth

Cluster 1 (blue), which includes *T. harzianum* strains Tr 4, Tr 10, Tr 11, Tr 13, Tr 15, Tr 19, Tr 21, Tr 24, Tr 25 and Tr 33 and *T. citrinoviride* Tr 33strain, is closer to the non-treated control (C).

Cluster 2 (green), including *T.harzianum* Tr 1, Tr 8, Tr 27 and Tr 30 strains, showed an effect between cluster 1 and cluster 3, indicating both the homogeneity of these strains and their distinct but moderate effect compared to the other ones. This intermediate effect suggests a performance between that of isolates in cluster 1 (close to the non-treated control (C)) and those in cluster 3 very far from the control C and which are the least efficient.

Cluster 3 (red) included *T.harzianum* Tr 44 and Tr16 strains, with similar characteristics which are negatively far from cluster 1, representing the group of strains with the lowest performance in biostimulation of wheat plant growth.

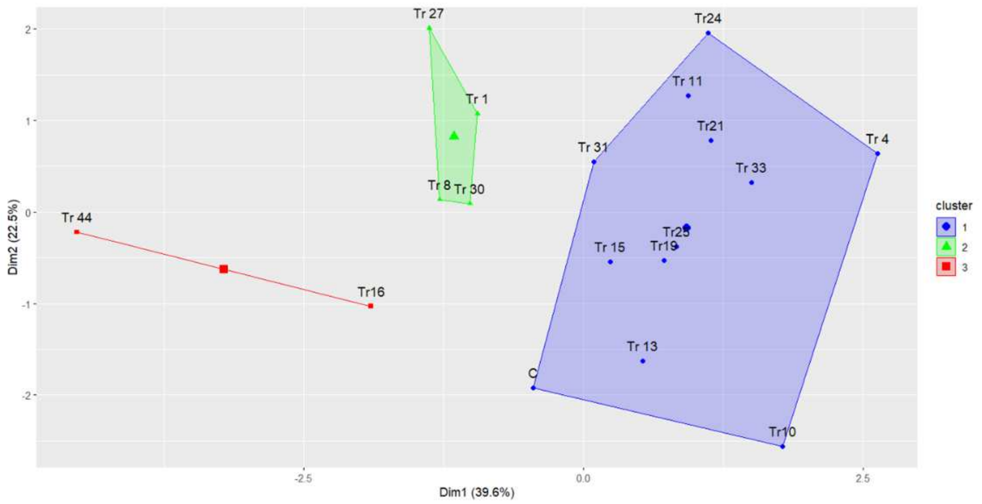

Figure 23: *Principal Component Analysis (PCA) of Trichoderma Strains Based on Their Biostimulant Effects*

As a biocontrol agent against *F. culmorum*

Cluster 1 (blue), which included *T. harzianum* Tr1, Tr10, Tr11, Tr19 and Tr21 strains, is closer to the healthy control (C), indicating a beneficial effect of biological control, characterized by better germination and reduced disease severity.

Cluster 2 (green), made up of *T. harzianum* Tr4, Tr8, Tr13, Tr15, Tr16, Tr25 and Tr44 strains, lies between clusters 1 and 3, indicating both the homogeneity of these isolates and their distinct but moderate effect compared to the other isolates. This intermediate effect suggests a performance between that of isolates in cluster 3, close to the inoculated control (C+F), and those in cluster 1, close to the healthy control (C).

Cluster 3 (red) includes isolates *T. citrinoviride* Tr 31 strain and *T. harzianum* Tr24, Tr27, Tr30 and Tr33 strains, with similar characteristics. Their proximity to the inoculated control (C+F) suggests that they had no significant effect on germination inhibition compared with the effect of *Fusarium* alone.

In comparison, the control inoculated with Fusarium (C+F) is close to the red cluster, suggesting that these strains have an effect similar to that of *Fusarium* alone, while the healthy control (C) is closer to the blue cluster, indicating that these isolates have a beneficial effect comparable to the total absence of the pathogen.

In conclusion, the strains in the blue group appear the most promising, being close to the healthy control, while those in the red cluster showed behavior similar to that of the infected control, suggesting less pronounced efficacy.

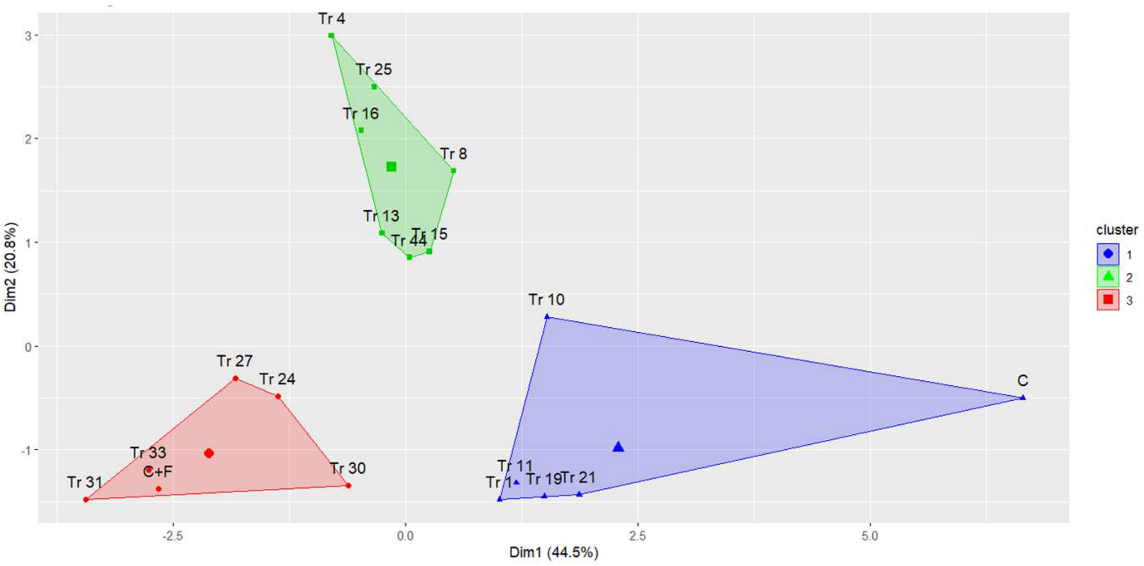

Figure 24: *Principal Component Analysis (PCA) of Trichoderma strains based on their biocontrol potential against Fusarium culmorum*

Dual-function *Trichoderma* isolates exhibiting both biostimulant and biocontrol activity against *F. culmorum*

Among the tested strains, and referring to clustering results, *T. harzianum* (Tr10, Tr 11, Tr 19 and Tr21) demonstrated the most promising dual action: not only did they significantly stimulate plant growth parameters, but they also effectively suppressed Fusarium crown rot (FCR) symptoms caused by *F. culmorum*.

Relationship between *Trichoderma* biostimulation and vegetative growth

Principal component analysis (PCA) reveals a strong positive correlation between vegetative growth parameters, notably root length (RL), leaf length (LL), and fresh and dry biomass at leaf (TrLFB, TrLDB) and root (TrRFB, TrRDB) levels. Arrows pointing in a similar direction, grouped in the same quadrant, indicate that these variables evolve in a coordinated fashion.

The close proximity of the RL, LL, TrRFB and TrLFB vectors suggests harmonious, synchronous growth of the above-ground and below-ground parts of seedlings treated with *Trichoderma* isolates. Isolates that stimulate root development also tend to promote leaf expansion and biomass accumulation, reflecting a consistent morpho-physiological response to the biostimulant effect.

Germination percentage (GP), although somewhat more distant from the other growth variables, remains positively correlated with all vegetative parameters, notably leaf length (LL) and leaf dry biomass (TrLDB). This indicates that seeds with greater germination capacity give rise to more vigorous seedlings

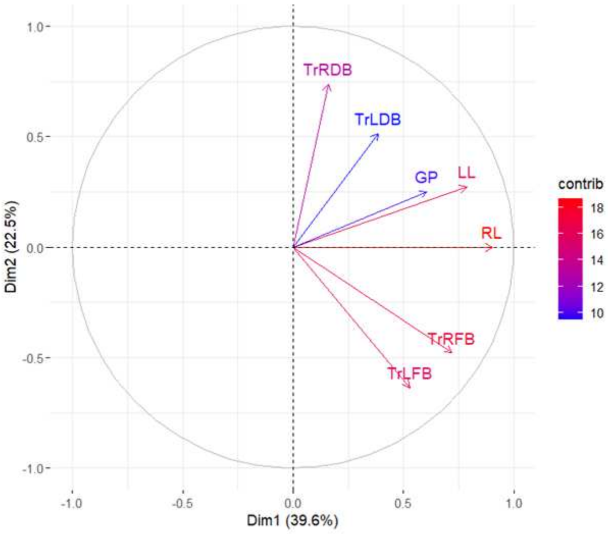

Figure25*: Correlations between the parameters studied: RL: Root length; LL: Leaf length; TrRFB: root fresh biomass; TrRDB: Roots dry biomass; TrLFB: Leaf fresh biomass; TrLDB: Leaf dry biomass.*

Relationship between biocontrol parameters and FCR disease severity

Correlation analysis using PCA was used to explore the relationships between the different variables measured during the study (Figure 25).

A strong positive correlation was found between parameters associated with vegetative growth, notably root length (RL), leaf length (LL), fresh leaf biomass (LFB), dry leaf biomass (LDB), as well as fresh root biomass (RFB) and dry root biomass (RDB). This consistency between the various morpho-physiological components reflects harmonious development dynamics: seedlings with a well-structured root system also tend to produce greater above-ground biomass.

Germination percentage (GP) shows a moderate positive correlation with growth variables, in particular RL, LL, LFB, LDB and RDB. This relationship indicates that seeds with a high germination rate generally give rise to seedlings with greater root and aerial vigor. Germinative vigor thus appears to be a reliable indicator of seedlings' initial development potential.

Similarly, indices of mycelial growth inhibition by *Trichoderma* isolates, whether under *in vitro* conditions (MGI-IV) or via the emission of volatile compounds (MGI-VOC), also show a moderate positive correlation with several growth parameters (RL, LL, LFB, LDB, RDB). This association could reflect a link between the efficacy of biocontrol agents and overall seedling health.

In contrast, disease severity (DS) is positioned opposite growth variables on the correlation circle, reflecting a marked negative correlation with GP, RL, LL, LFB, RFB and RDB. This indicates that the intensity of pathogen attack is inversely proportional to seedling vegetative vigour: the higher DS, the more root and leaf growth is compromised. This negative relationship reflects the hypothesis of a direct deleterious effect of the pathogen on the physiology of young plants.

Finally, the correlations between DS and fungal inhibition indices (MGI-IV, MGI-VOC) appear weak or non-existent, suggesting that the antifungal efficacy observed *in vitro* does not necessarily translate into an effective reduction in disease severity under more complex biological conditions.

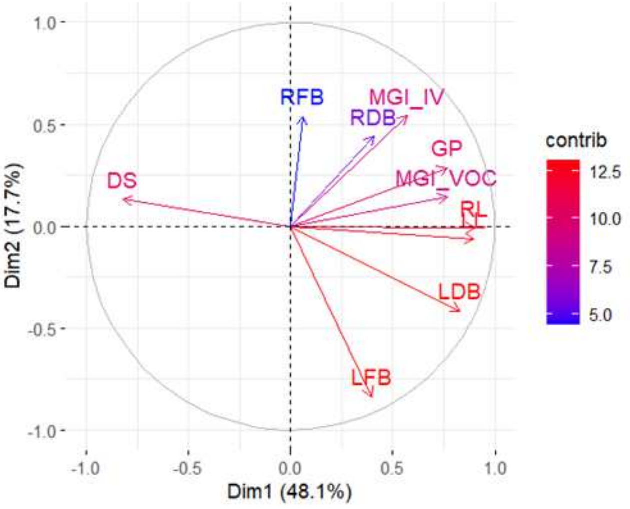

Figure26*: Correlations analysis using PCA between the parameters studied: GP: Germination percentage; DS disease severity; RL: Root length; LL: Leaf length; RFB: root fresh biomass; RDB: Roots dry biomass; LFB: Leaf fresh biomass; LDB: Leaf dry biomass; MGI-IV: Mycelia growth inhibition in-vitro; MGI-VOC: Mycelia growth inhibition- volatile compounds.*

Discussion

Species of the *Trichoderma* genus are widely distributed in agricultural soils around the world and play an essential ecological role, notably as biological control agents against numerous plant pathogens (Howell et al., 2003; Vinale et al., 2008; Saravanakumar et al., 2017; Tyskiewicz et al., 2022). Their mechanisms of action involved mycoparasitism, competition for nutrients and space, production of antifungal metabolites, and induction of systemic resistance in plants (Reino et al., 2007; Degenkolb et al., 2015; Li et al., 2018; Woo et al., 2023).

The distribution of *Trichoderma* species depends on many factors, including edaphic characteristics, climatic conditions and crop type (Papavizas et al., 1985; Hibar et al., 2004; Modrzewska et al., 2021). Several studies have highlighted the dominance of *T. harzianum* in different agroecosystems, attributed to its high adaptability and biological efficiency (Bunbury-Blanchette & Walker, 2019; Peng et al., 2021; Yao et al., 2023).

In this context, the present study, based on 32 soil samples collected from various cereal-growing regions of Tunisia, enabled the isolation and preliminary identification of 43 *Trichoderma* strains. Molecular analysis based on the ITS region sequencing revealed a marked predominance of strains assigned to *T. harzianum* complex (97.7%). In contrast, strain identified as *T. citrinoviride* were detected only in Béja region, suggesting a potentially restricted geographical distribution of this taxon. However, it is important to emphasize that identification based exclusively on the ITS region cannot unequivocally confirm species-level assignment within the *Trichoderma* genus, as ITS often lacks sufficient resolution for closely related species (Druzhinina et al., 2005; Cai and Druzhinina, 2020). Further studies integrating additional loci such as *tef1-α*, *rpb2*, are necessary for robust species delimitation (Chaverri et al., 2015; Atanasova et al., 2013).

Despite this limitation, the high frequency of isolates clustering with *T. harzianum* aligns with findings from other Mediterranean and semi-arid regions, where this taxon (or species complex) is frequently dominant (Bouanaka et al., 2021; Mulatu et al., 2022; Wang et al., 2022). Similarly, *T. harzianum* has been reported among the most abundant species in Tunisian soils (Sadfi-Zouaoui et al., 2009). Comparable patterns have been observed in Europe (Matarese et al., 2012) and North America, where Bunbury-Blanchette & Walker (2019) highlighted its prevalence across diverse soils, reflecting its remarkable ecological adaptability.

The detection of sequences related to *T. citrinoviride* while still requiring confirmation through multi-locus approaches may indicate the presence of less common *Trichoderma* taxa in specific agroecological zones such as Béja. This observation is consistent with reports by Ferrigo et al., (2020), who described moderate interspecific diversity in southern Europe, with some species being more prevalent in wetter soils. Similarly, Metz and Hausladen (2022) reported that *T. citrinoviride* and *T. intricatum* occurred at low frequencies, supporting our preliminary findings.

Overall, while our data suggests a clear dominance of isolates affiliated with *T. harzianum* alongside the possible occurrence of *T. citrinoviride*, these results should be interpreted with caution due to the limitations of ITS-based identification. Additional analyses using multi-locus sequence typing (MLST) and genomic approaches will be crucial to achieve more accurate species-level resolution and to better understand the ecological distribution of *Trichoderma* in Tunisian cereal soils.

These observations not only confirm the trends already identified in Tunisia but are also part of a wider international dynamic concerning the distribution of *Trichoderma* species according to soil types and climatic conditions. Taken together, these data reinforce the hypothesis that *T. harzianum* occupies a central position, thanks to its high adaptability, while other species, such as *T. citrinoviride*, appear to have a more localized distribution.

The predominance of *T. harzianum* in Tunisian soils could have significant implications for soil health management and the development of sustainable agricultural practices. A better understanding of the distribution of these species opens up interesting prospects for their use in biocontrol or soil fertility improvement strategies. However, molecular identification based only on the ITS region has certain limitations. Although several isolates show strong similarity to species such as *T. afroharzianum*, *T. lixii* or *T. intricatum*, their phylogenetic positioning groups them within the *T. harzianum* sensu lato complex, as observed in other work (Druzhinina et al., 2006; Kredics et al., 2014; Pedrero-Méndez et al., 2021; Klokočar-Šmit et al., 2018).

This highlights the need to use additional genetic markers, such as the tef1, cal or rpb2 genes, to improve taxonomic resolution (Degenkolb et al., 2015; Sarrocco et al., 2019; Pastuszak et al., 2021).

Furthermore, the phylogenetic structuring into three distinct clades observed in this study corroborates the findings of other research, indicating moderate but structured genetic diversity within *Trichoderma* isolates. The isolation of genetically distinct strains, such as *T. citrinoviride* (Tr31) or isolates related to *T. lixii* and *T. afroharzianum*, opens up new prospects for the selection of strains with high biocontrol potential (Akrami et al., 2011; Ommati & Zaker, 2012; Sui et al., 2022).

Consequently, this study not only enriches our knowledge of the specific diversity of *Trichoderma* in Tunisian cereal soils but also provides a valuable basis for the selection of promising isolates for incorporation into innovative microbial formulations, contributing to sustainable agriculture (Scott et al., 2023; Tyskiewicz et al., 2022).

On the other hand, the antifungal activity of *Trichoderma* isolates against *F. culmorum* has shown a high variability of efficacy between strains, which is widely reported in the literature (Papavizas et al., 1985; Howell, 2003; Vinale et al., 2008). *Trichoderma harzianum* Tr13 and Tr15 strains showed marked inhibition of mycelial growth, in line with the work of Hibar et al., (2004) and Kthiri et al., (2020), who attribute this efficacy to the production of antifungal metabolites and hydrolytic enzymes. Conversely, Tr11, Tr33, Tr30, Tr26 and Tr23 strains showed no significant difference with the control, which could reflect low antifungal activity or poor competitive interaction, as suggested by Bunbury-Blanchette and Walker (2019). Analysis of volatile compounds revealed that Tr24, Tr45 and Tr37 strains were the most inhibitory, while Tr42, Tr44 and Tr39 strains showed the lowest inhibition rates. These results corroborate those of Sarrocco et al., (2019), Reino et al., (2007) and Tyskiewicz et al., (2022), which highlight the importance of volatile organic compounds in long-distance antagonism. Compounds such as 6-pentyl-2H-pyran-2-one, well documented for their fungistatic action, are probably involved, as described by Vinale et al., (2008) and Thomas Degenkolb et al., (2015). This inter-strain variability in metabolite or VOC production is also confirmed by the work of Zlata Klokočar-Šmit et al., (2018), Pastuszak et al., (2021), Peng et al., (2021) and Bouanaka et al., (2021), who stress the importance of selecting the most effective strains according to the targeted pathosystem. Results showed that the effect of *Trichoderma* spp. strains on wheat growth and FHB severity varied considerably according to genotype. Although most strains did not significantly affect germination, a few, notably Tr27, Tr44, Tr8, Tr30 and Tr1 strains led to a decrease in germination rate, in line with the observations of Modrzewska et al., (2021) and Akrami et al., (2011) on the variability of effects depending on strain. Similarly, some strains such as Tr1, Tr11, Tr16, Tr21, Tr27, Tr44 and Tr31 significantly reduced leaf growth, with Tr44 displaying the most inhibitory effect, corroborating data from Bunbury-Blanchette & Walker (2019) and Yao et al., (2023) on possible antagonistic interactions between certain *Trichoderma* strains and young seedlings. On the other hand, several isolates such as Tr24, Tr4, Tr21, Tr10, Tr25, Tr13 and Tr33 stimulated root growth, which is in line with the work of Vinale et al., (2008) and Saravanakumar et al., (2017) demonstrating the ability of certain strains to produce metabolites promoting root architecture. In terms of biomass, significant increases in leaf fresh weight were noted for Tr33, Tr4, Tr24 and Tr31, while Tr44 showed the lowest values, reflecting the contrasting biochemical activity of the isolates, as highlighted by Howell (2003) and Reino et al., (2007). With regard to control of Fusarium head blight, isolates such as Tr10, Tr21, Tr11 and Tr19 significantly reduced disease severity, confirming their antagonistic potential reported by Hibar et al., (2004) and Bouanaka et al., (2021), while others, such as Tr33 and Tr31, aggravated symptoms, underlining the importance of careful selection of the strains used. Finally, although some isolates improved growth or biomass in the presence of *F. culmorum* (e.g. Tr11, Tr4, Tr10), others such as Tr31 showed deleterious effects, echoing the findings of Peng et al., (2021) and Sui et al., (2022) on the ambivalent role of certain strains depending on pathophysiological conditions and plant-microorganism interaction. These results underline the importance of a targeted approach to selecting the most suitable isolates, taking into account both their ability to promote growth and to reduce pathogen pressure.

Clustering and correlation analyses revealed marked functional variability between *Trichoderma* isolates, reflecting the complexity of their interactions with host plants and pathogens. This diversity is well documented in the literature and testifies to the ecological plasticity of the genus, capable of manifesting either biostimulant or bioprotective effects, or both (Vinale et al., 2008; Woo et al., 2023; Sarrocco et al., 2019). The isolates closest to the healthy control in multivariate analyses - notably Tr10, Tr11, Tr19 and Tr21 - appear to be the most promising, since they combine a significant improvement in vegetative growth with a notable reduction in Fusarium crown rot (FCR) symptoms. This dual effect has already been reported by Modrzewska et al (2021) and Bunbury-Blanchette & Walker (2019), who showed that certain strains of *T. harzianum* or *T. atroviride* could simultaneously enhance root development and activate plant defense mechanisms. Strong positive correlations between vegetative parameters - root length (RL), leaf length (LL), fresh and dry biomass of aerial and root parts - suggest a harmonious morpho-physiological response of seedlings to inoculation with certain isolates, reinforcing the idea that growth stimulation is not an isolated effect but results from a global remodeling of plant physiology (Peng et al., 2021; Yao et al., 2023). The potential contribution of bioactive compounds produced by *Trichoderma* - such as indoles, giberelic acid or cyclic peptides - could explain this synergy between root and aerial development (Reino et al., 2007; Thomas Degenkolb et al., 2015). Furthermore, the negative correlations observed between disease severity (DS) and growth parameters indicate that the impact of *F. culm*orum manifests itself as a direct inhibition of vegetative development, corroborating the observations of Hibar et al., (2004) and Scott et al., (2023), according to which

Fusarium pathogenicity not only affects infected tissues but also limits the allocation of resources to vegetative organs. However, the lack of a clear correlation between mycelial inhibition indices measured *in vitro* (MGI-IV, MGI-VOC) and reduced severity in planta revealed a frequent dissociation between *in vitro* efficacy and performance under complex biological conditions (Tyskiewicz et al., 2022; Klokočar-Šmit et al., 2018; Wang et al., 2022). This phenomenon can be explained by the fact that *in vitro* tests essentially capture the direct effect of antifungal metabolites, whereas in planta bioprotection also relies on the induction of systemic resistance (ISR), competition for ecological niches and nutrients, and interference in pathogen communication (Howell, 2003; Saravanakumar et al., 2017; Li et al., 2018). Other works, such as those by Bouanaka et al., (2021) and Akrami et al., (2011), have also highlighted that volatile compounds emitted by certain *Trichoderma* strains can inhibit fungal growth without necessarily leading to a significant reduction in symptoms on the plant, suggesting contextual regulation dependent on environmental conditions and the microbiota. In short, the biostimulant and bioprotective performance of *Trichoderma* isolates appears to be the fruit of a complex combination of mechanisms of action, which deserve to be evaluated in integrated systems and in semi-natural or field conditions to better anticipate their real effectiveness in a biological control or sustainable agriculture program.

Conclusion

This study revealed a moderate but structured diversity of *Trichoderma* species in Tunisian agricultural soils, dominated by *T. harzianum*, present in all the regions sampled, while *T. citrinoviride* was detected in only one region. *In vitro* evaluation showed considerable variability between strains in their ability to inhibit *F. culmorum*, with some (notably Tr13, Tr15 and Tr24) distinguished by marked antifungal activity. The impact on wheat growth also revealed contrasting effects: several isolates improved root growth and leaf biomass. Finally, under conditions of fungal stress, certain strains, such as Tr10, Tr11, Tr19 and Tr21, significantly reduced the severity of Fusarium crown rot. These results underline the potential of certain local *Trichoderma* strains such as biocontrol and biostimulation agents, while calling for careful selection to avoid negative effects.

Table S1. List of Trichoderma strains isolated from cereal soil in Tunisia during the two crop seasons (2020-2022).

Strains	***Trichoderma* species**	**Geographical origin**
Tr31	*Trichoderma citrinoviride*	Beja
Tr 1	*Trichoderma harzianum*	Sidi-bouzid
Tr2	*Trichoderma harzianum*	Sidi-bouzid
Tr3	*Trichoderma harzianum*	Sidi-bouzid
Tr4	*Trichoderma harzianum*	Sidi-bouzid
Tr5	*Trichoderma harzianum*	Sidi-bouzid
Tr6	*Trichoderma harzianum*	Jendouba
Tr7	*Trichoderma harzianum*	Jendouba
Tr8	*Trichoderma harzianum*	Jendouba
Tr9	*Trichoderma harzianum*	Jendouba
Tr10	*Trichoderma harzianum*	Jendouba
Tr11	*Trichoderma harzianum*	Jendouba
Tr12	*Trichoderma harzianum*	Jendouba
Tr13	*Trichoderma harzianum*	Bizerte
Tr14	*Trichoderma harzianum*	Bizerte
Tr15	*Trichoderma harzianum*	Bizerte
Tr16	*Trichoderma harzianum*	Bizerte
Tr17	*Trichoderma harzianum*	Bizerte
Tr18	*Trichoderma harzianum*	Bizerte
Tr19	*Trichoderma harzianum*	Bizerte
Tr20	*Trichoderma harzianum*	Bizerte
Tr21	*Trichoderma harzianum*	Bizerte
Tr22	*Trichoderma harzianum*	Bizerte
Tr23	*Trichoderma harzianum*	Bizerte
Tr24	*Trichoderma harzianum*	Bizerte
Tr25	*Trichoderma harzianum*	Bizerte
Tr26	*Trichoderma harzianum*	Bizerte
Tr27	*Trichoderma harzianum*	Bizerte
Tr28	*Trichoderma harzianum*	Beja
Tr29	*Trichoderma harzianum*	Beja
Tr30	*Trichoderma harzianum*	Beja
Tr33	*Trichoderma harzianum*	Beja
Tr36	*Trichoderma harzianum*	Beja
Tr37	*Trichoderma harzianum*	Beja
Tr38	*Trichoderma harzianum*	Beja
Tr39	*Trichoderma harzianum*	Beja
Tr40	*Trichoderma harzianum*	Beja
Tr41	*Trichoderma harzianum*	Beja
Tr42	*Trichoderma harzianum*	Nefza
Tr43	*Trichoderma harzianum*	Nefza

Tr44	*Trichoderma harzianum*	Nefza
Tr45	*Trichoderma harzianum*	Nefza
Tr46	*Trichoderma harzianum*	Nefza

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General Conclusion

This PhD thesis was structured in three different activities, with the main aim to investigate the prevalence, impact, and potential control methods for *Fusarium* species in wheat cultivation in Tunisia.

The first activity allowed for a detailed assessment of the incidence and mycotoxin contamination in wheat crops. The results demonstrated that *Fusarium* contamination is predominantly observed at the crown level, with *F. culmorum* being the most prevalent species. Notably, contamination at the grain level was significantly lower, indicating that the disease, while affecting stems and crowns, does not always spread to the ears. Moreover, the research revealed a significant correlation between Fusarium contamination and mycotoxin (DON) accumulation, emphasizing the potential threat posed by Fusarium to wheat quality and yield (Chapter II).

The second activity was carried out to assess the impact of *F. culmorum* on wheat yield and quality, specifically in terms of agronomic parameters and mycotoxin accumulation. Inoculation with *F. culmorum* led to a significant decrease in wheat plant density, tiller density, and kernel density, resulting in notable yield losses. The study also demonstrated that *Fusarium* inoculation increased DON accumulation, especially in the crown. Additionally, the performance of durum wheat varieties in response to Fusarium inoculation varied significantly, highlighting the potential for selecting resistant varieties to mitigate the impact of Fusarium infection (Chapter III).

Finally, the third part of the study focused on the characterization of *Trichoderma* species and their potential use in biological control and plant growth promotion. The results revealed a moderate but structured diversity of *Trichoderma* in Tunisian cereal-growing soils, with *T. harzianum* being the dominant species, detected in all sampled regions, whereas *T. citrinoviride* was limited to a single site. *In vitro* assays showed considerable variability among isolates in their antagonistic potential against *F. culmorum*. Several isolates also enhanced wheat growth by promoting root elongation and leaf biomass accumulation. Also, under fungal stress conditions, some strains significantly reduced the severity of Fusarium crown rot. These findings highlight the dual potential of certain local *Trichoderma* isolates as biocontrol and biostimulant agents, emphasizing the need for careful screening to select the most effective and consistent candidates (Chapter IV).

In conclusion, the results of this thesis contribute to a deeper understanding of *Fusarium* species distribution, impact, and potential control measures in Tunisian wheat production. The findings underline the need for integrated management strategies, including the use of resistant wheat varieties and biocontrol agents, to mitigate the effects of *Fusarium* diseases and ensure the sustainability of wheat production in Tunisia. Further research is essential to optimize these strategies and enhance their effectiveness in combating Fusarium-related challenges in agriculture.