Università degli Studi di Sassari

Innovative approaches to the management of mycotoxin-producing fungi

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Abstract

Fusarium and *Aspergillus* pose significant challenges for agriculture and medicine due to their increasing resistance to synthetic fungicides. These fungi reduce crop yields and quality, in addition to producing dangerous mycotoxins. Furthermore, they may infect immunocompromised humans and exhibit invasive growth patterns. Therefore, it is critical to seek innovative and long-term solutions to limit the resistance of these fungi. The current study focused on the identification of natural fungicides and inhibitors of mycotoxin biosynthesis in *Aspergillus* and *Fusarium* fungi. Various approaches were tested, including the use of phenolic compounds, plant extracts, and microorganisms. Bioprospecting phenols and their potential for sustainable trichothecene and *Fusarium* growth control in wheat were documented by reviewing scientific literature. However, since phenol derivatization can enhance antifungal activity, we also investigated the changes in cinnamic acid structure via esterification and etherification, and thereby obtained derivatives with enhanced antifungal activity against *Fusarium* spp. of clinical and agricultural interest. Since plants contain phenolic compounds with the potential to inhibit mycotoxins, five Mediterranean aqueous plant extracts (i.e., chestnut flower, cistus, eucalyptus, orange peel) were tested for inhibition of *Aspergillus carbonarius* (Bainier) Thom. rot and ochratoxin A (OTA) biosynthesis in grape berries. This study also tested four *Bacillus* strains (AngB1, BV, F33 and 54) for their inhibitory effects on *Fusarium culmorum* (Wm.G. Smith) Sacc. in wheat. When applied as seed coating, all strains reduced foot and root rot symptoms in wheat (by 77 to 97%). *Bacillus* sp. BV proved to be the most efficient strain, and was further tested. It was sprayed onto wheat spikes in order to evaluate its inhibitory effect on Fusarium head blight and deoxynivalenol, and demonstrated an equally marked antifungal effect on *F. culmorum* and deoxynivalenol. Future research can build on the findings of this thesis, toward reconsideration of the use of synthetic fungicides,

since the focus is now on controlling economically important pests via an integrated pest management approach.

Keywords: *Aspergillus*; *Fusarium*; ochratoxin; deoxynivalenol; biofungicides.

Declaration

I, the undersigned Wiem Chtioui, declare that this dissertation is the original report on my doctoral research at the University of Sassari, it has been written by myself and has not been submitted or presented, in whole or in part, for the award of any other academic degree or diploma elsewhere.

Wiem Chtioui

(2020-2023)

To the loving memory of my grandmom Fatma,
who inspired me and taught me the value of education.

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Abbreviations

OTA	Ochratoxin A
FAO	Food and Agriculture Organization
DAS	Diacetoxyscirpenol
STO	Scirpentriol
MAS	4-Monoacetoxyscirpenol
NEO	Neosolaniol
DON	Deoxynivalenol
3-ADON	3-Acetyldeoxynivalenol
15-ADON	15-Acetyldeoxynivalenol
NIV	Nivalenol
4-ANIV	Fusarenone-X
IARC	International Agency for Research on Cancer
AFB1	Aflatoxin B1
HBV	Hepatitis B Virus
EU	European Union
FB1	Fumonisin B1
FB2	Fumonisin B2
JECFA	Joint Expert Committee on Food Additives
TDI	Tolerable Daily Intake
EFSA	European Food Safety Authority
BW	Body Weight
GAP	Good Agricultural Practices
BCAs	Bio-Control Agents
FHB	Fusarium Head Blight
DMIs	Demethylation Inhibitors
QTL	Quantitative Trait Loci
HCAs	Hydroxycinnamic Acid Derivatives
FRR	Foot and Root Rot
PKS	Polyketide Synthase
Otα	Ochratoxin α
FSSC	*Fusarium solani* species complex
FOSC	*Fusarium oxysporum* species complex
FFSC	*Fusarium fujikuroi* species complex
FIESC	*Fusarium incarnatum-equiseti* species complex
FCSC	*Fusarium chlamidosporum* species complex
FDSC	*Fusarium dimerum* species complex
FSAMSC	*Fusarium sporotrichioides* species complex
ZEA	Zearalenone
FPP	Farnesyl Pyrophosphate
TDN	Trichodiene

CAL	Calonectrin
a_w	Water Activity
ROS	Reactive Oxygen Species
UDP	Uridine diphosphate
GST	Glutathione S-Transferase
PAL	Phenylalanine Ammonia-Lyase
CHLO	Chlorogenic Acid or 5-O-Caffeoylquinic Acid
CA	Caffeic acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
NAD	Nicotinamide Adenine di Nucleotide
NADH	B-Nicotinamide Adenine di Nucleotide
SDH	Succinate Dehydrogenase
ATP	Adenosine Triphosphate
DFA	Ferulic Acid
4-HBA	4-Hydroxybenzaldehyde
CDs	Cyclodextrins
CMC	Carboxymethyl Cellulose
PEG	Polyethylene Glycol
α-CD	Six-Cycloamylose Units
β-CD	Seven-Cycloamylose Units
γ-CD	Eight-Cycloamylose Units
OSA	Octenyl Succinate
THC	Tetra Hydrocurcumin
PDP	Pyranocoumarin
FMM	*Fusarium* Minimal Medium
MIC	Minimal Inhibitory Concentration
LD_{50}	Lethal Dose 50
TRB	Fungicides Terbinafine
AmB	Amphotericin B
NOESY	Nuclear Overhauser Effect Spectroscopy-NMR
NMR	Nuclear magnetic resonance spectroscopy
FDA	Food and Drug Administration
GRAS	Generally Recognized as safe
TLC	Thin-Layer Chromatography
CFU	Colony-Forming Units
DMSO	di Methyl Sulfoxide
PDA	Potato Dextrose Agar
IAC	Immunoaffinity Column
PTFE	Polytetra-Fluor Ethylene
HPLC	High-Performance Liquid Chromatography
LOQ	Limit of Quantification
YEPD	Yeast Extract Peptone Dextrose

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Introduction

1.Mycotoxins in agriculture

Molds are fungi, and although over 100,000 species of fungi are known to date, few actually produce the toxic chemical substances or secondary metabolites known as mycotoxins.

The term "mycotoxin" derives from the Greek word *mycos*, meaning fungus, and the Latin word *toxicum* meaning poison (Agriopoulou et al., 2020; Pittet, 1998). Although these molecules are not involved in fungal growth, they often represent virulence and pathogenicity factors in plant diseases (Hof, 2008).

Mycotoxins have detrimental health effects on domestic animals, which then raises concerns about their impact on human health. They are thought to have first infected human food when humans began cultivating and storing crops, probably around 10,000 years ago (Pitt & Miller, 2017). Throughout history, there have been signs of their presence and impact on animal and human health. Ergotism, commonly known as "St. Anthony's fire," was induced by the ingestion of rye containing the ergot alkaloids generated by the fungus *Claviceps purpurea* (Fr.) Tul. (Beardall & Miller, 1994; Schiff, 2006). A delayed harvest due to the Second World War resulted in grains severely infected with trichothecenes generated by *Fusarium* spp. in Siberia. People who ate the grain eventually developed a variety of nonspecific diseases, and death rates reached 10% (Manahan, 2001). It was also reported that 100,000 turkeys perished in the UK in 1962 from what was known as "Turkey X disease". This incident was subsequently connected to aflatoxins produced by *Aspergillus flavus* Link (Binder, 2007). These few examples demonstrate the acute impact of high loads of single mycotoxins on both human and animal health. However, long-term exposure to low concentrations of mycotoxins

can also cause chronic toxicities, with non-specific symptoms that are difficult to attribute to mycotoxins.

To date, around 400 different forms of mycotoxins were identified (Ülger et al., 2020). However, just six groups of mycotoxins are commonly found in food: deoxynivalenol/nivalenol, zearalenone, ochratoxins, fumonisins, aflatoxins, and patulin (Alshannaq & Yu, 2017). *Fusarium*, *Penicillium*, *Aspergillus*, and *Alternaria* are the most prevalent mycotoxigenic genera (Greeff-Laubscher et al., 2020).

These fungi can cause diseases in humans, animals and plants due to their invasive growth, which is common and lethal in immunocompromised organisms, or through the ingestion of contaminated food or feed (Awuchi et al., 2021).

Mycotoxins can cause substantial economic losses in livestock farming, which range from animal death to reduced productivity and increased costs of veterinary and human healthcare. In the event of severe contamination, total losses occur when the product is considered unfit for consumption and thus rejected by the market, and must therefore be destroyed (Wu, 2007).

According to the Food and Agriculture Organization (FAO), approximately 25% of the world's crops are contaminated by mycotoxins each year, causing annual losses of approximately 1 billion metric tons of food and food products. However, this level of contamination appears to be greatly underestimated, since Eskola et al. (2020) report that 60%-80% of agricultural commodities contain detectable levels of mycotoxins. They are a prominent topic in the field of food safety, as demonstrated by the fact that over 20,000 studies about mycotoxins have been published since 1960.

2. Major mycotoxins in food products

Mycotoxins that contaminate foods and feeds belong mainly to the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* (Haque et al., 2020). While *Fusarium* and *Alternaria* species mainly infect and propagate in growing crops, *Aspergillus* and *Penicillium* species typically grow on foods and feed held in storage (Alshannaq & Yu, 2017).

2.1. *Fusarium* mycotoxins

Trichothecenes, zearalenone, and fumonisins are produced by *Fusarium* species (Ekwomadu et al., 2021). The following section outlines their main producers, their structure, and the symptoms related to their ingestion.

2.2. Trichothecenes

Trichothecenes are sesquiterpenoid alcohol esters arranged around a trichothecane tricyclic ring, with a double bond at C9-C10 and an epoxide at C12-C13. Based on their chemical properties and the fungi that produce them, trichothecene compounds are classified into four major groups: A, B, C, and D. Groups A and B contain trichothecenes produced by *Fusarium* spp. (Chtioui et al., 2022) (**Figure 1**).

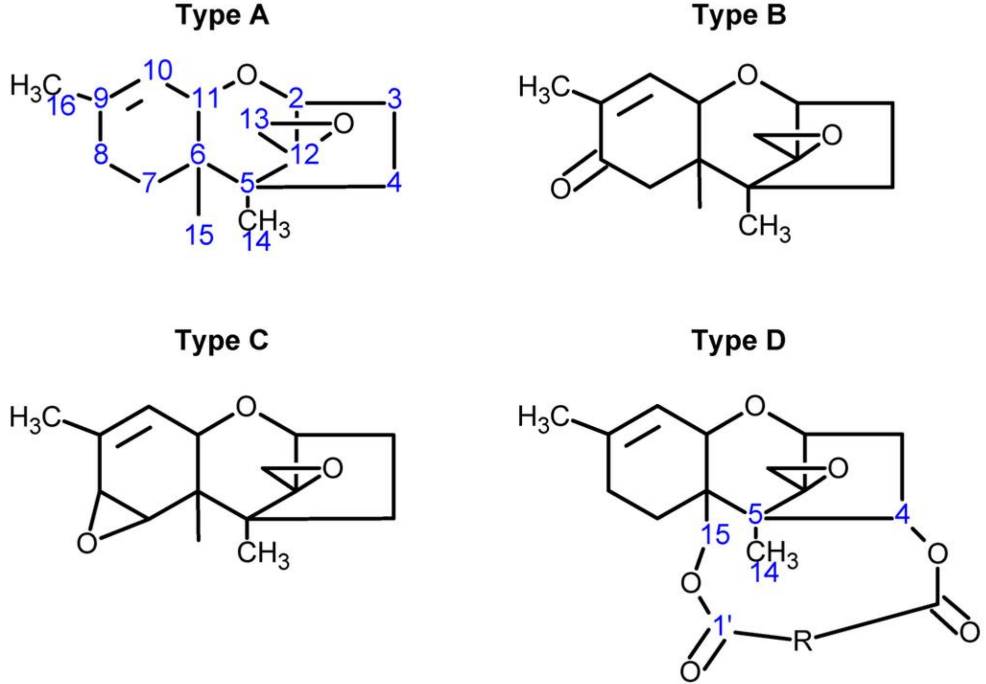

Figure 1. Chemical structure of trichothecenes A, B, C and D (Shank et al., 2011)

T-2 and HT-2 toxins, diacetoxyscirpenol (DAS), scirpentriol (STO), 4-monoacetoxyscirpenol (MAS), and neosolaniol (NEO) belong to type A trichothecenes. *Fusarium sporotrichioides* Sherb., *Fusarium sambucinum* Fuckel, *Fusarium poae* (Peck) Wollenw., *Fusarium langsethiae* Torp and Nirenberg., and *Fusarium equiseti* Corda (Sacc.) are the primary producers of Type A trichothecenes (Janik et al., 2021).

Deoxynivalenol (DON), the acetyl derivatives (3-ADON and 15-ADON), together with nivalenol and fusarenone-X (4-ANIV), are examples of Type B trichothecenes with a C-8 keto group. *Fusarium culmorum* (W.G. Smith), *Fusarium graminearum sensu stricto* (Schwabe), and *Fusarium crookwellense* L.W. Burgess are the primary producers of Type B trichothecenes in cereals (Chen et al., 2019).

Trichothecenes have been demonstrated to induce a variety of acute and chronic symptoms in experimental and livestock animals, including growth retardation, reproductive abnormalities,

immune system suppression, feed refusal (anorexia), vomiting, bleeding, diarrhea, and death. Monogastric animals are the most vulnerable to these toxins, while ruminant mammals and fowl can withstand higher concentrations. In addition, trichothecenes in the diet have been linked to nausea, diarrhea, abdominal pain, and fever in humans (Polak-Śliwińska & Paszczyk, 2021).

2.3. Zearalenone

Fusarium graminearum (teleomorph *Gibberella zeae*) is the main producer of zearalenone, but this compound can be also produced by *F. culmorum*, *F. sporotrichioides*, *Fusarium semitectum* Berk. and Ravenel, and *F. equiseti* (Ropejko & Twarużek, 2021). Zearalenone is an estrogenic mycotoxin. The molecule includes a lactone group and a free C-4 hydroxyl group, which allow it to bind to the estrogen receptor. This translates into diseases like hyperestrogenism in animals, particularly in pigs. There are numerous zearalenone derivatives, some of which are more estrogenic than the mother molecule, such as α-zearalenol, α- and β-zearalenol (Zhang et al., 2018) (**Figure 2**).

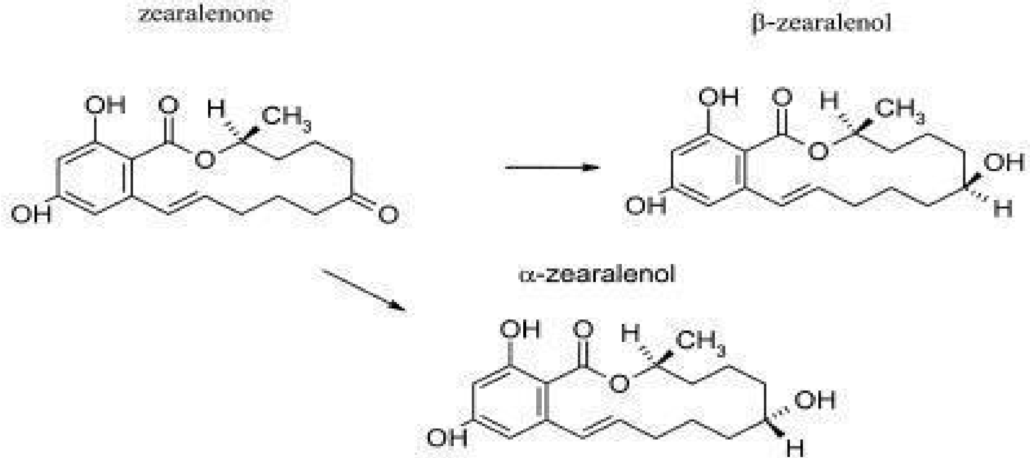

Figure 2. Chemical structure of zearalenone and its reduced metabolites, α- and β-zearalenol (El-Nezami et al., 2002)

2.4. Fumonisins

Fumonisins (notably FB1, FB2; **Figure 3**) are mostly produced by *Fusarium verticillioides* (Sacc.) Nirenberg, and *Fusarium proliferatum* (Matsush.) Nirenberg**.** In structure, fumonisins are comparable to sphingolipid long-chain bases, such as sphinganine and sphingosine. This property is closely related to their toxicity mechanism, which involves the inhibition of sphingolipid biosynthesis (Soriano & Dragacci, 2004). Exposure to fumonisins has been linked to a wide range of diseases in animals, including liver cancer in rats, leukoencephalomalacia in horses, and pulmonary edema in pigs, as reviewed by Voss et al. (2007).

Fumonisin B_1

Fumonisin B_2

Figure 3. Chemical structure of fumonisins B 1 and B 2 (Zain, 2011)

2.5. *Aspergillus* and *Penicillium* mycotoxins

Ochratoxin A and aflatoxins are mainly produced by species in the genera *Aspergillus*. Patulin is produced by species in the genera *Penicillium*.

2.5.1. Ochratoxin A

Several *Aspergillus* and *Penicillium* species produce ochratoxins and citrinin. *Aspergillus ochraceus* G. Wilh. and *Penicillium verrucosum* Dierckx are the two main ochratoxin A (OTA) producers. These fungi are widespread and contaminate animal feed and human food. *Aspergillus* spp. mainly produce ochratoxin at high temperatures and humidity, while *Penicillium* spp. may produce ochratoxins at temperatures as low as 5 °C (Wang et al., 2016).

The International Agency for Research on Cancer (IARC) classifies OTA as a probable human carcinogen (Group 2B). The most serious health consequences of ochratoxin contamination are nephrotoxicity, carcinogenicity, teratogenicity, immunotoxicity, and possibly also neurotoxicity (Malir et al., 2016).

2.5.2. Aflatoxins

Aflatoxins are furanocoumarins that are primarily generated by *Aspergillus* species, most notably *A. flavus*, and *Aspergillus parasiticus* Speare (Khan et al., 2021; Wu et al., 2009). These colonize crops in tropical and subtropical climates worldwide, and can also produce aflatoxins during food storage, transit transportation, and processing.

Aflatoxin B1 (AFB1) is the most poisonous, mutagenic, and carcinogenic of the 18 forms of aflatoxins produced by *A. flavus* strains (Kumar et al., 2017). High doses of aflatoxin cause acute aflatoxicosis, which is responsible for severe gastrointestinal symptoms and, in some cases, death. Hundreds of instances of aflatoxicosis have been reported in Africa in recent years. Aflatoxin exposure may also be linked to childhood stunting and immunosuppression. It is also linked to liver cirrhosis: aflatoxin and hepatitis B virus (HBV) exposure may work together to significantly increase the risk of cirrhosis. At present, approximately 5 billion

people worldwide are in danger of chronic aflatoxin exposure from their food (Benkerroum, 2020).

2.5.3. Patulin

Patulin is produced by a variety of *Penicillium* species, mainly by *Penicillium expansum* Link. This compound is toxic to many biological systems, including bacteria, mammalian cell cultures, higher plants, and animals, although its role in disease transmission in animals and humans remains unknown. Patulin has a lactone structure and causes cancer in mice when injected intradermally; it is a public health concern due to its probable carcinogenic properties (Zhong et al., 2018).

3. Main commodities affected

Many food commodities may be contaminated by mycotoxins. These include cereals (Lee & Ryu, 2017), meat (Pleadin et al., 2021), seafood (Tolosa et al., 2019), dairy products (Benkerroum, 2016), vegetable and fruits (Nan et al., 2022), juice/alcoholic beverages (Carballo et al., 2021), and even public drinking water (Mhlongo et al., 2019).

Of these commodities, cereals are particularly important, as they represent two-thirds of the world's food energy intake and constitute a staple food for over 4 billion people. Cereals provide 46% of the calory intake in Africa and 26% in Europe (FAO, 2017). However, cereal crops can be contaminated by a wide range of mycotoxins, including *Fusarium* trichothecenes. DON is frequently connected with wheat, T-2, and HT-2 toxins with oats, and fumonisins with maize (Carvajal-Moreno, 2022). The grains most typically infected by zearalenone are maize and wheat (Leslie et al., 2021). Furthermore, *Fusarium* mycotoxins have been found in cereal-derived products such as flour, malt, soybean, and beer (Mousavi Khaneghah et al., 2019).

Although OTA is present in a variety of foods (e.g., coffee beans, dried vine fruits, wine, grape juice, spices, and animal-based foods), 60% of the total dietary exposure to OTA is due to cereals (Marin et al., 2013). In Algeria for example, approximately 70% of wheat samples tested positive to OTA (Leslie et al., 2021).

Aflatoxins also contaminate staple foods, such as corn, sorghum, wheat, and rice. In addition, oilseeds (soybean, peanut, sunflower, and cotton seed), spices (chili pepper, black pepper, coriander, turmeric, and ginger), and tree nuts (pistachio, almond, walnut, coconut, and Brazil nut) are among the crops most frequently contaminated by aflatoxins (Kumar et al., 2021). Aflatoxin M1 can also be found in the milk of animals fed on contaminated feed (Flores-Flores et al., 2015).

Finally, patulin is typically found in decaying apples and apple products; apples and apple juice infected with molds are major human dietary sources of patulin, which can also be found in moldy fruits, grains, and other food (Zhong et al., 2018).

4. Regulations

Reducing mycotoxin contamination in food and feed is an economic and health priority (Leslie et al., 2021). Thus, mycotoxin levels are frequently regulated at the national level and in international trade agreements.

The European Union (EU) imposes maximum concentrations of AFB1, DON, ZEA, OTA, and the sum of fumonisin B1 (FB1) and B2 (FB2) for foods, and advised limits have been introduced for T2-HT2.

4.1. Trichothecene

The European Commission established maximum contamination levels for DON in cereal-based foods in 2005 (EC No. 856/2005), which were revised in 2007 (EC No. 1126/2007) (European Commission, 2007a). The EU restrictions establish a maximum level of 1250 µg/kg in unprocessed common wheat and 1750 µg/kg in unprocessed durum wheat for human consumption (EC No. 1126/2006), at 1750 µg/kg for unprocessed oats and at 750 µg/kg for oat-derived products for human consumption (European Commission, 2007b).

4.2. Zearalenone

The regulatory limit for ZEA is 100-200 µg/kg in unprocessed cereals, 75 µg/kg in processed cereals, 20 µg/kg in processed cereal foods, and 50 µg/kg in cereal snacks (EFSA, 2014). EU authorities have also set the maximum levels allowed in food items for children and babies at 20 µg/kg in foods, 50 µg/kg in cereals and corn-based snacks, and 200 µg/kg in unprocessed maize (EFSA, 2014).

The Joint Expert Committee on Food Additives (JECFA) has set a preliminary maximum tolerable daily intake (TDI) of 0.5 µg/kg body weight, while the European Food Safety Authority (EFSA) sets a lower TDI of 0.25 µg/kg body weight (EFSA, 2014; JECFA & WHO, 2000).

4.3. Fumonisins

Many countries have set regulatory limits for fumonisins. In the EU, the maximum level for total FBs (FB1 + FB2) ranges from 200 µg/kg of body weight (BW) for processed maize-based foods and baby foods for infants and young children to 2000 µg/kg of BW for unprocessed maize (Terciolo et al., 2019). Recently, EFSA established a TDI for FBs of 1.0 µg/kg BW per

day (EFSA, 2018). For example, the guidance level was fixed at 60 mg/kg for maize and maize products and at 5 mg/kg for complete swine feed (EFSA, 2018).

4.4. Ochratoxin A

Maximum OTA levels have been established for raw cereals (5 μg/kg) and cereal-derived products (3 μg/kg) (EC Regulation No. 472/2002) (European Commission, 2002), roasted coffee (5 μg/kg), soluble coffee (10 μg/kg), and wines, musts, and grape juices (2 μg/kg) (EC Regulation No. 123/2005) (European commission, 2005). Maximum OTA levels have also been set for baby meals and processed cereal-based foods for infants and young children (0.5 μg/kg) (EC No 683/2004) (European Commission, 2004).

4.5. Aflatoxins

EU regulations regarding maximum aflatoxin levels for cereals, excluding maize, are 4 μg/kg, while the level for maize subjected to further processing before human consumption is 10 μg/kg.

In the case of peanuts, EU regulation thresholds range from 2 μg/kg for AFB1 and 4 μg/kg for total aflatoxins (sum of AF B1 + B2 + G1 + G2) (European Commission, 2006). The European Commission has also set strict limits for AFM1 in milk. EU legislation sets the limit in milk at 0.05 μg/kg for adult consumption, and 0.025 μg/kg for food products aimed at babies and young children (European Commission, 2010).

5. Control strategies

Because of their proven toxicity, mycotoxin levels in foods should be reduced to the lowest possible levels, and efficient strategies to reduce mycotoxins start by preventing them from entering the food chain.

5.1. Agronomic practices

Food Standards Agencies, FAO, and EFSA have published guidelines based on good agricultural practices (GAP), which include crop rotation, irrigation, tillage, and the use of resistant varieties, in addition to planning a pre-harvest strategy to reduce mycotoxins in cereals. These measures could be applied to *Fusarium* mycotoxins in grains and cereal-based products. Crop rotation with non-cereals was found to significantly reduce *F. graminearum* infection in wheat, and therefore mycotoxin concentrations (Bernhoft et al., 2012). Moreover, it was found that the cropping system and the choice of preceding significantly influences mycotoxin prevalence and concentration of Type A trichothecenes in cereal commodities (Kolawole et al., 2021).

Good agricultural practices are also valid for reducing *Aspergillus* mycotoxins in food and feed. Hocking et al. (2007) observed that *Aspergillus* inoculum could be reduced in grapes by maintaining constant soil moisture and reducing the fall of dead berries. It has been shown that the use of resistant hosts can contribute to reducing the volume of primary pathogen inoculum (Orrù et al., 2021).

5.2. Use of biocontrol agents (BCAs)

Biocontrol involves using beneficial microorganisms, such as filamentous fungi, bacteria or yeasts to control plant pathogens. It is a recognized environmentally friendly method for controlling pathogens in agriculture (Sarrocco et al., 2019). Some species of *Trichoderma*,

Pseudomonas, *Streptomyces,* and *Bacillus* are valuable candidates for biocontrol (Palazzini et al., 2007; Zhao et al., 2014). They can reduce fungal infection through parasitism, antibiosis, biotransformation of mycotoxins, or biocompetition (Pellan et al., 2021).

Among the valid candidates for biocontrol of cereal diseases, *T. gamsii* T6085 was found to inhibit the growth of both *F. graminearum* and *F. culmorum* causal agents of Fusarium head blight (FHB) through antagonistic mycoparasitism (Baroncelli et al., 2016; Matarese et al., 2012; Sarrocco et al., 2013). Regarding the use of bacteria in biocontrol of fungi, Mattei and co-workers found that coating seeds with *Streptomyces* sp. (DEF39) reduced *F. graminearum* infection by 49% (Mattei et al., 2022). Similarly, a combination of *B. velezensis* RC218 and chitosan reduced FHB in wheat by 54%, and the accumulation of DON by 64.5% (Palazzini et al., 2022).

On the other hand, various biological agents for regulating OTA in food items have been proposed, including the use of yeasts, bacteria, or fungi to reduce fungal growth or OTA levels. Yeasts are interesting candidates for *Aspergillus* control. Non-fermenting yeast strains (i.e *Cyberlindnera jadinii* 273 and *Candida friedrichii* 778) and low-fermenting yeast strains (i.e *Candida intermedia* 235 and *Lachancea thermotolerans* 751) were reported to reduce the growth and sporulation of *Aspergillus carbonarius* (Bainier) Thom. and to have OTA-adsorption properties (Farbo et al., 2018; Fiori et al., 2014). Jiang et al. (2014) found that *B. subtilis* was able to reduce OTA production by *A. carbonarius* in table grapes.

However, there are some important limits to biocontrol, such as low delivery mechanisms, and a lack of successful large-scale applications (Teixidó et al., 2022).

5.3. Use of synthetic fungicides

Synthetic fungicides are largely used in agriculture to control diseases, including FHB (Mesterházy, 2003). Triazole-based products (e.g., tebuconazole, metconazole, or prothioconazole), which are demethylation inhibitors (DMIs), are widely used chemicals for controlling *Fusarium,* and the combined use of multiple DMIs was found to increase their efficacy. In fact, combining prothioconazole with tebuconazole resulted in a 52% FHB reduction compared with the non-treated control (Paul et al., 2008). However, use of DMIs does not provide complete protection against FHB. There is also rising concern about emerging resistance of *F. graminearum* isolates to triazoles in Europe (Klix et al., 2007).

On the other hand, when strobilurins were tested *in vitro* for FHB control, the results were not always satisfactory (Dubos et al., 2011). In some cases, strobilurins were found to stimulate DON accumulation in field conditions (Blandino et al., 2006; Ellner, 2005).

With regard to *Aspergillus* in viticulture, several fungicides, such as cyprodinil and fludioxonil, have been shown to be effective against fungal colonization and OTA formation (Varga & Kozakiewicz, 2006). However, fungicides must be used with caution because some, such as carbendazim, have been shown to diminish fungal flora while stimulating OTA production (Medina et al., 2007).

In addition, the use of fungicides presents a risk to human health and the environment. EU Directive 2009/128/EC (EU 2009/128/EC) imposes strict regulations on the use of pesticides in order to limit their impact on consumers' health and the environment. For these reasons, it is important to develop new and sustainable agricultural tools that respond to the constant public concern for safe, high-quality, and pesticide-free foods and feeds (Sarrocco & Vannacci, 2018).

5.4. Cultivar selection

One way to reduce fungal damage and mycotoxin accumulation is to use resistant cultivars. Plant breeding strategies are among the most promising and effective approaches to cope with the mycotoxin problem in the short to long term, and are unquestionably one of the most important pillars of integrated disease management (Gauthier et al., 2015).

Plant resistance to *Fusarium* and mycotoxin production is a complex system. Wheat has five resistance forms, which are transferable to barley and maize. Mechanisms linked with one of these five types of resistance can be host specific.

Type I resistance in wheat and barley protects against floret infection. Type II resistance restricts infection propagation within the host (Schroeder et al., 1963; Steiner et al., 2017). Type III regards grain infection resistance, while Type IV is linked to tolerance and yield maintenance. Finally, Type V resistance encompasses all mechanisms of mycotoxin accumulation resistance (Mesterházy, 1995; Mesterházy, 2002; Miller et al., 1985).

Wheat disease resistance to several *Fusarium* species is governed by the species' nonspecific Quantitative Trait Loci (QTL) (Leslie et al., 2021). For this reason, recent biochemical research has attempted to decode the metabolic defenses contributing to FHB resistance and minimal mycotoxin accumulation in wheat. Genetic techniques are being applied in order to identify and define QTL for FHB resistance.

In addition, metabolomic approaches are now providing strong prospects for plant breeding by identifying resistant biomarker metabolites, and offer the advantage of integrating genetic background and environmental effects (Gauthier et al., 2015).

A vast number of metabolites have been identified as potentially functioning in cereals to prevent toxigenic *Fusaria* and minimize mycotoxin accumulation. These metabolites include fatty acids, amino acids derivatives, carbohydrates, amines and polyamines, terpenoids, and phenylpropanoids and are derived through primary and secondary plant metabolism (Gauthier et al., 2015).

Despite the significant progress made in understanding the genetics of resistance in *durum* wheat (*Triticum turgidum* subsp. *durum* (Desf.) Husn.), no commercial varieties are resistant to FHB. Most lines presented after breeding are only moderately resistant to FHB (Boutigny et al., 2008).

5.5. Use of natural compounds

Plants contain a wide set of chemicals that contribute to their phenotypic flexibility and allow them to escape from stressors (Kaplan et al., 2008; Stout et al., 2005). These include – among others – phenolic acids, flavonoids, polyphenols, and stilbenoids.

Phenolic chemicals are responsible for the antioxidant capacity of fruits and vegetables, and play a vital part in plant defense mechanisms. Resistant plants show a hyperproduction of phenolic-type chemicals, which confirms that these compounds play an important role in a plant’s self-defense activity.

Approaches using phenolic compounds to reduce mycotoxin contamination could be interesting. Phenolic compounds may also act on the pathogenic and mycotoxigenic potential of mycotoxin-producing fungi, instead of acting on the saprophytic phase, thereby reducing the risk of cross-resistance (Chtioui et al., 2022; Pani et al., 2014).

6. Case study 1: *Fusarium*

6.1. *Fusarium* genera

Fusarium is a well-known genus of economically important plant pathogenic fungi, and belongs to the phylum Ascomycota, subphylum Pezizomycotina, class Sordariomycetes, order Hypocreales, and family Nectriaceae. *Fusarium* genera number around 1,500 species, some of which produce mycotoxins.

Fusarium fungi are associated with crop spoilage and mycotoxin contamination in agricultural production in the world's temperate and cold regions (e.g. central, and northern Europe, and North America) (Pinotti et al., 2016).

F. culmorum and *F. graminearum* are among the most economically significant *Fusarium* species affecting small-grain cereals.

6.1.1. *Fusarium culmorum* (W.G. Smith) Sacc. has been identified as causing some of the major wheat diseases worldwide. This pathogen can cause foot and root rot (FRR) and FHB on numerous small-grain cereals, particularly wheat and barley. It is widespread in cool climates but is also widely reported in the Mediterranean basin, especially in years when moist conditions occur during the flowering and kernel-filling stages (Scherm et al., 2013). Morphologically, *F. culmorum* produces a dense aerial mycelium, which is initially white but later becomes typically yellow or pink. Genetically, *F. culmorum* (**Figure 4**) is a haploid pathogen that reproduces asexually by producing conidia that infect plants via the florets. Macroconidia of *F. culmorum* are short and blunt, slightly curved, 3- to 4-septate, with thick walls and rounded apical cells. There are no microconidia, but many chlamydospores. To date, no teleomorph has been reported for *F. culmorum*.

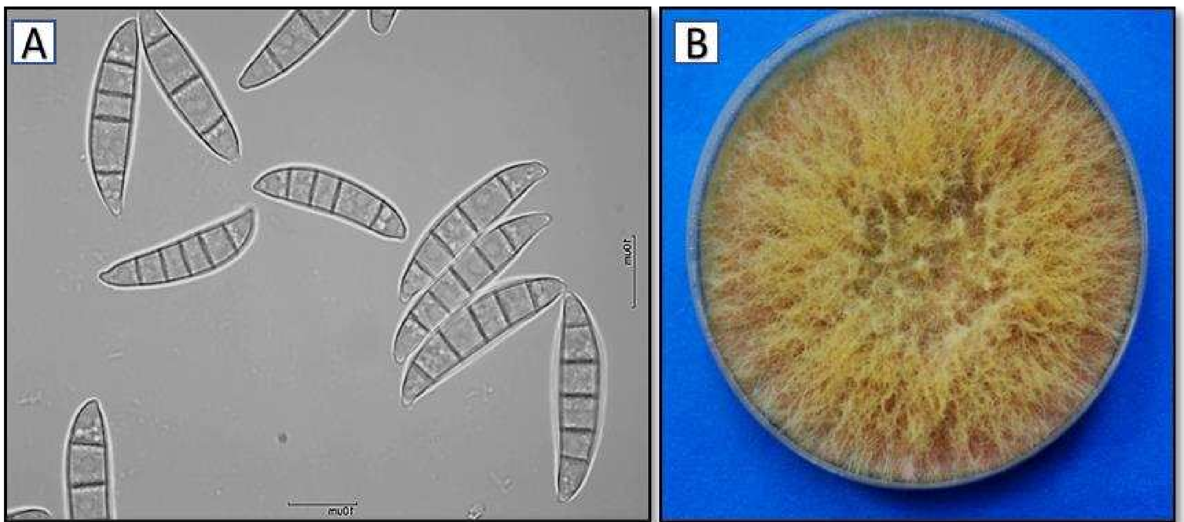

Figure 4. *F. culmorum* (A) macrocondia; (B) Mycelium on Potato Dextrose Agar (Source: Prof. Virgilio Balmas)

6.1.2. *F. graminearum* is the anamorph (conidial stage) of *Gibberella zeae*. *F. graminearum* is ranked fourth among the top ten fungal pathogens, based on its scientific and economic impact (Dean et al., 2012). *F. graminearum* causes FHB and *Gibberella* ear rot, two important diseases of wheat, barley and maize. Furthermore, *F. graminearum* can also produce Type B trichothecenes, which accumulate in grains (Moretti et al., 2017). It survives the winter as saprophytic mycelium in crop residues from previous cultivation. Its conidia and perithecia-carrying ascospores develop in spring under moist weather conditions, and these infect flowering wheat spikelets. Conidia or ascospores germinate and enter the spikelet through natural openings such as stomata and other vulnerable sites (Trail, 2009). During this initial stage, the fungus develops morphological structures comparable to appressoria and infection cushions.

Microscopically, the asexual spores of *F. graminearum* are described as multiseptate fusiform macroconidia up to 25-50 x 3-4 µm in size (Booth, 1971) (**Figure 5**). Macroconidia overwinter in the soil and/or plant waste and re-infect in the spring (Bai & Shaner, 2004). The pigment on the reverse plate of PDA is red. It generates sexual spores (ascospores) of 19-24 x 3-4 µm

during the homothallic sexual phase. Both sexual and asexual spores play a key role in generating the infection, which has devastating effects on crop plants.

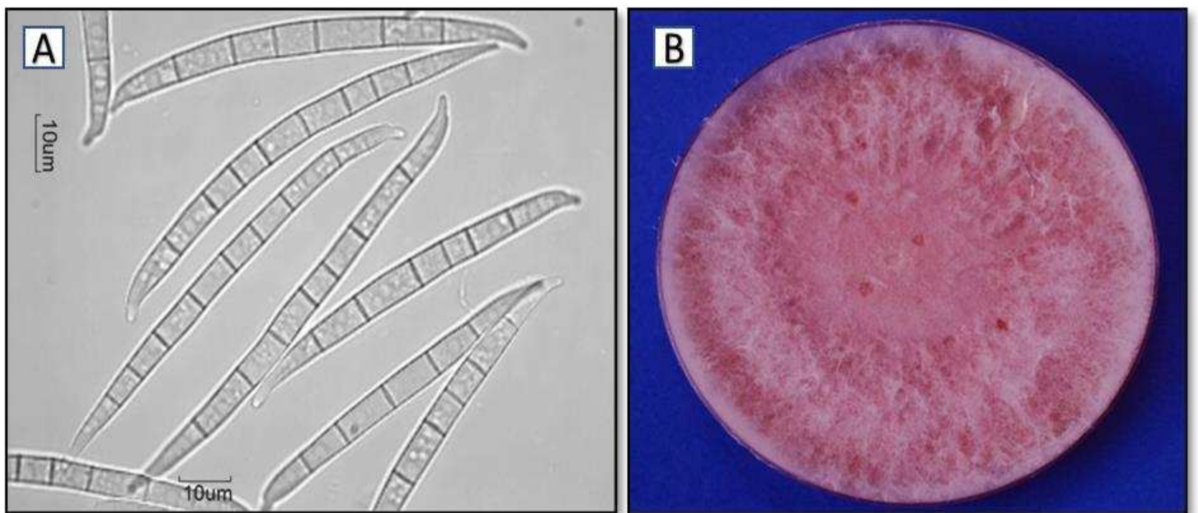

Figure 5. *F. graminearum* (A) macroconidia; (B) Mycelium on Potato Dextrose Agar (Source: Prof. Virgilio Balmas)

6.2. *Fusarium* trichothecene biosynthesis

The trichothecene biosynthesis pathway has been extensively studied, and understanding its control is critical for developing measures to reduce mycotoxin buildup.

The key genes for trichothecene production (*TRI1-TRI16*) are arranged into three clusters. Many enzymatic changes convert the major metabolic product (farnesyl diphosphate) to a toxic sesquiterpene epoxide (McCormick et al., 2011). In addition to *TRI*5 – the first gene encoding the trichodiene synthase, essential to transform farnesyl diphosphate in trichodiene – the main *TRI* cluster includes *TRI*8, *TRI*7, *TRI*3, *TRI*4, *TRI*5, *TRI*11, and *TRI*13 genes, which encode for 5 enzymes synthesizing the mycotoxin skeleton, the transcriptional regulators TRI6 and TRI10, a transport protein TRI12, and two genes (*TRI*9 and *TRI*14) with uncertain function (Proctor et al., 2018) (**Figure 6**).

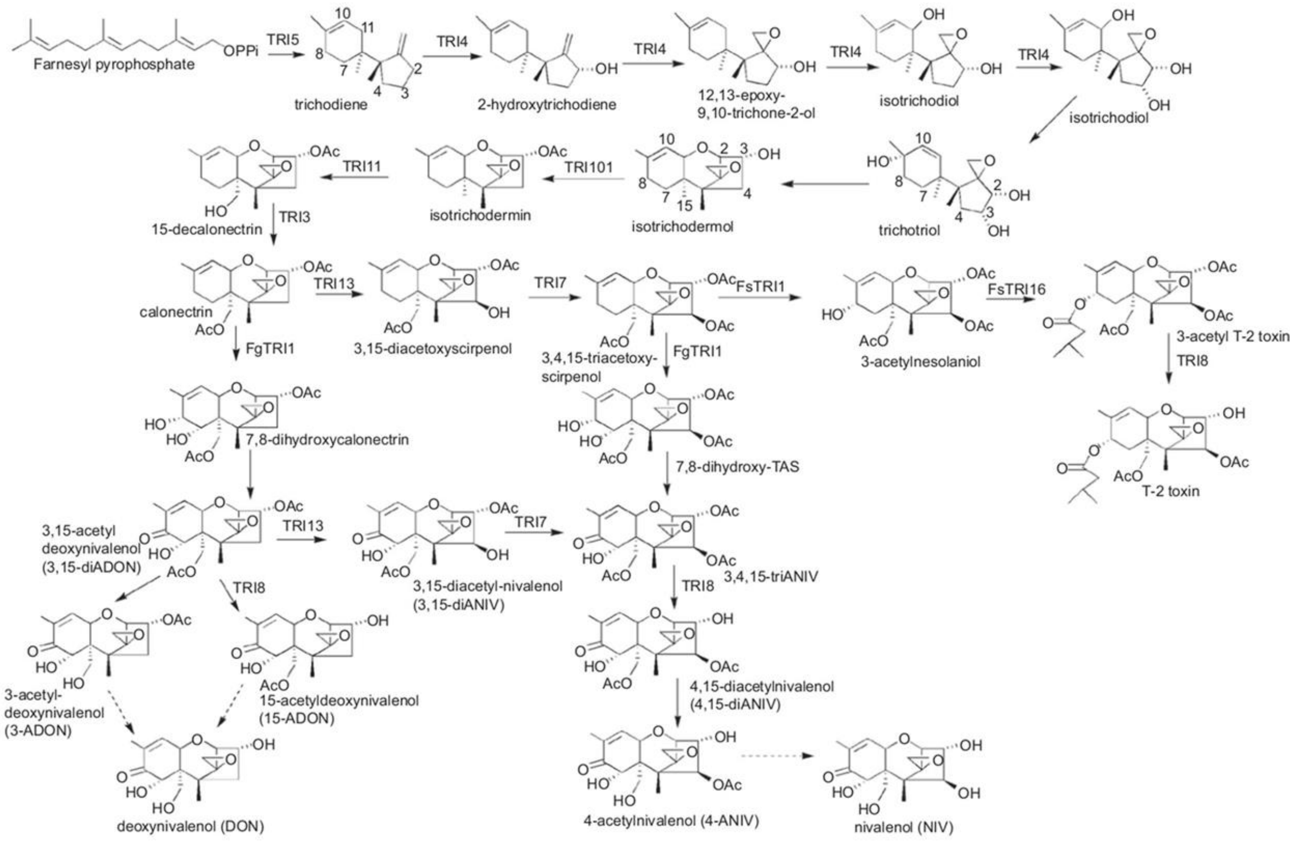

Figure 6. Trichothecene biosynthesis pathway (Alexander et al., 2009)

The differences in chemical structures are related to differences in gene functioning within the cluster. TRI13 and TRI7 are responsible for hydroxylation and acetylation of C-4 in NIV-producing *Fusarium* strains. These genes are not functional in DON-producing isolates, resulting in the absence of the hydroxyl group in C-4 (Lee et al., 2002). The removal of the acetyl group from C-3 or C-15 positions in the gene responsible for DON esterification (*TRI8*) results in the synthesis of acetylated derivatives (3ADON or 15ADON) (Alexander et al., 2011). As a result, distinct chemotype lineages, such as 3ADON, 15ADON, and NIV chemotype (nivalenol, 4 acetylnivalenol) are described among *Fusarium* isolates (Miller et al., 1991). The evolutionary history of most of the trichothecene biosynthesis genes is discordant with the species phylogeny, but it may affect the fitness and toxicity of the isolates *in planta* (Ward et al., 2002).

7. Case study 2: *Aspergillus*

7.1. *Aspergillus* genera

Aspergillus species are among the most abundant and widespread fungi. *Aspergillus* genera are composed of 4 subgenera and 19 sections recognized for a total of 339 identified species. It is also one of the most economically significant fungal genera. *Aspergillus* are mostly saprophytic, feeding on decomposing plant materials (Moretti & Susca, 2017). Several *Aspergillus* species are capable of invading living plant tissues; the majority of the species are storage molds on plant products.

7.2. *Aspergillus* mycotoxins

The main mycotoxins produced by species belonging to *Aspergillus* genus are AF (B1, B2, G1, G2), OTA, sterigmatocystin, cyclopiazonic acid, penicillic acid, citrinin, cytochalasin E, verruculogen, and fumitremorgin A and B (Perrone & Gallo, 2017). Among these, the most important are AF, OTA, and fumonisins.

7.3. Ochratoxin A and synthesis pathway

Ochratoxin A is a polyketide-derived secondary metabolite that contains a dihydrocoumarin moiety coupled to an l-β-phenylalanine. The chemical name of ochratoxin is: l-phenylalanine-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyrane-7-yl)carbonyl]-(*R*)-isocoumarin (El Khoury & Atoui, 2010).

In contrast to other significant mycotoxins, little is known about the OTA biosynthesis route in *Aspergillus* and *Penicillium* species. The isocoumarin group is assumed to be a pentaketide produced from acetate and malonate via a polyketide synthesis pathway. However, a polyketide synthase (PKS) is considered as a crucial enzyme in the biosynthesis of OTA. According to Huff & Hamilton (1979), OTA biosynthesis involves three major steps. The first step is the

polyketide synthesis of ochratoxin α (Otα) using mellein through a polyketide synthase. The second step involves acyl activation, which includes mellein methylation and its oxidation to 7-carboxy-mellein (=Otβ). Otα is then produced through chlorination by chloroperoxidase. This component is subsequently converted to a mixed anhydride via ATP. The second precursor, phenylalanine, is produced via the shikimic acid pathway, then activated by ethyl ester to participate in the subsequent acyl displacement reaction.

In the third stage, these active precursors are linked via a synthetase, yielding OTC, an ethyl ester of OTA: The final step in this proposed biosynthetic pathway is de-esterification by an esterase or transesterification (El Khoury & Atoui, 2010) (**Figure 7**).

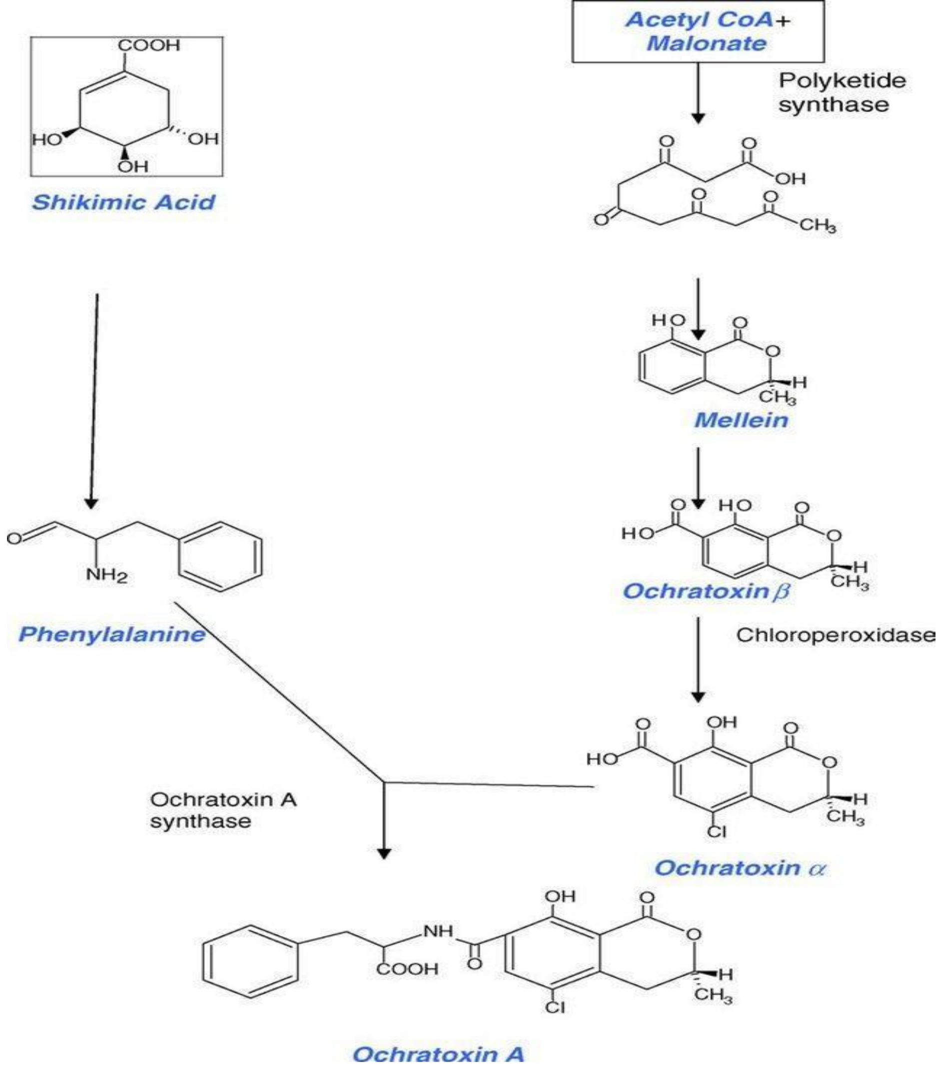

Figure 7. OTA biosynthesis pathway (Ringot & Chango, 2010)

8. *Fusarium* and *Aspergillus* spp. in human hosts and chemotherapy

Fusarium and *Aspergillus* are transkingdom pathogens, being able to infect both plants and humans (Gauthier & Keller, 2013). Invasive fungal infections are an important emerging global health issue, and cause over one million deaths each year worldwide (Bongomin et al., 2017; Brown et al., 2012).

Invasive aspergillosis infections are especially dangerous for immunocompromised patients (Kullberg & Oude Lashof, 2002). The principal causal agents of aspergillosis are *Aspergillus fumigatus* Fresen., *A. flavus*, *Aspergillus terreus* Thom, and *A. Niger* Tiegh.They cause variable symptoms, which range from minor and superficial infections to life-threatening and invasive illness with a mortality rate of up to 80% (Vahedi-Shahandashti & Lass-Flörl, 2020).

Fusariosis is the second most common fungal infection in humans after aspergillosis (Al-Hatmi et al., 2018; Guarro, 2013). Onychomycosis (**Figure 8A**) and keratitis (**Figure 8B**) are the most common *Fusarium* infections in immunocompetent patients (Homa et al., 2013). Invasive fusariosis is characterized by widespread skin lesions, positive blood cultures, and a poor prognosis, and is apt to infect immunocompromised patients with hematological diseases.

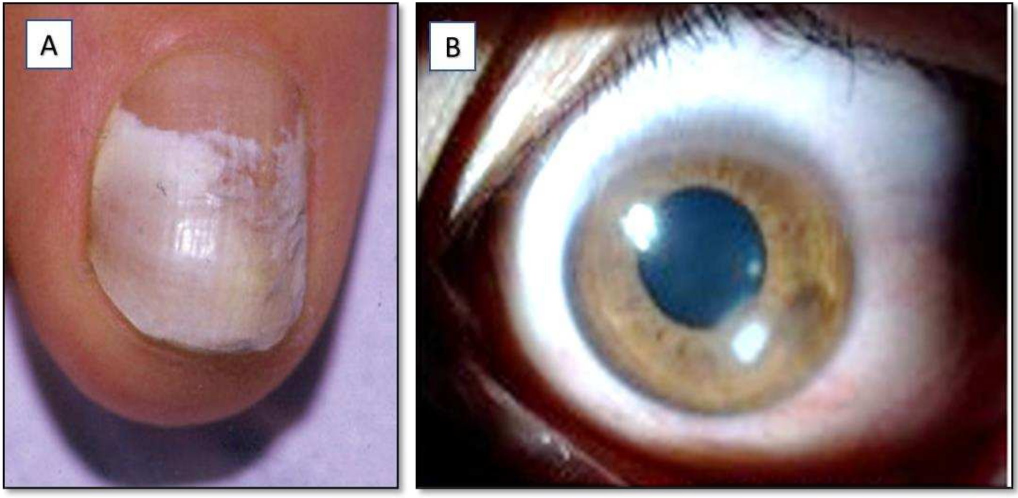

Figure 8. *Fusarium* symptoms on human host **(A;** Source: Prof. Virgilio Balmas**)** Onychomycosis**;** Keratitis **(B;** (Boral et al., 2018)**)**

Fusarium species that cause disease in humans belong to seven species complexes: *Fusarium solani* species complex (FSSC), *Fusarium oxysporum* species complex (FOSC), *Fusarium fujikuroi* species complex (FFSC), *Fusarium incarnatum-equiseti* species complex (FIESC), *Fusarium chlamydosporum* species complex (FCSC), *Fusarium dimerum* species complex (FDSC), and *Fusarium sporotrichioides* species complex (FSAMSC) (van Diepeningen et al., 2014).

Although *Fusarium* and *Aspergillus* infections are important public health concerns, the development of antifungal drugs has been slow, meaning that there are few therapeutic options available (Roemer & Krysan, 2014). The four major classes of antifungal agents currently used in the treatment of systemic mycoses are polyenes, azoles, echinocandins, and flucytosines (Gintjee et al., 2020). However, these antifungals have some important constraints, including toxicity, drug-drug interactions, variable pharmacokinetics, and low bioavailability, and the emergence of drug resistance has resulted in additional limits on their use (Patterson et al., 2016).

Fusarium is resistant to older azoles (e.g., itraconazole and fluconazole) and echinocandins, with variable resistance to triazoles and amphotericin B. Recent guidelines have recommended amphotericin B and voriconazole for localized and disseminated fusariosis. Posaconazole and terbinafine are two additional drugs that exert some activity against *Fusarium* species (Al-Hatmi et al., 2016).

Similarly, the prevalence of azole-resistant *Aspergillus* isolates has increased significantly, leading to therapeutic failures (Zoran et al., 2018). While the prevalence of azole-resistant clinical isolates of *Aspergillus* spp. has reached 30% in EU countries, it ranges between 0.6% to 11.2% outside the EU (Gonçalves et al., 2016). Therefore, there is an urgent need to develop novel strategies for the control of clinical *Fusarium* and *Aspergillus*.

Since plant extracts can be effective against human fusarioses (Zabka et al., 2014), researchers are at present focusing on the identification and characterization of numerous anti-fungal compounds derived from natural sources that have multi-target bioactivities (Amblard et al., 2007; Sarrica et al., 2018).

The present PhD research project has explored innovative approaches to the development of promising alternatives to conventional fungicides for the management of *Fusarium* and *Aspergillus*. It represents progress toward the development of new fungicides with different modes of action, thus reducing the risk of cross-resistance.

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Project

Fusarium and *Aspergillus* are important genera in agriculture, causing mycotoxigenic contamination of food and feed that leads to economic losses. They are also emerging and globally spreading agents of a large spectrum of human diseases, and have increased resistance to the azole antifungals currently used. Therefore, they constitute a major challenge in both agriculture and medicine. This thesis is an attempt to identify innovative and sustainable approaches to the control of mycotoxigenic *Fusarium* and *Aspergillus* fungi in both agricultural and medical contexts. The work consists of six chapters:

The first chapter includes an introduction about *Fusarium* and *Aspergillus*, their toxins and toxicity on human health.

The second chapter is a review paper published in *Toxins*, entitled, 'Bioprospecting Phenols as Inhibitors of Trichothecene-Producing *Fusarium*: Sustainable Approaches to the Management of Wheat Pathogens'. Here we cover the recent and most relevant research related to the use of natural phenolic compounds as antifungal and anti-mycotoxigenic agents on *Fusarium*. We cover the mechanisms of action of major exogenous phenolic inhibitors, their structure-activity interaction, and their combined use with other natural and/or conventional fungicides in mycotoxin modulation. We also discuss the literature related to the use of high-throughput analysis tools and their role in the discovery of key signaling molecules in mycotoxin modulation. In addition, we discuss the development of sustainable formulations enhancing potential inhibitors' efficacy.

The third chapter of this thesis is a paper published in *Molecules*, entitled 'Prenylated Trans-Cinnamic Esters and Ethers against Clinical *Fusarium* spp.: Repositioning of Natural

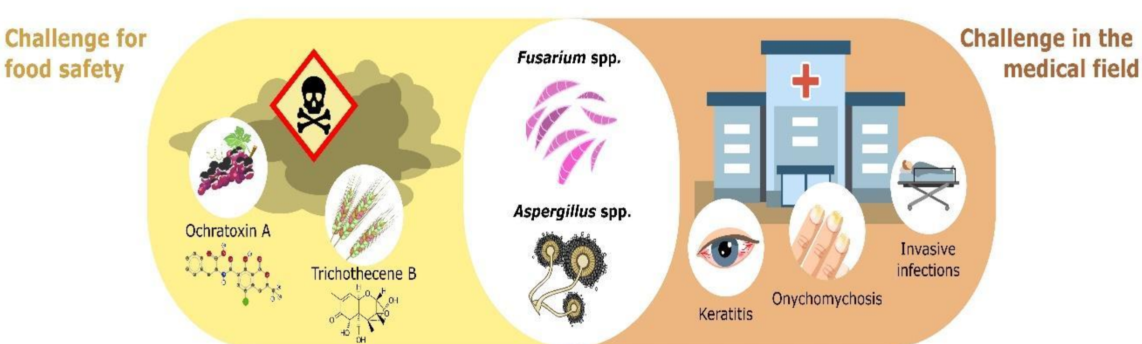
Compounds in Antimicrobial Discovery'. The aim of this work is to explore the fungicidal activity of an array of naturally-occurring cinnamic acids and their ester and ether derivatives on six representative strains of clinically and agronomically relevant *Fusarium* spp. in order to test their structure-activity relationship and overcome the growing problem related to the emerging cross-resistance of *Fusarium* in human and plant hosts.

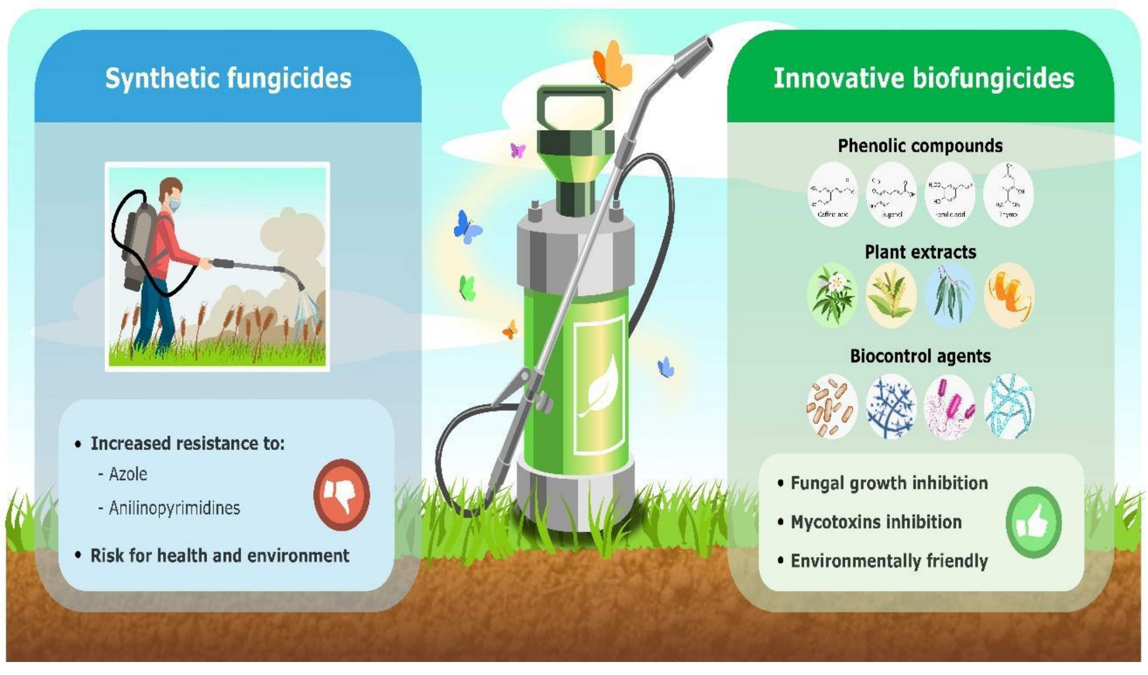
The fourth chapter of the thesis has been submitted to the *International Journal of Food Microbiology* and is entitled 'Plant extracts as biocontrol agents against *Aspergillus carbonarius* growth and ochratoxin A (OTA) production on grapes'. It is the outcome of an ongoing collaboration with the Polytechnic Institute of Bragança, Portugal. The goal is to explore the application of a set of natural plant extracts grown in the Mediterranean area and rich in phenolic compounds as alternatives treatments to synthetic fungicides in order to contain OTA contamination and prevent *A. carbonarius* contamination of grape.

The fifth chapter of this thesis explores another component of biological control, i.e. the use of microorganisms. We examined the use of bacteria belonging to the genera *Bacillus* spp. as biocontrol agents against *Fusarium*; the literature describes them as antimicrobial factories, and we explored their effect on *Fusarium* growth and on its mycotoxins. One of the challenging aspects of this study was to explore the duality of *Bacillus* as a biocontrol agent of *Fusarium* and as a biostimulant of wheat growth.

The final chapter is dedicated to the discussion and conclusion of the achievements of this project.

Graphical Abstract





Chapter 2

Summary
Bioprospecting phenols as inhibitors of trichothecene-producing *Fusarium*: sustainable approaches to the management of wheat pathogens
Abstract
1. Introduction
2. Trichothecenes: biosynthesis and regulation
3. Role of trichothecene detoxification in wheat resistance against *Fusarium*
4. Major plant phenolic compounds and their effect on *Fusarium*
5. Antifungal activity of exogenous phenolic compounds on *Fusarium* vegetative growth
6. Inhibition of trichothecene biosynthesis by exogenous phenolic compounds
7. Effect of the combination of phenolic compounds with other natural products or conventional fungicides
8. Sustainable formulations for bioprospecting phenolic compounds
9. Structure-activity interactions
10. Conclusions and future trends
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Summary

There is an increasing body of evidence from investigations regarding the fungicidal properties of phenols that has linked them to plant resistance against fungal pathogens. In agriculture, pathogenic fungi are notoriously responsible for economic and food losses; in particular, toxigenic fungi pose a major risk to food safety. However, despite attempts to control these pathogens, the development of resistance to the active ingredients in synthetic fungicides is now a major challenge confronting agriculture and medicine. In recent decades, research has investigated several phenolic compounds, which have given encouraging *in-vitro* and *in-vivo* results for inhibition of *Fusarium* growth and trichothecene biosynthesis. Chapter 2 reviews and summarizes a wide range of phenolic compounds with regard to their chemical structure, mode of action, and whether they can be used in combination with natural and synthetic fungicides in *Fusarium* management. To overcome some limits of phenols, such as their low bioavailability, solubility or toxicity, innovative formulations including encapsulation in biomatrices are proposed. However, since phenolic compounds are correlated to the resistance of wheat to *Fusarium*, it is envisioned that the current trend to use -omics approaches and bioinformatics will reveal more about the mechanism of action of phenolic compounds and thereby enable better understanding of their structure activity features.

Review

Bioprospecting Phenols as Inhibitors of Trichothecene-Producing *Fusarium*: Sustainable Approaches to the Management of Wheat Pathogens

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Abstract: *Fusarium* spp. are ubiquitous fungi able to cause Fusarium head blight and Fusarium foot and root rot on wheat. Among relevant pathogenic species, *Fusarium graminearum* and *Fusarium culmorum* cause significant yield and quality loss and result in contamination of the grain with mycotoxins, mainly type B trichothecenes, which are a major health concern for humans and animals. Phenolic compounds of natural origin are being increasingly explored as fungicides on those pathogens. This review summarizes recent research activities related to the antifungal and anti-mycotoxigenic activity of natural phenolic compounds against *Fusarium*, including studies into the mechanisms of action of major exogenous phenolic inhibitors, their structure-activity interaction, and the combined effect of these compounds with other natural products or with conventional fungicides in mycotoxin modulation. The role of high-throughput analysis tools to decipher key signaling molecules able to modulate the production of mycotoxins and the development of sustainable formulations enhancing potential inhibitors' efficacy are also discussed.

Keywords: phenolics; *Fusarium*; wheat; Fusarium head blight; trichothecene mycotoxins; cereals; food safety; fungicides

Key Contribution: The aim of this review is to systematize information on the antifungal and anti-mycotoxigenic activity of natural phenolic compounds against *Fusaria* and to elucidate their potential contribution as a sustainable control strategy in modern agriculture.

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1. Introduction

Fusarium spp. are found in various ecosystems, including agricultural soils, where they have a relevant impact on cereal crops [1–5]. Among the most important cereal diseases, fusarioses are incited by a complex of toxigenic species of the genus *Fusarium* [6]. Depending on the cereal type and the geographic area, the range of *Fusarium* species present may differ. *Fusarium culmorum* (W.G. Smith) Sacc., *Fusarium graminearum sensu stricto* (Schwabe), and *Fusarium pseudograminearum* O'Donnell and Aoki are considered as main pathogens of wheat [7–12]; whereas other species that are detected less frequently include *Fusarium acuminatum* Ellis and Everhart, *Fusarium avenaceum* Fr. (Sacc.), *Fusarium langsethiae* Torp and Nirenberg [13], *Fusarium poae* (Peck) Wollenw., and *Fusarium tricinctum* (Corda) Sacc. The presence of one or more *Fusarium* species also depends on other factors such as previous crops, management of cultural residues, environmental conditions, and cultivation techniques.

Fusarium head blight (FHB) is caused on wheat and other small grains, mainly by *F. graminearum* and *F. culmorum* [4,14] (Figure 1).

Figure 1. Symptoms of Fusarium head blight on durum wheat spikes (**A**–**D**) and kernels affected by fusariosis (**F**,**G**) compared to healthy kernels (**E**).

These fungi reduce yields and cause quality losses due to the production of mycotoxins [2,15]. *F. graminearum* and *F. culmorum* may produce zearalenone (ZEA) and type B trichothecenes; these include deoxynivalenol (DON) and its two acetylated forms: 3 acetyl-deoxynivalenol (3-ADON chemotype) and 15-acetyl-deoxynivalenol (15-ADON chemotype), as well as nivalenol (NIV chemotype) [16,17] and its acetylated form 4-acetylnivalenol or fusarenone-X (4-ANIV chemotype) [18]. DON is by far the most frequently detected mycotoxin in cereal grains worldwide [19–22], with incidences ranging from 50% in Asia to 76% in Africa [23].

Trichothecenes may occur in food and feed at high concentrations and have toxic effects on plants and animals [24–26]. They are phytotoxic in wheat, causing chlorosis, inhibition of root elongation, and dwarfism [27]. Moreover, livestock exposure to mycotoxins, including trichothecenes, may be responsible for direct production losses, such as milk production decrease in dairy cattle, but also indirect losses, as a consequence of reduced liver function immune responses, epithelial barrier function, and reproductive capacity [21,28]. Trichothecenes are also a cause of public health concern: these compounds elicit many adverse effects in humans, among which the most relevant are emesis, nausea, anorexia, abdominal pain, growth suppression, diarrhea, hemorrhage, and immunotoxicity [29,30].

Fusarium mycotoxins are among the most relevant causes of concern regarding chronic toxicity of natural food and feed contaminants and pose critical challenges in food toxicology [23,31]. Consequently, maximum contamination levels acceptable for DON in cereal-based food were set by the European Commission in June 2005 (EC no. 856/2005) and revised in July 2007 (EC no. 1126/2007) and by amending regulations [32–36]. These limits were fixed at 1250 μg/kg in unprocessed common wheat and 1750 μg/kg in unprocessed durum wheat for human consumption in the European Union (EC no. 1126/2006).

The efficient containment of *Fusarium*-associated disease and the reduction in food and feed trichothecene contamination poses a major challenge and requires integrated management approaches, spanning from the choice of tolerant cultivars, the adoption of crop rotation strategies, reduced nitrogen application, management of crop residues, and seed coating with biocontrol agents or antifungal compounds [23,37–39].

Fungicides bearing an azole unit are widely used in agriculture for the control of *Fusarium* species and their mycotoxins as they are generally inexpensive, have a broad spectrum of action and long stability [40]. Azoles inhibit the ergosterol biosynthesis pathway by blocking the sterol α-demethylase [41]. Despite their efficacy, though, if used incorrectly, they may induce a selective pressure on fungal populations, favoring the appearance of resistant mutants [42–45]. The frequent use of fungicides of the triazole

family is also associated with a shift in the FHB-causing *Fusarium* species, e.g., by increasing the frequency of *F. avenaceum* (Fr.) Sacc. and *F. poae* while decreasing the population of *F. culmorum* and *F. graminearum* [46]. Studies on the *Fusarium* population showed the proliferation of highly aggressive strains and chemotypes, with high resistance to certain fungicides [46,47]. For example, a more aggressive and toxigenic *Fusarium asiaticum* O'Donnell, T. Aoki, Kistler and Geiser 3-ADON population has now replaced the previous NIV population in China. Similarly, in North America, a highly toxigenic population mainly formed by 3-ADON isolates of *F. graminearum* is replacing the existing 15-ADON population [47].

On the other hand, azole fungicides do not always warrant the decrease in mycotoxins in food and feed [48]. Under certain conditions, they may act as stress factors resulting in the induction of toxin biosynthesis [49–53]. Increased mycotoxin biosynthesis may take place when fungicides are distributed below the recommended dosage [54,55] or if they show differential fungicidal control of mixed FHB pathogen populations [56]. Moreover, chemical fungicides pose adverse effects on human health and on different components of the ecosystems, including water, soil, and non-target organisms [57,58].

Increasing efforts are now devoted to the design of alternative approaches to replace synthetic fungicides, particularly new classes of compounds capable of limiting the pathogenic and/or the mycotoxigenic potential of *Fusarium* spp., or able to enhance natural resistance mechanisms in the host plant [59,60]. For example, antioxidants have attracted considerable attention as they play a crucial role in the natural defense response of plants to oxidative stress caused by fungal invasion, and a strong, specific inhibitory activity was demonstrated for plant antioxidants (e.g., phenolic and polyphenolic compounds) against trichothecene-producing strains of *F. graminearum* and *F. culmorum* [61–63].

The objective of this review is to summarize the potentialities and limits of naturally occurring phenolic compounds as inhibitors of *Fusarium* spp. of agricultural interest, with emphasis on trichothecene producers affecting cereals: after a brief introduction on the structure and biosynthesis of trichothecene mycotoxins, the role of endogenous phenolic compounds in wheat defense reaction during fungal attack will be described. Then, the inhibitory effects of exogenous natural phenols on *Fusarium* vegetative growth and mycotoxin production will be illustrated. Finally, the possibility to design different combinations of phenolics and other natural compounds with improved activity against pathogenic *Fusaria* will be discussed.

2. Trichothecenes: Biosynthesis and Regulation

Trichothecenes are esters of sesquiterpenoid alcohols positioned around a trichothecane tricyclic ring characterized by a double bond at C9–C10 and an epoxide at C12–C13 [64]. Trichothecene compounds are divided into four main groups, namely A, B, C, and D, based on their chemical properties and on the producing fungi. Trichothecenes synthesized by *Fusarium* spp. are included in groups A and B (Figure 2, Scheme 1).

TRICHOTHECENES

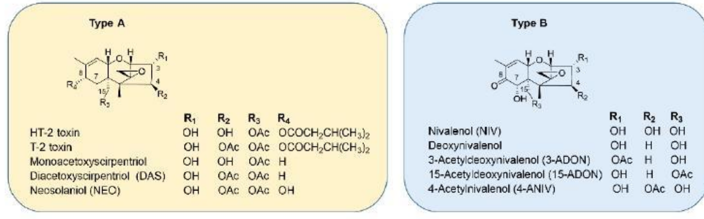
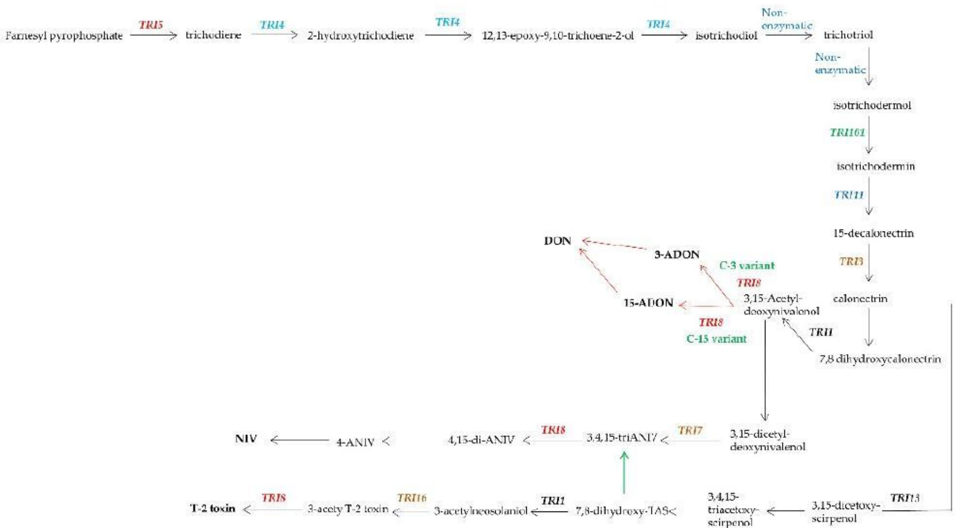


Figure 2. General structure of trichothecenes A and B.



Scheme 1. Trichothecene biosynthesis pathway.

Type A trichothecenes include: T-2 toxin and HT-2 toxin, diacetoxyscirpenol (DAS), scirpentriol (STO), 4-monoacetoxyscirpenol (MAS), and neosolaniol (NEO). These metabolites are mainly produced by *Fusarium sporotrichioides* Sherb., *Fusarium sambucinum* Fuckel, *F. poae*, *F. langsethiae*, and *Fusarium equiseti* Corda (Sacc.) [39,65,66]. Type B trichothecenes are characterized by a C-8 keto group and include: deoxynivalenol (DON), the acetyl derivatives (3-ADON), and 15-ADON), as well as nivalenol and fusarenone-X (4-ANIV) [18,67,68]. These compounds are predominantly produced in cereals by *F. culmorum*, *F. graminearum*, and *Fusarium crookwellense* L.W. Burgess, P.E. Nelson and Toussoun [17,69–72]. Types C and D share the presence of a carbonyl group attached to C-8 of the sesquiterpenoid backbone of trichothecenes. The presence of additional 7, 8 epoxides allows differentiation between type C from the other types. Type D contains a macrocyclic ring that connects C-4 and C-15 of the sesquiterpenoid backbone [67].

The precursor of trichothecene biosynthesis is the farnesyl pyrophosphate (FPP), an intermediate of the mevalonate pathway with a backbone of 15 carbon atoms [21,73]. The biosynthetic enzymes needed for trichothecene production are encoded by at least 15 *TRI* genes, which are located at three different loci on different chromosomes in *F. graminearum*: a 12-gene core *TRI* cluster, two genes at the *TRI1-TRI16* locus, and the single-gene *TRI101* locus [18,68,74]. Trichothecene production is driven by the expression of the *TRI5* gene, encoding the key biosynthesis enzyme trichodiene synthase, which cyclizes FPP to trichodiene (TDN), the first step in trichothecene biosynthesis [75,76]. TDN is then converted to calonectrin (CAL) following nine reactions that are sequentially catalyzed by TRI4 (a key multifunctional CYP58 family cytochrome P450 monooxygenase allowing four consecutive oxygenation steps in trichothecene biosynthesis, converting TDN to isotrichotriol), TRI101 (C-3 acetyltransferase), TRI11 (C-15 hydroxylase), and TRI3 (15-O-acetyltransferase). The reaction steps are found in *Fusarium* species producing type A trichothecenes (T-2 toxin and HT2) and type B trichothecenes (NIV and DON). In DON producers, CAL is hydroxylated at both the C-7 and C-8 positions by the cytochrome P450 monooxygenase TRI1 and deacetylated by the esterase TRI8 [77], leading to the formation of either 3-ADON or 15-ADON, followed by DON. A sequence variation in the coding region of the trichothecene biosynthetic gene *TRI8* was reported in *Fusarium* spp., indicating that differential activity of the TRI8 protein determines the 3-ADON and 15-ADON subchemotypes in *Fusarium* [78].

All these reaction steps catalyzing FPP to CAL are shared among *Fusarium* species that produce type A trichothecenes (T-2 toxin and HT2) and type B trichothecenes (NIV and DON). Two alternative pathways for NIV biosynthesis were observed for *F. graminearum*, involving either the TRI13-TRI7-TRI1-TRI8 pathway (and the CAL as a substrate) or the TRI13-TRI7-TRI8 pathway (with the 3, 15-ADON as the initial substrate) [79,80]. Depending on the type of trichothecenes produced, different chemotypes have been described: chemotype I, producing DON and/or its acetylated derivatives (3-ADON and 15-ADON), and chemotype II, producing nivalenol (NIV) and/or 4-acetyl-NIV [19].

Similar to other secondary metabolites, mycotoxins may be over-produced in response to external stresses, e.g., oxidative, nutritional, or light stress, as well as other environmental factors, such as pH, temperature, water activity, exposure to fungicides or plant secondary metabolites [81,82]. Temperature and water activity (a_w) are the primary environmental factors influencing mycotoxin production by several *Fusarium* species [49,83–85]. For instance, *F. culmorum* and *F. graminearum* grow optimally at 15–25 °C in an a_w range of 0.98–0.99. Optimum DON production is situated at a_w = 0.97–0.99 for *F. culmorum* and at a_w = 0.98–0.99 for *F. graminearum*, all with an optimal temperature range of 15–25 °C [83].

Control of trichothecene production is driven by various transcriptional regulators involved in basal metabolic functions [86], e.g., the Pac transcription factor, which governs fungal responses to environmental changes such as pH [87–89] the velvet complex involved in response to light [90], and the *F. graminearum* FgAp1 factor, playing a role in response to oxidative stress [91]. Understanding the effect of each of these factors is essential to predict and prevent mycotoxin development.

During infection, plant cells respond to mycotoxin presence by a hypersensitive reaction that triggers the generation of reactive oxygen species (ROS), such as H_2O_2 and superoxide [24]. The oxidative properties of H_2O_2 modulate trichothecene biosynthesis [92,93] and induce increased expression of *TRI* genes [94,95]. However, *Fusarium* response to oxidative stress may vary depending on the ability to activate antioxidant defense responses and on the chemotype of the isolate: in vitro production of DON and 15-ADON by *F. culmorum* and *F. graminearum* chemotype I isolates can be enhanced upon H_2O_2 treatment, whereas NIV and 4-ANIV production by chemotype II isolates is reduced [96]. Similarly, differences in the detoxification ability were reported in the two chemotypes: isolates in chemotype I, when exposed to oxidative stress, react by increasing the catalase activity, resulting in a higher H_2O_2-degrading ability [96].

3. Role of Trichothecene Detoxification in Wheat Resistance against *Fusarium*

The ability of both *F. culmorum* and *F. graminearum* to spread in wheat is dependent on their potential to produce larger amounts of DON in culture [97,98] or in infected tissues [97–100], although this correlation is not always linear [97,101,102]. Trichothecenes also play an important role as virulence factors by inhibiting defense mechanisms activated by the plant [9]. Mutants of *F. graminearum* in which the ability to produce DON is impaired are able to infect but not to spread within the host plant [103–106].

Genetic improvement of wheat varieties by breeding or transgenesis to select wheat varieties resistant or partially resistant to *Fusarium* spp. is definitely the most sustainable approach to reduce the occurrence of these fungi and the contamination of grain with mycotoxins [82,107]. Plant resistance to FHB is a highly complex quantitative trait controlled by multiple genes [107–110]. The differing susceptibility of wheat cultivars to infection by *Fusarium* spp. is associated with different levels of mycotoxin contamination. This variability results from breeding programs, as well as agronomic and environmental cultivation conditions in individual countries [111]. Moreover, while the mechanisms by which abiotic stress may influence wheat resistance traits toward *Fusarium* spp. are still largely unknown, it is generally acknowledged that wheat would be more susceptible to *Fusarium* infection under future climate change conditions [112,113].

In wheat, two types of resistance to FHB were first described by Schroeder and Christensen [114]: type I (resistance to initial infection) and type II (resistance to fungal

spread within the host tissue). Out of approximately 500 quantitative trait loci (QTLs) for FHB resistance mapped so far in wheat, most refer to type I and type II resistance, indicating their key role in controlling FHB. Some of these QTLs have been successfully applied in marker-assisted selection to improve FHB resistance [110,115].

Three additional types of resistance were defined: type III (ability to resist kernel infection); type IV (plant tolerance to infection and to the presence of DON and other secondary metabolites); and type V (resistance to the accumulation of mycotoxins in grain by converting them into non-toxic derivatives or by impeding the generation of toxic metabolites [39,116,117].

Given the key role of DON as a virulence factor for *Fusarium*, resistance to DON through detoxification or modulation mechanisms is considered as an innate component of FHB resistance. Kluger et al. [118] described the various metabolic routes involved in the detoxification of DON and reported a correlation between the efficiency of detoxification and a QTL for FHB resistance called *Fhb1*. Due to its pivotal role in wheat FHB resistance, *Fhb1* has been the subject of extensive map-based cloning studies to identify the causal gene. An early study has shown that *Fhb1* is involved in the conversion of DON into non-toxic DON-3-glucoside (D3G) [119]. Later, the *Fhb1* locus has been cloned from the resistant wheat cultivar Sumai 3 and shown to encode a chimeric lectin with two agglutinin domains relevant in carbohydrate binding. This protein also contains an ETX/MTX2 domain involved in pore forming, named PFT (pore-forming toxin-like) [120]. However, Yang et al. [121] found that TaPFT is also present in a number of highly FHB-susceptible wheat accessions, leading to reconsider the identity of *Fhb1*. Cloning of *Fhb1* has shown that its DON-detoxifying ability is not associated with PFT activity but rather with a putative uridine diphosphate (UDP)-glucosyltransferase that is also located on the chromosomal region introgressed from the cultivar Sumai 3 [120]. *Fhb1* was recently identified as an atypical disease resistance gene by two independent studies [122,123] reporting on the map-based cloning of *Fhb1*. In both papers, a critical deletion in the same gene coding for a reticulum histidine-rich calcium-binding-protein gene (His; also called HRC) was identified as the key determinant of *Fhb1*-mediated resistance to FHB in bread wheat. However, while these authors acknowledged the role of *Fhb1* in FHB resistance, they reached diverging conclusions on the causative allele: Su et al. [123] hypothesized that the *Fhb1*-mediated resistance is caused by a loss-of-function mutation; whereas Li et al. [122] concluded that this deletion results in a gain of function. Lagudah and Krattinger [124] explained the findings reached by these two apparently contradictory concurrent studies by conjecturing that the critical deletion may generate a dominant-negative effect. *Fhb2* is located on chromosome 6BS and confers enhanced type II FHB resistance [125,126]. Metabolomic and transcriptomic analyses of a recombinant inbred line carrying the *Fhb2*-resistant allele highlighted increases in defense-related compounds (phenylpropanoids, lignin, glycerophospholipids, flavonoids, fatty acids, and terpenoids), along with significant induction of genes encoding receptor kinases, transcription factors, signaling as well as mycotoxin detoxification proteins [127].

In the same QTL region, different putative defense-associated genes were identified, such as 4-coumarate: CoA ligase, callose synthase, basic helix loop helix transcription factor, glutathione S-transferase, ABC transporter-4, and cinnamyl alcohol dehydrogenase, suggesting that DON detoxification and cell wall reinforcement may be concurrently driven by *Fhb2*-regulated genes, thereby limiting the colonization of the wheat spike by the pathogen [127].

Fhb5 is linked to a glutamate-gated ion channel, which is capable of triggering Ca^{2+} influx for early defense signaling in response to FHB [128,129].

The gene *Fhb7* from *Thinopyrum elongatum*, a wild relative of wheat used in breeding programs to improve cultivated wheat, encodes a glutathione S-transferase (GST). When introgressed into wheat backgrounds, *Fhb7* confers broad resistance to both FHB and *Fusarium* crown rot by detoxifying trichothecenes through de-epoxidation [130].

Manadalà et al. [131] demonstrated the efficacy of the barley *HvUGT13248* expressed in both bread wheat and durum wheat. The transgenic durum wheat displayed much greater DON-to-D3G conversion ability and a considerable decrease in total DON + D3G content in flour extracts, while the transgenic bread wheat exhibited a UGT dose-dependent efficacy of DON detoxification.

4. Major Plant Phenolic Compounds and Their Effect on *Fusarium*

Phenolic compounds contain at least one hydroxylated aromatic ring, with the hydroxyl group attached directly to the phenyl unit representing the core of the molecule. More oxygenated functionalities can be present and distributed in the other positions of the phenolic ring. Phenyl, aryl, aliphatic rings, and aliphatic chain, often containing hydroxylated functionalities, can be bound to the parent phenolic ring (Figure 3).

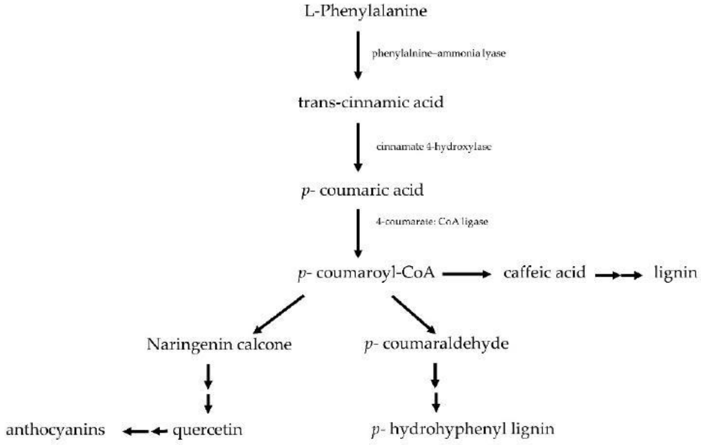
HO R

R = phenyl
aryl
aliphatic chain
aliphatic ring

Figure 3. Schematic structure of the bioprospecting phenols.

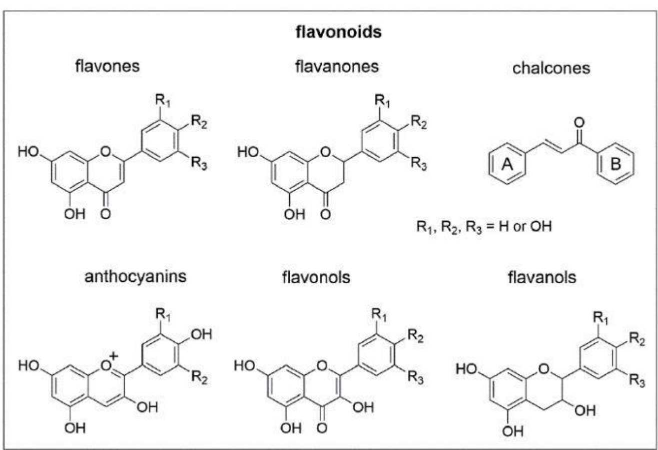
They contribute to various traits, such as pigmentation and resistance to pathogens in plants [132–134], and are generally present in food, spices, or food preservatives or belong to the list of generally recognized as safe (GRAS) compounds [135].

Phenolic compounds derive from the phenylpropanoid pathway [136], and their production is driven by phenylalanine ammonia-lyase (PAL), which converts phenylalanine into *trans*-cinnamic acid. This phenolic acid undergoes other enzymatic transformations, yielding a broad range of related phenylpropanoids [137] (Scheme 2).



Scheme 2. Phenylpropanoid pathway.

They are chemically divided into two groups (Figure 4): flavonoid phenylpropanoids, including flavones, flavonols, flavanones, flavanols, anthocyanins, and chalcones; and non-flavonoid phenylpropanoids such as stilbenes, lignans, and phenolic acids [63] (Figure 4).



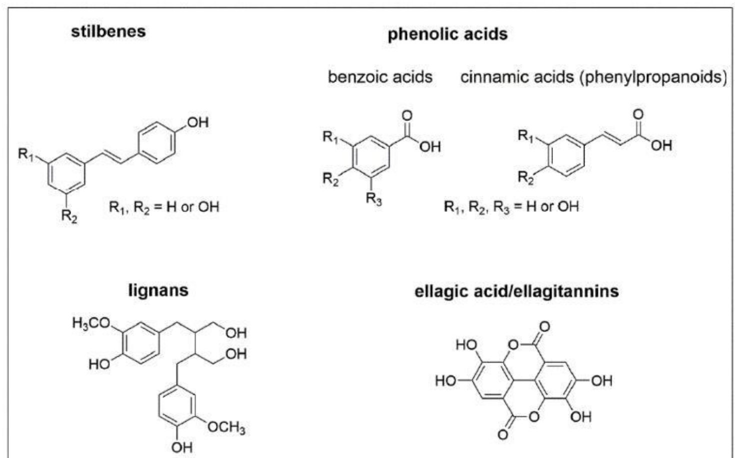


Figure 4. Classes of polyphenols.

The majority of phenolic compounds are bound to the cell wall [138], which suggests their contribution to the preformed general defense system against potential pathogens [139,140]. The main role of flavonoids in plant defense mechanisms depends on their antioxidant properties [141–144], allowing them to quench ROS generated by both the pathogen and the plant during the infection process [145]. In addition, flavonoids, similarly to other non-flavonoid compounds such as cinnamic acids, are thought to take part in the reinforcement of plant cell walls and act as a physical barrier against fungal infection [146]: they protect plant cell wall integrity by hampering the activity of plant cell wall-degrading enzymes secreted by pathogens. Flavonoids are also known for their inhibitory activity toward fungal spore development, hyphal elongation, and fungal biofilm formation [109,147].

Phenolic acids form one of the main classes of non-flavonoid phenylpropanoids. Based on the number and position of hydroxyl groups on the aromatic ring, they can be divided

into two main groups: the hydroxybenzoic acids and the hydroxycinnamic acids [148] (Figure 5).

HYDROXYBENZOIC ACIDS

p-hydroxybenzoic acid protocatechuic acid gallic acid syringic acid

HYDROXYCINNAMIC ACIDS

p-coumaric acid caffeic acid ferulic acid sinapic acid chlorogenic acid

Figure 5. Structure of some representative phenolic acids with reported antifungal activity.

Benzoic acid derivatives include gallic, *p*-hydroxybenzoic, syringic, protocatechuic, and vanillic acids, while caffeic, chlorogenic, *p*-coumaric, ferulic, and sinapic acids are included in the group of cinnamic acid derivatives (Figure 5). Cereals contain phenolic acids in both soluble (free) and insoluble (cell-wall-bound) forms [149]. Soluble phenolic acids include either free acids or esterified to sugar conjugates, whereas insoluble phenolic acids are conjugated to several polysaccharides and to lignin through ester and ether bonds. The soluble forms are compartmentalized within the vacuoles, while the insoluble forms are incorporated in cell walls [63,150–152].

Species, cultivar, and environmental conditions determine phenolic richness and composition in cereal grains through both constitutive and induced biosynthesis [153]. They likely reduce mycotoxin accumulation in plants, including trichothecenes [154] and fumonisins [155–157]. It is generally acknowledged that the fungus-plant interaction involves oxidative stress with the production of radical oxygen species (ROS) that enhance the biosynthesis of mycotoxins. The antioxidant metabolites present in cereal grains can play a crucial role in the resistance to *Fusarium* and in the production of mycotoxins [60,63,142]. Among phenolic acids, cinnamic acid derivatives accumulated in the kernel and well known as antioxidants are considered as the main contributors to FHB resistance [59,109,153,154]. Reactive oxygen species (ROS) are generated by fungi during their metabolic activity playing a crucial role when phytopathogenic fungi interact with plant cells. Gallic acid, a widespread plant metabolite, exhibits antioxidant activity interfering with ROS as a scavenging agent and produces cell apoptosis in the organism that generates ROS. In virtue of the metal-chelating properties of gallic acid due to the presence of hydroxyl groups in the aromatic ring, gallic acid might promote radical production exhibiting pro-oxidant activity. This behavior may appear in some conditions that depend on the concentration of the acid and in the presence of transition metals (i.e., Cu^{2+} and Fe^{2+}). Pro-oxidant activity can accelerate damage to sensitive parts of the cell such as DNA, proteins, carbohydrates molecules, provoking the death of the organism [158].

5. Antifungal Activity of Exogenous Phenolic Compounds on *Fusarium* Vegetative Growth

Phenolic acids are common metabolites in plants and exert toxic effect on diverse fungi, including *Fusarium* species [60–63,109,132,143,150–152,159]. In cereal grains such as wheat, corn, rice, barley, sorghum, rye, oat, and millet, the predominant phenolic acids include

ferulic acid, dimers of ferulic acid, *p*-hydroxybenzoic acid, sinapic acid, cinnamic, and vanillic acid [62,63,109,143,153,159]. A higher concentration of phenolic acids was observed in *Fusarium*-resistant wheat and corn plants than in susceptible ones, thus identifying these compounds as biomarkers of plant resistance [60,132,143]. The antifungal effect of phenolic acids was assayed in vitro by artificial amendment of each compound to the pathogenic fungi. According to the species of *Fusarium* on which the exogenous phenolic compounds are tested and on their concentration level, different antifungal activity was observed [153,160,161].

The bioactivity of phenolic compounds mainly depends on their ability to affect cellular membranes, with consequent impairment of cellular ionic homeostasis, acidification of vacuolar and cytosolic pH, and ultimately the destruction of structural cellular integrity [162–166].

Chlorogenic acid or 5-O-caffeoylquinic acid (CHLO), generated by the esterification of caffeic acid (CA) with quinic acid, is a cinnamic acid derivative (Figure 5). It is one of the most widespread soluble phenolic compounds in the plant kingdom and represents a key component of the plant defense mechanism against *Fusarium* [33,143,164,167]. CHLO was found to be the main phenolic acid that *F. graminearum* is likely to cope with when it infects the ear [168,169]. Gauthier et al. [33] tested CHLO and one of its hydrolyzed compounds in vitro on both *F. culmorum* and *F. graminearum* at concentrations close to the physiological amount previously quantified in kernels by Atanasova-Pénichon et al. [169]. Both chlorogenic and caffeic acids reduced fungal growth. CHLO showed a moderate antifungal effect with LC_{50} values > 10 mM, while caffeic acid was significatively more toxic. However, there is great variability in sensitivity to phenolic acids among *Fusarium* strains [33]. When comparing results obtained in the same conditions by Gauthier et al. [33] and Ponts et al. [59], it appears that *F. culmorum* strains (LC_{50} between 8.8 and 10 mM) are likely less susceptible to caffeic acid than *F. graminearum* (LC_{50} between 4 and 10.1 mM) [63]. Lately, Gauthier et al. [170] investigated caffeic acid (0.5 mM) on *F. avenaceum* at different pH conditions in liquid medium: caffeic acid inhibited only 10% of the growth at pH = 6 while at pH = 3, the fungal biomass was increased upon exposure.

Similarly, ferulic acid has a remarkable antifungal effect on *Fusarium* species. Boutigny and coworkers reported that ferulic acid reduces fungal biomass of *F. culmorum* by 39% at 2.5 mM and by 85% at 5 mM [154], whereas Pani et al. [161] found a significant inhibition of *F. culmorum* at the concentration of 0.5 mM. Ferulic acid is also reported to inhibit fungal growth in *F. graminearum*: 0.7 mM of ferulic acid reduced fungal growth by 50%, while 0.5 mM had no significant effect, albeit inhibitory concentrations are often strain dependent [59].

The fungistatic effects of phenolic acids on *F. graminearum* were ranked in ascending order of toxicity as follows: chlorogenic acid < *p*-hydroxybenzoic acid < caffeic acid < syringic acid < *p*-coumaric acid < ferulic acid: therefore, cinnamic-derived acids appear as more toxic compared to benzoic acid-derived ones [59,154,161].

Under certain conditions, fungal biomass can be increased by sublethal doses of ferulic, caffeic, or coumaric acid [170]. For example, ferulic acid applied at 0.5 mM induced an increase in fungal biomass of *F. langsethiae*, while at 1 mM, it reduced its growth [171]. In contrast, some phenolic acids display moderate effects on fungal growth: *p*-hydroxybenzoic acid has a minor effect at concentrations > 15 mM, reducing by 50% the growth of *F. graminearum* [59,153].

Two phenylpropanoids, zingerone (4-(3-methoxy-4-hydroxyphenyl)-butan-2-one) and dehydrozingerone (Figure 6), are constituents of *Zingiber officinale*, structurally and biologically related to curcumin, with marked antifungal and antibacterial activity [172–174].

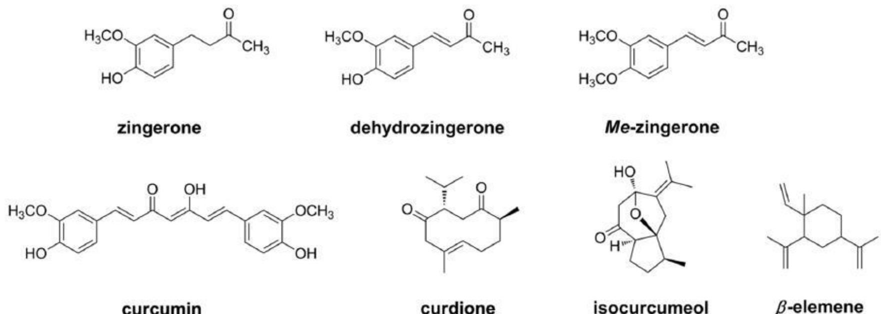


Figure 6. Structure of different compounds derived from *Zingiber officinale* and *Curcuma longa*.

Pani et al. [161] studied comparatively the antifungal effect of dehydrozingerone and Me-zingerone on *F. culmorum*: both reduced fungal growth by >50% at 1.5 mM, but dehydrozingerone retained its inhibitory effect at 1 and 0.5 mM, whereas Me-zingerone had a stimulating effect on vegetative growth when applied at 0.5 mM. Tested at 0.5 mM, zingerone reduced by 83% the fungal growth and by 33% the DON production [161].

Chen et al. [175] tested different compounds derived from *Curcuma longa*, including curdione, isocurcumenol, curcumenol, curzerene, β-elemene, curcumin (Figure 6), germacrone, and curcumol at 0.5 mg/mL, by calculating the percent inhibition of mycelial growth in the untreated control. All these compounds displayed an inhibitory effect toward *F. graminearum*. Curdione showed an inhibitory rate of > 50%. The inhibitory effect of curdione in combination with isocurcumenol and β-elemene (tested at 0.25 mg/mL for each component) was 100%, while curdione combined with curcumin, curzerene, curcumenol, curcumol, and germacrone allowed inhibition rates of 93.6%, 88.9%, 82.7%, 63.6%, and 56.4%, respectively. Their toxicity involved fungal cell membrane disruption and inhibition of ergosterol biosynthesis, respiration, succinate dehydrogenase (SDH), and NADH oxidase activity [175].

Significant antimicrobial activity against pathogenic microorganisms has also been reported for thymol [5-methyl-(1-methylethyl) phenol], a natural monoterpene phenol found primarily in thyme, oregano, and tangerine peel [176]. Gao et al. [177] studied the hyphal growth, the conidial production, and germination of 59 isolates of *F. graminearum* under thymol treatment: the mean EC_{50} value for *F. graminearum* was 26.3 μg/mL. The molecular structure of thymol is responsible for its ability to dissolve and accumulate within the cell membrane, causing its destabilization, which has been related to the disruption of proton transfer efficiency [178]. In *F. graminearum*, the antifungal activity of thymol has been related to cell membrane damage as a consequence of lipid peroxidation and the disturbance of ergosterol biosynthesis [177]. Accordingly, thymol is reported to induce damages on the membrane integrity and the cell wall of other microorganisms such as *Candida* sp. [178], *Saccharomyces cerevisiae* [179], and *Bacillus cereus* [180].

Magnolol (1,5,5′-diallyl-2,2′-dihydroxybiphenyl), a natural hydroxylated biphenyl isolated from *Magnolia officinalis*, displays a wide range of biological activities [181]. Tested at 1.5 mM, magnolol exhibits a marked fungicidal activity in vitro toward *F. culmorum*; while a progressive decline in its activity has been observed at 1.0, 0.5, and 0.25 mM, the antifungal effect of magnolol remains significant at 0.25 mM [166,182]. Magnolol interacts with ergosterol in the cell membrane, inducing a partial disruption of its structure [183]: cell wall components, such as β-1,3-glucans, have been proposed as potential targets of magnolol, similarly to fungicides belonging to the echinocandin family [184]. Incidentally, magnolol is also potentially applicable to control human fusarioses: when tested on a collection of representative isolates of *Fusarium oxysporum* Schlechtend. emend. Synd. and Hans., *Fusarium solani* (Mart.) Sacc. and *Fusarium verticillioides* (Sacc.) Nirenberg of

clinical and ecological concern, magnolol displayed a fungicidal activity similar to that shown by fluconazole (1–50 μg/mL), a fungicide widely used in treating fungal infections on humans [165]. Honokiol showed an even stronger antifungal activity than its isomer magnolol at 0.5 mM against *Fusarium* spp. [165]. The role of honokiol as an activator of mitochondrial ROS by dysfunction and depolarization of mitochondrial membrane potential in *C. albicans* has been highlighted [185]. Honokiol is also thought to burden the high content of pro-oxidant iron ions in yeast by sequestration [186]. Some differences between magnolol and honokiol in safety and toxicology have been reviewed by Sarrica et al. [187].

The efficacy of flavonoids as inhibitors of fungal growth has been referred to as their ability to react with nucleophilic amino acids in fungal proteins [188]. Compared to LC_{50} values described for phenolic acids, those detected for flavones and flavanones against *Fusarium* species, including *F. culmorum* and *F. graminearum*, are substantially weaker.

The promising ability of flavonoids to inhibit spore development and mycelium elongation of plant pathogens has been the subject of some studies [146,189]. Unsubstituted flavones and flavanones (with LC_{50} values comprised between <0.05 and 1.6 mM against *Fusarium* species, including *F. culmorum* and *F. graminearum*) display a higher antifungal activity than hydroxylated flavones (e.g., flavonol), with an LC_{50} in the 2.9–4.8 mM range [63]. Medical research has also focused on flavonoids as potential alternatives to synthetic drugs against human fungal pathogens displaying resistance to commonly used antifungal agents (e.g., triazoles).

Benzoxazinoids, a group of secondary metabolites present in several cereals, such as rye, wheat, and maize, play a key role as allelochemicals in the defense against predators and pathogen infection [190]. Their antifungal activity has been reported [181–193], and their role in wheat resistance to *Fusarium* spp. is being increasingly highlighted [134].

Inhibition of colony growth, of cell wall and membrane constituents (such as ergosterol and glucosamine), and alterations in enzyme activity with a consequent reduced biomolecular synthesis are all indicators of mechanisms involving the inhibition of cell multiplication. As previously mentioned, the inhibitory behavior of phenolic compounds depends on their ability to disrupt the integrity of the plasma membrane and to induce mitochondrial dysfunction, leading to metabolic stagnation [154,194]. For example, curcumin may disrupt the synthesis of critical proteins and enzymes, leading to inhibition of *F. graminearum* growth: this compound downregulates D-glyceraldehyde 3-phosphate: NAD^+ oxidoreductase (GAPDH); moreover, it inhibits the biosynthesis of ergosterol and suppresses the activity of B-nicotinamide adenine dinucleotide (NADH) oxidase and succinate dehydrogenase (SDH), thereby interfering with the tricarboxylic acid cycle as well as inhibiting adenosine triphosphate (ATP) synthesis in the mitochondria [175]. Ferulic acid, instead, acts on the cell membrane, inducing significant changes in intracellular ATP concentrations, a decrease in the intracellular pH, cell membrane hyperpolarization, a reduction in cell membrane integrity, and ultimately evident morphological alterations. Gallic acid exhibits both antioxidants as well as pro-oxidant features, displaying a double-edged sword behavior, which turns it into an efficient apoptosis-inducing agent [158].

Quite regrettably, despite the powerful antimicrobial potential of these compounds, their poor delivery and bioavailability, coupled to the scarce stability, especially in the case of curcumin, do not allow them to reach the biological target at the bioactive concentration in plants.

6. Inhibition of Trichothecene Biosynthesis by Exogenous Phenolic Compounds

From a human health perspective, the main issue to consider in cereal protection is the capability of *Fusarium* to synthesize mycotoxins. Several phenolic compounds are able to modulate the production of mycotoxins in vitro in *Fusarium* species. However, their effect is highly variable depending on the class of mycotoxins, on the fungal species, the applied concentration as well as on the experimental conditions [153]. Some phenolics may even increase the biosynthesis of secondary metabolites in *Fusarium* spp.; therefore, it is essential to carefully consider each individual case: a partial inhibition of fungal growth is not

necessarily correlated with the impairment of mycotoxin biosynthesis since the fungistatic activity could trigger secondary metabolic routes as a response to stress [195].

Cinnamic acid derivatives, such as ferulic acids, caffeic, *p*-coumaric, chlorogenic, and sinapic acid, are all efficient inhibitors of trichothecene mycotoxins produced by *F. graminearum* and *F. culmorum* [63,154].

Increased concentrations of ferulic acid reduce substantially most analyzed mycotoxins [153,196]. Bily et al. [150] reported a 57% inhibition of trichothecene production by *F. graminearum* in media supplemented with 0.25 mM ferulic acid. Moreover, antioxidant phenolic acids (e.g., ferulic acid) proved highly inhibitory toward both type A and type B trichothecenes [153,154], thereby suggesting a link with the evidence that accumulation of ferulic acid is positively correlated to *Fusarium* resistance in wheat varieties [153]. Ferulic acid inhibited the in vitro production of 3-ADON by 16–30% in *F. graminearum* and *F. culmorum* when applied at 0.5–1.0 mM [161,166]. This compound was also found to exert a transcriptional control, reducing the expression of key biosynthetic genes, namely *TRI5*, *TRI6*, and *TRI12* [82,154,197]. In the course of other studies, ferulic acid proved a powerful phenolic acid with anti-mycotoxigenic effects against various *Fusarium* species, including *F. graminearum*, *F. verticillioides*, *F. poae*, *F. langsethiae*, and *F. sporotrichioides* [63,153,171,198]. This compound and its dimeric forms play a key role in cereal resistance to *F. graminearum* and to DON accumulation and may also contribute to improving resistance to the infection by *F. avenaceum* and the associated contamination with enniatins [62,109,150,170]. The presence of dimeric forms of ferulic acid (DFAs) in the wheat kernel pericarp is associated with *F. graminearum* and *F. culmorum* resistance [62,150]. The main forms of DFAs are 8-5′-diferulic acid benzofuran, 8-0-4′-diferulic acid, 8-5′-diferulic acid and 5,5′-diferulic acid. DFAs are produced by coupling reaction of ferulate monomers catalyzed by peroxidase during cell wall deposition, conferring hardness to pericarp and resistance to fungal penetration. Fungal esterases and other hydrolytic enzymes attack the plant and induce the release of free DFAs from the plant cell wall polysaccharides. High concentrations of free DFAs during the plant-fungus interaction contribute to the inhibition of trichothecene biosynthesis by *Fusarium* [62].

Caffeic acid showed an inhibitory effect toward trichothecene: when tested at 1.0 mM, it led to complete inhibition of 3-ADON without affecting the mycelial growth of *F. culmorum* [161]. Similarly, 0.5 mM caffeic acid decreased the synthesis of type B trichothecenes by *F. graminearum*, whereas no significant effect on mycelium development was observed [86]. The ability of these compounds to impair mycotoxin production with no significant effects on fungal growth may be particularly useful for achieving mycotoxin control without applying selection pressure on resistant mutant populations [199]. Nonetheless, despite much evidence on the inhibitory effect of both ferulic and caffeic acid on trichothecene production by *Fusarium*, Ponts et al. [59] and Etzerodt et al. [200] highlighted a stimulating effect of these compounds on trichothecene biosynthesis. This could be explained by differences in strains, culture medium, and in vitro conditions of the experiment, reflecting fluctuating contexts in the delivery and bioavailability of the exogenous molecule.

Sinapic acid displays both antioxidant and antibacterial effects and plays an intriguing role as a preservative in foods [201,202]. Furthermore, it has been proposed as a resistance biomarker metabolite in cereals against *Fusaria* [203]. Kulik and coworkers [164] tested different levels of sinapic acid on both *F. culmorum* and *F. graminearum* under in vitro conditions, finding that exogenous application of this compound decreases the production of trichothecenes by both species, leading to 73.2–97.7% reduction at 3.6 mM. The expression of *TRI4*, *TRI5,* and *TRI10* genes was inhibited by sinapic acid, whereas an increase in ergosterol biosynthesis was observed. Thus, sinapic acid may bear the potential for its ability to limit mycotoxin contamination in food and feed [164].

Eugenol is another phenylpropanoid compound extracted from different plants with antifungal bioactivity toward *Fusarium* spp. [204–206]. Tested in vitro at 1.0 mM, eugenol induced complete inhibition of 3-ADON with no effects on vegetative growth in *F. culmorum* [161].

Similarly, the natural acetophenone apocynin (0.5 mM) reduced DON production of *F. graminearum* by 90% [166] and significantly reduced 3-ADON in *F. culmorum* without affecting fungal growth [161]. Both eugenol and apocynin proved efficient inhibitors of trichothecene also in field tests, albeit their bioactivity was transient and limited to the first post-inoculation stages [207].

Several studies illustrated the effect of flavonoids on mycotoxin production. Brown et al. [208] observed the ability of flavones to inhibit trichothecene production through the modulation of cytochrome P-450 monooxygenase-catalyzing conversion of TDN. Takahashi-Ando et al. [209] revealed that TRI4 is the potential target site of flavone and furanocoumarin in the inhibition of trichothecene biosynthesis. Bollina and Kushalappa [210] showed that naringenin and quercetin (Figure 7) induced complete inhibition of trichothecene biosynthesis in *F. graminearum* at early stages of incubation in artificial media. Bilska et al. [211] tested various amounts of exogenous flavonoids on different strains of *F. graminearum* and *F. culmorum*. Most flavonoids reduce trichothecene biosynthesis, but their effect depends on the fungal strain, the flavonoid compound, and its concentration. Quercetin was the most efficient compound, leading to a significant reduction (78.2% to 99.8%) in the accumulation of trichothecene, and the inhibition occurred at the transcriptional level. These data also confirm the role of the antioxidant activity on trichothecene inhibition: in virtue of differences in the structural feature and polarity existing between quercetin and naringenin, quercetin exerts a protective effect against bulk lipid oxidation, whereas naringenin fails.

Figure 7. Structure of some phenols and flavonoids with inhibitory activity toward trichothecene biosynthesis.

The balance between lipophilicity and antioxidant activity can be a key factor in predicting the capacity of a phenolic compound to inhibit mycotoxin production. The ability of a compound to cross the fungal membrane lipids is mandatory to exert its antifungal/inhibitory activity. Fungal cultures are a peculiar system where both lipidic and emulsion systems coexist. In such a multicomponent environment, different physicochemical parameters, such as temperature, light, or pH, have a direct effect on lipophilicity and on the antioxidant capacity of phytochemicals. Therefore, correlating theoretical antioxidant potential and lipophilicity values with experimental data is far from being straightforward [161]. Nonetheless, the hypothesis that antioxidant properties of cereal metabolites can play a critical role in their anti-mycotoxigenic activity is consistent with the postulated activating effect of oxidative stress on the biosynthesis of mycotoxins [63]. Montibus et al. [212] emphasized the modulation of fungal secondary metabolism by oxidative stress

and the enhancement of mycotoxin production, including DON, after exposure to reactive oxygen species. Thus, due to their ability to quench oxygen free radicals, antioxidant metabolites may reduce or suppress upstream signals such as oxidative stress that modulate toxin biosynthesis.

The toxicity of phenolic acids can also be linked to their interaction with various intra- and extracellular fungal enzymes, including phenol oxidases and several hydrolytic activities [63,213,214]. Moreover, Passone et al. [215] mentioned that antioxidant compounds interfere with mycotoxin production, probably indirectly via their capacity to perturb the membrane function and modify its permeability.

7. Effect of the Combination of Phenolic Compounds with Other Natural Products or Conventional Fungicides

Phenolic compounds isolated from natural sources present valuable antifungal properties, but their efficacy as inhibitors of mycotoxins and fungal growth is often strain and molecule dependent [33,154]. The scarce stability and/or solubility of the compound may also play a putative role. A possible strategy to improve their bioactivity is to combine natural compounds with other phenolic acids or benzo analogs or with conventional fungicides, resulting in the enhancement of antifungal activity against fungi [216].

In clinical practice, the synergetic use of antifungals is becoming popular to avoid resistance and reduce the required dosage of specific drugs [217]. Different studies described the efficacy of this method in containing *Candida* spp., a major group of fungal pathogens in humans [218–221]. By following the same approach, Dzhavakhiya and coworkers [216] found that the activity of azole and strobilurin fungicides can be significantly enhanced through their co-application with certain natural products against several economically important plant pathogenic fungi: thymol emerged as a potent chemosensitizing agent when combined with azoxystrobin on *Bipolaris sorokiniana* Shoemaker, *Phoma glomerata* (Corda) Wollenw. and Hochapfel, *Alternaria* sp. and *Parastagonospora nodorum* (Berk.) Quaedvl., Verkley and Crous at a non-fungitoxic concentration [216]. In addition, difenoconazole applied in combination with thymol significantly enhanced antifungal activity against *B. sorokiniana* and *P. nodorum*, while tebuconazole combined with 4-hydroxybenzaldehyde (4-HBA), 2,3-dihydroxybenzaldehyde inhibited the growth of *F. culmorum* at a significantly higher level than the fungicide alone [216].

Also, the combination of phenolic molecules and other natural compounds with differing modes of action may improve the inhibitory efficacy, as they could act in synergism with a multitarget effect [222]. For instance, Siranidou et al. [223] reported a synergistic antifungal effect of *p*-coumaric with ferulic acid in reducing the mycelial growth of *F. culmorum*. An equimolar combination of propyl gallate and thymol tested at a final concentration of 0.25 mM proved a strong inhibitor of trichothecenes both in vitro and in plants [224]. Oufensou et al. [166] tested an equimolar solution of thymol and magnolol, which had an additive effect on *F. graminearum*, possibly due to the different mode of action of the two compounds, or/and to the ability of one compound of the mixture to cross the fungal membrane, thereby improving the delivery of the other compound. Accordingly, plant extracts including various phenolic compounds and terpenes were highlighted as promising antifungal agents, the efficacy of which was attributed to a potential synergistic effect of the different components [225,226]. Recently, Montibus et al. [226] investigated the effect of maritime pine sawdust, a by-product from the industry of wood transformation, which includes, among other bioactive molecules, 11 compounds belonging to three families of phenolics, namely phenolic acids, lignans, and flavonoids, on various strains of *F. graminearum*. Pine sawdust tested at 500 mg/L proved extremely efficient, leading to a total inhibition of trichothecene production, with no fungal biomass reduction, for five out of six strains of *F. graminearum* tested.

Several compounds have different behavior in vitro and in plants. In vitro, the fungus is closely in contact with the potential inhibitor, whereas in plants, the effect of the compound is weaker due to the need to reach fungal cells within the colonized plant

tissues. Lipophilicity and antioxidant activity of the inhibitor and composition of the carrier solution are key elements to magnify the effect of the potential inhibitor in plants. Phenolic compounds may be combined with essential oils to improve their bioavailability. The hydrophobicity of essential oils enables a better partition of phenolic compounds within the lipids of the cell membrane and mitochondria, thereby increasing their permeability and ultimately leading to the release of intracellular constituents [227,228] and to interference with many biological processes [229]. Wang et al. [230] showed that *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. exposed to clove oil exhibits morphological and ultrastructural alterations, confirming the disruption of the fungal cell wall and of the endomembrane system, increased permeability, and loss of intracellular constituents. Therefore, investigations on the essential oils as co-formulants open a new scenario in the antifungal strategy, even though the reproducibility and stability of the essential oil mixture represent two elements of weakness in this approach. Nevertheless, essential oils are gaining popularity as safe and effective antifungal agents, in combination with other naturally occurring phenol exhibiting a different mode of action. For instance, Ochoa-Velasco et al. [231] reported antifungal effects of carvacrol and thymol below their MIC values against *F. verticillioides* and *Rhizopus stolonifer* (Ehrenb.) Vuill.

The ability to increase aqueous solubility is definitely a valuable aid to resolve the solubility problems of hydrophobic compounds, especially if the bioactive compound should be applied on the canopy by spray method. In this case, a right compromise between lipophilicity of the compound and wettability and complexation ability of the delivery composition is of paramount importance for the efficiency of the inhibitor/fungicide.

8. Sustainable Formulations for Bioprospecting Phenolic Compounds

Formulation technology plays a crucial role in the efficacy of the potential phenolic inhibitors: without a proper formulation, even the most effective compound is worth nothing [232]. Nowadays, formulation technology offers a wide choice of molecules, polymers, and materials; nevertheless, the search for sustainable agrochemical formulations is still open and strict rules concerning the safety of humans, animals, and the environment must be taken into account [233,234]. Many bioprospecting phenolic compounds are not water soluble or water dispersible [171], and this is a major drawback since the most common mode of delivery of any active ingredient into a crop is via spray applications of an aqueous solution or treatment of seeds with aqueous emulsions. Although many efforts have been devoted to the search for phenolic compounds effective against *Fusarium* spp., the medium used to solubilize the compound often lacks in safety for eventual application in the field. Dimethyl sulfoxide, acetone, and ethanol are the most common solvents used in vitro to assess the effectiveness of phenols. Usually, the concentration of the compound in these solvents ranges between 0.05 and 0.5 mM, which represent the minimum concentrations that allow the solubilization of the compound by preventing toxic effects due to the solvent present in the aqueous solution (1–10%) [153].

Tween 20, a non-ionic surfactant, improved dispersion of a hydrophobic curcumin derivative in aqueous solution, thereby allowing the aspersion of the aqueous fungicidal solution onto phytopathogenic fungi [235]. Tween 20 is a polysorbate containing lauric acid and 20 repeat units of polyethylene glycol distributed across four different chains. A nano and micro-emulsion of thymol and Tween 20 was used in combination with sunflower oil favoring the dispersion of the phenol in wheat plants [236]: complete FHB inhibition was achieved at 0.5% thymol, but phytotoxic effects were observed [236]. Among commercial polysorbates, Tween 20 is allowed in feed at a maximum concentration of 5000 mg/kg without any safety concern, while Tween 80 is generally used in vitro assay at the concentration of 10% (v/v) [237].

Other biomatrices recovered from waste of industrial activity or produced on a large scale have been assayed as safe formulating agents: collagen, chitosan, starch, cyclodextrins (CDs), carboxymethylcellulose (CMC), polylactic acid, polyethylene glycol (PEG) [232]. Water-soluble microcapsules made of a blend of collagen hydrolysates, CMC, and thyme

oil were applied as a film on wheat seeds surfaces [238]: the authors investigated only the preparation of the microcapsule and their characterization in terms of water content, shelf-life stability, and release of the active ingredients.

CDs, cyclooligosaccharides obtained as by-products of starch degradation, are now produced by effective biotechnological processes in α-CD (six-cycloamylose units), β-CD (seven-cycloamylose units), and γ-CD (eight-cycloamylose units) [232] separately. Structurally, CDs are constituted by an amphiphilic torus with a hydrophobic interior cavity able to host lipophilic molecules. Due to a truncated conical shape and an external hydrophilic surface, CDs can form water-soluble inclusion complexes with lipophilic molecules or activate strong interactions with them, facilitating the delivery/solubilization of the molecule in aqueous solutions. Due to these properties and their non-toxicity, CDs have been largely used in medicine, food, and materials, and promising carriers in antifungal formulations were proposed [239]. The synergistic effect of CDs with phenol-based essential oils was observed against fungal pathogens [240,241]. Although phenolic molecules activate strong interactions with CDs, only few examples appeared in literature as emulsifier for in vitro antifungal assay [161,182] and as formulating agents in agriculture against *F. graminearum* and *F. culmorum* [207,242]. All the available examples are based on the use of 3 mM aqueous solution of β-CD as an emulsifier agent of phenols.

Efforts were devoted to the preparation of technologically advanced biomaterials where the fungicide is embedded in nanoparticles or hydrogels. These formulations improve the shelf-life of the fungicide, its delivery, and permeability through the fungal membrane, maintaining safety and health criteria. Among the naturally occurring biomatrices, nanosized chitosan particles and hydrogel chitosan-based are now considered suitable formulating agents [232].

Loron et al. [243] tested the starch octenylsuccinate (OSA-starch) and the chitosan as a matrix for the encapsulation of the curcumin derivative tetrahydrocurcumin (THC), demonstrating the antifungal and anti-mycotoxigenic properties of the encapsulated particles against *F. graminearum*. Both starch and chitosan spray-dried particles seemed to better protect THC and to extend its time of release, even though THC-loading aspects should be taken into account.

An ideal formulation should be inexpensive, environmentally sustainable, easy to distribute, and should present a shelf-life long enough for proper storage. Coating-forming agents may contribute to enhancing the properties of formulations. Although many promising bioformulations appeared in literature, unfortunately, no biomatrices have yet reached an advanced stage of development and commercialization to be applied in agriculture.

9. Structure-Activity Interactions

As previously mentioned, some phenolic compounds are reported as strong inhibitors of mycotoxin production without any effect on the fungal biomass [244]. Identifying molecules with specific molecular targets in the trichothecene biosynthesis pathway with no fungitoxic effects would be highly desirable to reduce the selective pressure on fungal populations, hence limiting the onset of resistant mutants. Given the fact that DON acts as a virulence factor, its inhibition may reduce the infection process and the development of the disease symptoms.

An early molecular docking study was carried out with the trichothecene 3-O-acetyltransferase TRI101 as the target protein. The ligand, a phenyl derivative of pyranocoumarin (PDP) extracted from *Psoralea corylifolia* seeds, showed a strong affinity toward the TRI101 by inhibiting the acetylation mechanism of the trichothecene and leading to the destruction of the "self-defense mechanism" of *Fusarium* sp. [245].

Pani et al. [182] investigated the mechanism of trichothecene inhibition by focusing on the binding mode of diverse naturally occurring phenols. Docking analyses were performed onto a 3D atomic-level protein model of the *F. culmorum* trichodiene synthase TRI5, based on the crystal structure of *F. sporotrichioides* TRI5 [246]. Docking analyses identified two sites (named site 1 and site 2) located on the surface of TRI5 *F. culmorum* as privileged sites for

phenol-based hydrophobic ligands inhibiting trichothecene biosynthesis in vitro. Phenols with a long aliphatic chain and in dimeric form (i.e., hydroxylated biphenyls) interact simultaneously with sites 1 and 2. Propyl gallate, ellagic acid, magnolol, eugenol, and the eugenol dimer bind preferentially to sites 1 and 2 and far from the catalytic domain. With few exceptions, no-charged phenols interact with the same set of amino acids identified as: Gln68, Thr69, Tyr76, Trp298, Leu300, Cys301, Asp302, Ala303, His308, Phe329, Ala333, Gly336, Ala337, Val338, Trp343.

Aiming to provide further insight into the understanding of structure-activity relationship, Maeda et al. [244] have identified NPD352 [testosterone 3-(O-carboxymethyl)oxime amide-bonded to phenylalanine methyl ester], a TDN inhibitor identified from a chemical library of the RIKEN Natural Product Depository by chemical array screening using a recombinant trichodiene synthase tagged with hexahistidine (rTRI5) as a target protein. Unfortunately, the high lipophilicity of NPD352, its high molecular weight, and the high cost of production do not permit the development of the compound for a straightforward application in agriculture. The author also highlighted that, by optimizing the steroid skeleton, so to minimize endocrine perturbation and by modifying the side chains of the aromatic amino acids for higher activity, effective natural-like inhibitors of trichothecene biosynthesis may be developed in the future [244].

Another computational study on TRI5 protein has been recently carried out by Oufensou et al. [224]. A set of 15 naturally occurring compounds belonging to cinnamic acids, gallic esters, terpenes, phenylpropanoids, and 1 phenylethanone was selected for docking onto TRI5. Based on this protein model, the binding capacity of the selected compounds and of NPD352 [244] with the TRI5-inorganic pyrophosphate model (TRI5-PPi) was studied by comparing the most populated sites with those evaluated when the same compounds were docked with TRI5 containing the substrate (i.e., farnesyl pyrophosphate (FPP)). The five sites previously identified by Pani et al. [182] in the TRI-PPi model were also confirmed for the tested phenolic compounds, thereby confirming sites 1 and 2 as the privileged ones. Notably, NPD352 interacted with the same sites and with the same set of amino acids, providing further proof of the reliability of the in silico TRI5 model.

Recently, several computational analyses have been introduced for the prediction of drug targets in *F. graminearum* [247,248]. However, not enough effort for discovering novel natural drugs has been reported because of the unavailability of the crystal structure of drug targets.

10. Conclusions and Future Trends

Fusarium mycotoxins are an important challenge in agriculture worldwide, particularly in the cereal and grain production sector. Control of *Fusarium* spp. is largely based on the use of fungicides bearing an azole unit that is used for both plant and human therapy, as they are generally inexpensive, share a broad spectrum of action and long stability. In recent years, large-scale abuse of azoles in agricultural settings has been blamed as a major driver of the increasing resistance phenomena that are also being reported in human pathogenic fungi [40,249]. These concerns will certainly lead to drastic restrictions in the use and availability of active ingredients to cope with phytopathogenic fungi. Under such circumstances, natural phenolic compounds are becoming increasingly attractive, as they prove potent antifungal agents, when applied singly or in combination, with less or no toxic effect and differing mechanisms of action. Phenolic compounds with low/moderate molecular weight are rapidly metabolized by the natural microflora, thereby providing an essential alternative to industrial agrochemicals, which are often detrimental to the environment. Most of these compounds are widely used as food additives, as they are commercially available at a reasonable price [250].

The antifungal and anti-mycotoxigenic activity of different cinnamic acids, acetophenones, benzaldehydes, benzoic acids, phenylpropanoids, or hydroxylated biphenyls, has been widely reported over the last decades. Yet, their efficacy appears dose and strain dependent, often leading to inconsistent results, especially when moving from the lab

to the field. Additional studies are required to highlight their in vivo activity, toxicity, and bioavailability through the design of sustainable formulations. This shall pave the way for the selection and identification of new fungal targets and possibly of new "anti-mycotoxin" molecules with no fungicidal effect, aiming to reduce the selection of resistant mutants [251].

The recent improvements in analytical platforms using integrated high-throughput technology, such as transcriptomics, proteomics, microbiomics, and metabolomics, providing multi-level omics data, may help to further identify relevant factors governing mycotoxin production and shall improve significantly our understanding of the mode of action of natural bioactive molecules to be used as new eco-friendly targets to mitigate the issue of food and feed contamination. A combination of ^{1}H NMR and LC-QTOF-MS analyses tools have been implemented by Atanasova-Pénichon et al. [86] to explore the interdependence between the biosynthetic pathway of DON and the central metabolism, comparing the exo- and endo-metabolomes of *F. graminearum* grown in different culture media amended with phenolic compounds as toxin-inducing or -repressing conditions. Metabolome alterations induced by DON-producing *Fusarium* have also been evidenced aiming to the characterization of key plant metabolites that may contribute to resistance to fusarioses or interfere with DON accumulation [109,252–254].

Despite our growing understanding, it is evident that further research will continue to more accurately define the food safety risks management associated with new eco-sustainable molecules with different mechanisms of action and to shed light on the factors contributing to the success of these versatile and interesting compounds as plant protectants. Given the importance of phenolics in industry, food safety, and human health, and the growing interest in understanding the regulation and expression of the fungal secondary metabolome, this field is likely to represent a fertile prairie for the next generation of researchers.

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Chapter 3

Summary
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Supplementary materials

Summary

The derivatization of phenolic compounds is documented as influencing their antifungal efficiency against *Fusarium*. This study investigated whether the functionalization of cinnamic acids through esterification and etherification can modulate their antifungal activity against a set of *Fusarium* species. Ten prenylated derivatives were obtained by introducing a prenylated chain on the hydroxy group of trans cinnamic acid, and these were then tested in *Fusarium* minimal media and for their LD_{50} and MIC. Of the tailored cinnamic acid derivatives tested in this study, esters were seen to be the most efficient derivatives and showed improved antifungal efficiency compared to the mother molecules. In particular, *p*-coumaric acid 3,3′-dimethyl allyl ester 13 had the greatest antifungal efficiency, with comparable efficacy to the synthetic fungicide terbinafine. The findings of this study not only offer insights into improving the bioactivity of cinnamic acid via modification of their structure, but also open up perspectives regarding the use of prenylated cinnamic acids in *Fusarium* management.

Article

Prenylated *Trans*-Cinnamic Esters and Ethers against Clinical *Fusarium* spp.: Repositioning of Natural Compounds in Antimicrobial Discovery

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Abstract: Onychomycosis is a common nail infection mainly caused by species belonging to the *F. oxysporum*, *F. solani*, and *F. fujikuroi* species complexes. The aim of this study was to evaluate the in vitro susceptibility of six representative strains of clinically relevant *Fusarium* spp. toward a set of natural-occurring hydroxycinnamic acids and their derivatives with the purpose to develop naturally occurring products in order to cope with emerging resistance phenomena. By introducing a prenylated chain at one of the hydroxy groups of *trans*-cinnamic acids **1–3**, ten prenylated derivatives (coded **4–13**) were preliminarily investigated in solid Fusarium minimal medium (FMM). Minimal inhibitory concentration (MIC) and lethal dose 50 (LD_{50}) values were then determined in liquid FMM for the most active selected antifungal *p*-coumaric acid 3,3′-dimethyl allyl ester **13**, in comparison with the conventional fungicides terbinafine (TRB) and amphotericin B (AmB), through the quantification of the fungal growth. Significant growth inhibition was observed for prenylated derivatives **4–13**, evidencing ester **13** as the most active. This compound presented MIC and LD_{50} values (62–250 μM and 7.8–125 μM, respectively) comparable to those determined for TRB and AmB in the majority of the tested pathogenic strains. The position and size of the prenylated chain and the presence of a free phenol OH group appear crucial for the antifungal activity. This work represents the first report on the activity of prenylated cinnamic esters and ethers against clinical *Fusarium* spp. and opens new avenues in the development of alternative antifungal compounds based on a drug repositioning strategy.

Keywords: onychomycosis; mycoses; *Fusarium* spp.; drug development; antifungal activity; phenolic inhibitors; hydroxycinnamic acid derivates; *p*-coumaric acid 3,3′-dimethyl allyl ester

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1. Introduction

Phenolics are among the most desirable food components because of their excellent antioxidant activity and nutraceutical properties. They also find wide-ranging application in medicine and agriculture in virtue of their antimicrobial, anti-inflammatory, and antitumoral activities [1,2]. Among the phenolic compounds, cinnamic acids are a group of aromatic carboxylic acids formed through the biochemical route of shikimate pathway, leading to the synthesis of lignin, the polymeric material that provides mechanical support to the plant cell wall [3,4]. *p*-Coumaric acid **1**, caffeic acid **2**, and ferulic acid **3** are the most common cinnamic acids, consisting of a *trans*-α, β-unsaturated carboxylic chain bonded to a phenol, catechol, and guaiacyl unit, respectively. They possess three distinctive

structural motifs that may possibly contribute to the free radical scavenging capability of these compounds (Figure 1).

Figure 1. Chemical structures of *p*-coumaric acid **1**, caffeic acid **2**, and ferulic acid **3**.

The presence of an electron donating group on the benzene rings provides the additional property of terminating free radical chain reaction. The carboxylic acid group with a conjugated C-C double bond provides additional quenching sites for free radicals. These features of cinnamic acids are reflected in many biological processes. Cinnamic acids, the main constituents of plant defense, prevent the effects of reactive oxygen species (ROS) formed during fungal infection [5]. The carboxylic acid group of cinnamic acid can act as an anchor by which the compound binds to the lipid bilayer, providing some protection against lipid peroxidation, a process spread out across mammal and human cells [6].

Cinnamic acids **1**–**3** are widely distributed in fruits (e.g., apple, pear, grape, orange, tomato, and berries), vegetables (e.g., bean, potato, and onion), and cereals (e.g., maize, oat, and wheat bran) [7]. They occupy a key place as intermediates in the synthesis of pharmaceuticals, dyes, flavorings, cosmetics, thermoplastics, and materials [8,9]. Because of their high-promoting health capacities and commercial value and given the availability of cinnamic acids **1**–**3** in different plants, extracting processes from biomass are also well studied even though the main accessibility of these compounds comes from synthetic or microbial processes [10,11].

Drug repositioning implies the identification of novel biological targets for natural-occurring compounds that may find application in fields where safety and efficiency are still lacking. One approach relies on slight structural modification of the natural compound, aiming to enhance its biological activity toward a specific target. When the structural modification of the compound concerns the introduction of a natural unit to the molecule framework, the final compound acquires a natural-like feature. Often, such slight structural modifications are devoted to improving bioavailability and selectivity of the parent compound [12–16].

Onychomycosis is a chronic fungal nail infection mainly caused by *Fusarium* spp., particularly those belonging to three specie complexes: *F. oxysporum* (FOSC), *F. solani* (FSSC), and *F. fujikuroi* species complex (FFSC) [17–21]. *F. oxysporum* Schlechtend. emend. Snyder & Hansen; *F. solani* (Mart.) Sacc.; *F. petroliphilum* (Q.T. Chen & X.H. Fu) Geiser, O'Donnell, D.P.G. Short, & N. Zhang; *F. keratoplasticum* Geiser, O'Donnell, D.P.G. Short, & Ning Zhang; *F. falciforme* (Carrión); and *F. verticillioides* (Saccardo) Nirenberg are the most representative species responsible for onychomycosis; moreover, they also infect skin and hair and are considered as emerging pathogens from superficial mycoses as dermatomycoses. *Fusarium* spp. are increasingly reported among the world population. Besides dermatomycoses or onycomycoses, they are responsible for disseminated infections, particularly in patients undergoing cancer therapy or those affected by immunological deficiency [22–24].

Systemic antifungals are the most effective treatment, with meta-analyses showing mycotic cure rates of 76% for terbinafine, 63% for itraconazole with pulse dosing, 59% for itraconazole with continuous dosing, and 48% for fluconazole [25,26]. The use of these agents is discouraged in patients suffering from liver, renal, or heart disease, and in those receiving medications with which there may be significant drug–drug interactions [27]. Terbinafine (TRB) belongs to the allylamine class of synthetic antimycotic agents, and inhibits the squalene epoxidase, a key enzyme involved in the early phase of the ergosterol biosynthetic pathway [28]. Amphotericin B (AmB) is a broad-spectrum antifungal agent belonging to the polyene class; its mechanism of action targets membrane function by

forming channels in the fungal cell membrane, hence allowing ions and organic compounds of the cytoplasm to escape [29].

Susceptibility to fungicides among different *Fusarium* spp. may vary greatly, and clinical *Fusaria* showing multiple resistance to most applied antifungal drugs are increasingly being reported [30–32]. Numerous factors have been cited to explain the lack of response to therapy, such as nonadherence to treatment, incorrect diagnosis, or advanced disease [25]. Additionally, antimycotic prophylaxis in high-risk patients may enhance selective pressure, which favors multidrug-resistant fungi, including *Fusaria* [33]. This urges a massive investment in the development of novel agents to treat emerging and resistant fungi [34].

Considering the antimicrobial activity of cinnamic acids **1**–**3** [35], their low toxicity, and large market availability, the aim of this work was to investigate these compounds against a selection of representative strains belonging to the *F. oxysporum* (FOSC), *F. solani* (FSSC), and *F. fujikuroi* (FFSC) species complexes; namely, *F. oxysporum*, *F. solani F. keratoplasticum*, and *F. verticillioides*.

Aiming to improve the bioavailability and to enhance antimicrobial activity, we transformed cinnamic acids **1**–**3** in esters and ethers using short, medium, and long prenylated chains as alcoholic unit. The efficiency of prenylated phenols in crossing bacterial and fungal membranes [36], as well as their role in exerting antimicrobial activity [37], are generally acknowledged. *O*-prenylated phenols are secondary metabolites of plants. Even though they have been considered for years as biosynthetic intermediates of the most widespread *C*-prenylated derivatives, recently, *O*-prenylated chains are assuming a key role in the bioactivity of molecules into which they are embedded [38,39]. We thus predicted that the preparation of prenylated ethers and prenylated esters of cinnamic acids **1**–**3** (Figure 2) could offer an alternative to the conventional antifungal drugs used against clinical *Fusaria*, enhancing the antimycotic effect of the parent acid.

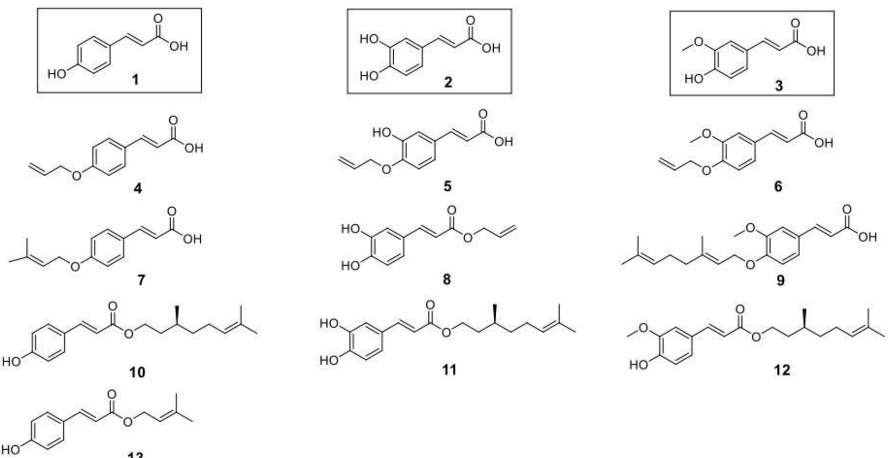

Figure 2. Chemical structure of the tested compounds numbered from **1** to **13**.

An in vitro assay of clinical isolates of *Fusarium* spp. grown on solid Fusarium minimal medium (FMM) amended with compounds **1**–**13** was carried out and a structure–activity relationship was described. Minimal inhibitory concentration (MIC) and lethal dose 50 (LD_{50}) values were then determined in liquid FMM for the most active selected antifungal compound, in comparison with the conventional fungicides terbinafine (TRB) and amphotericin B (AmB). The presence of a *O*-prenylated chain in natural occurring cinnamic acids

may share a different mode of action compared to conventional antifungal drugs and offers a successful example of drug repositioning.

2. Results

2.1. Chemistry

Prenylated esters **4–7** and **9** and prenylated ethers **10–13** were prepared, starting from the corresponding cinnamic acid **1–3** and, according to the reaction, allyl, 3,3′-dimethyl allyl (prenyl), geranyl, and citronellyl bromide or alcohol. Because of the contemporary presence of two hydroxyl groups in the starting hydroxy cinnamic acid, different synthetic approaches were applied when ether or ester was the final product.

In order to functionalize selectively the phenolic-OH group with a prenylated chain, esterification of the carboxylic group was mandatory (Scheme 1).

Scheme 1. Synthesis of hydroxyl cinnamic ethers **4–7** and **9** and esters **10–13** and **14–16**.

Ethers **4–7** and **9** were obtained by the Williamson reaction, starting from the corresponding methyl ester and the appropriate alkenyl bromide under basic conditions and further ester hydrolysis. Yields varied in the range of 53 and 85%. Methyl esterification of acids **1–3** was carried out under acid conditions under microwave treatment.

For compound 5, the selectivity of etherification reaction at the *p*-phenolic-OH was confirmed by Nuclear Overhauser Effect Spectroscopy-NMR (NOESY) experiment and by comparison of NMR spectra with the corresponding methyl ester reported in the literature (see Materials and Methods for references).

Esters **10–13** were prepared with yields ranging between 37 and 47%, starting from the corresponding parent acid (i.e., 4-hydroxy cinnamic acid **1**, caffeic acid **2**, and ferulic acid **3**) via activation of the carboxylic group with ethyl chloroformate and triethylamine and further addition of the appropriate prenylated alcohol (Scheme 1) and hydrolysis of the phenyl ethyl carbonate.

Compound **8** was obtained via Fischer esterification of acid **2** using allyl alcohol as solvent under acidic conditions as reported in the literature. Compound **13** was also obtained in 87% yield by enzymatic transesterification of the corresponding ethyl ester, in turn achieved by the microwave method, as well as 3,3′-dimethyl allyl alcohol, in mild conditions.

The purity of all new compounds was judged to be >98% by ^{1}H-NMR spectral determination.

The remarkable different lipophilicity estimated, as the value of the logarithm of the partition coefficient of compounds **1–13** for *n*-octanol/water (LogP), allowed us to evaluate the possible influence of this property in the antifungal activity of each compound (Table 1).

Table 1. Values of the logarithm of LogP of compounds **1–13** for *n*-octanol/water, estimated by ChemBioDraw Ultra 13.

Compound	LogP	Compounds	LogP	Compounds	LogP
1	1.15	**6**	2.37	**11**	4.51
2	1.42	**7**	3.04	**12**	4.78
3	1.54	**8**	2.11	**13**	3.04
4	2.50	**9**	4.44		
5	2.11	**10**	4.90		

As expected, in all compounds studied, the lipophilicity increases as the number of carbon atoms in the prenylated chain increases. Among the three natural occurring acids, the lipophilicity changes in the following order: ferulic acid **3** > caffeic acid **2** > *p*-coumaric acid **1**. The trend is also retained both in the series of geranyl esters **10** > **12** > **11** and in the series of allyl ethers **4** > **6** > **5**. No difference in LogP value resulted in caffeic acid derivatives when the *p*-phenolic-OH or the carboxylic functionalities were protected by an allyl group (i.e., compounds **5** and **8**) or by a 3,3′-dimethyl allyl chain (i.e., compounds **7** and **13**).

2.2. *Antifungal Activity of the Parent Compounds* **1–3** *and Derivatives*

The three naturally occurring hydroxycinnamic acids **1–3** and their ethers and esters derivatives **4–13** (Figure 2) were used in a preliminary in vitro screening to compare their antifungal activity against six *Fusarium* spp. clinical isolates associated to onychomycosis in a hospital in Milan (Italy) (Table 2).

Table 2. List of *Fusarium* spp. isolates tested in this study.

Species/Species Complex/Sequence Type (ST)	NRRL n.[a]	PVS-Fu n.[b]	Diagnosis	Isolate Source	Date
F. oxysporum/FOSC/ST33	46603	89	Onychomycosis	Toe	2004
F. oxysporum/FOSC/ST33	46606	91	Onychomycosis	Toe	2005
F. keratoplasticum/FSSC/ST2bb	46443	93	Dermatomycoses	Foot	2004
F. solani/FSSC/ST5aa	44903	96	Onychomycosis	Toe	2006
F. verticillioides/FFSC	46599	87	Onychomycosis	Toe	2007
F. verticillioides/FFSC	46442	115	Onychomycosis	Toe	2005

[a] NRRL n. Collection number of Agricultural Research Service (ARS); [b] PVS-Fu n. Collection number of Dipartimento di Agraria, Sezione Patologia Vegetale ed Entomologia, Sassari, Italy.

In the first screening, carried out on solid FMM, exposure to cinnamic acids **1–3** did not determine any significant reduction of mycelium fungal growth compared with the untreated control, with the exception of caffeic acid **2**, which induced a slight inhibition on *F. oxysporum* 89 colony diameter (Figure S1).

In the case of FOSC and FFSC, we noted a significant inhibition of vegetative growth upon exposure to all the compounds derived from cinnamic acids **1–3** compared with the untreated control, except for compound **5**, which did not induce any significant reduction of colony diameter in the two FOSC isolates. FOSC isolates were particularly sensitive (>53% and >56% of inhibition for *F. oxysporum* 89 and *F. oxysporum* 91, respectively) to compounds **4**, **7**, **8**, **9**, and **13**, whereas in the case of FFSC, the most effective compounds were **5**, **8**, **9**, **10**, **11**, and **13**. In both species complexes, compound **13** was by far the most effective inhibitor of fungal growth, leading to complete inhibition of the two *F. verticillioides* isolates (Figures S1–S3).

The two representative isolates of the FSSC displayed a different level of sensitivity to compounds **4–13**: compounds **4**, **7**, **10**, **11**, and **13** determined >25% inhibition of radial growth on *F. keratoplasticum* 93, whose vegetative growth on solid FMM was completely inhibited by compound **13**, whereas *F. solani* 96 was only partially inhibited by compounds **10**, **11** (36–30% inhibition, respectively), and **13** (63% inhibition; Figure S2).

These preliminary data demonstrate unequivocally that compound **13** has the highest antifungal activity towards all *Fusarium* strains investigated (Figures S1–S3). Citronellyl *p*-coumaric ester **10** was the second most effective inhibitor towards FSSC and FFSC strains, while FOSC representative isolates proved more sensitive to compound **8**. Compound **9** was more effective on FOSC and FFSC representatives compared with FSSC ones. The inhibitory activity of compound **9** towards FOSC isolates was comparable to that displayed by the ethers **4** and **7**, presenting a shorter prenylated chain. Overall, the ester functionality in the cinnamic acid derivatives appears more efficient in conferring inhibitory properties to the tested compounds compared with the ether one.

2.3. Determination of the Minimal Inhibitory Concentration (MIC) and Lethal Dose 50 (LD_{50}) for p-Coumaric acid 3,3′-Dimethyl Allyl Ester 13

In a further screening performed on FMM liquid medium, the minimal inhibitory concentration (MIC) and the lethal dose 50 (LD_{50}) of compound **13** and of two conventional fungicides were determined (Table 3). *p*-Coumaric acid 3,3′-dimethyl allyl ester **13** confirmed its remarkable good antifungal activity already shown in solid FMM compared with TRB and AmB, with an MIC range comprised between 125 and 250 µM in FOSC (Figures S4 and S5), 62 and 125 µM in FSSC (Figures S6 and S7), and 125 and 500 µM in FFSC (Figures S8 and S9) representative isolates. With respect to the LD_{50}, the most effective compound was TRB (LD_{50} ranging from 2.0 to 64 µM), followed by AmB (LD_{50} ranging from 1.0 to 67.5 µM) and ester **13** (LD_{50} ranging from <7.8 to 125 µM), respectively, for almost all strains. In the case of *F. verticillioides* 115, which was less sensitive to AmB (LD_{50} 8.40–16.8 µM) than to TRB (LD_{50} 2.0–4.0 µM) and **13**, the latter was able to reduce 50% of the fungal growth at a concentration <7.8 µM (Table 3, Figure S9).

Table 3. In vitro susceptibility of strains belonging to the three *Fusarium* species complexes, isolated from onychomycosis and dermatomycosis against the selected compound *p*-coumaric acid 3,3′-dimethyl allyl ester **13** and the two conventional drugs TRB [b] and AmB [c].

Species/Species Complex/Sequence Type (ST)	[a] PVS-Fu n.	Ester 13		[b] TRB		[c] AmB	
		[d] MIC (µM)	[e] LD_{50} (µM)	MIC (µM)	LD_{50} (µM)	MIC (µM)	LD_{50} (µM)
F. oxysporum/FOSC/(ST33)	89	>125–250	31–62	>256	8–16	>135	16.8–33.7
F. oxysporum/FOSC/(ST33)	91	>125–250	62–125	>256	16–64	>135	33.7–67.5
F. keratoplasticum/FSSC/(ST2bb)	93	62	<7.8	128–256	2.0–4.0	33.7–67.5	>2.1–4.2
F. solani/FSSC/(ST5aa)	96	62–125	<7.8	64–128	2.0–4.0	4.2–2.1	1.0
F. verticillioides/FFSC	87	500	62–125	>256	2.0–4.0	>135	2.1–4.2
F. verticillioides/FFSC	115	125–250	<7.8	>256	2.0–4.0	>135	8.4–16.8

[a] PVS-Fu n. Collection number of Dipartimento di Agraria, Sezione Patologia Vegetale ed Entomologia, Sassari, Italy; [b] TRB: terbinafine; [c] AmB: amphotericin B; [d] minimal inhibitory concentration (MIC) range determined visually according to mOD absorbance values (nm) detected after 48 h of incubation at 25 °C; [e] lethal dose 50 (LD_{50}) range determined visually according to mOD absorbance values (nm) detected after 48 h of incubation at 25 °C.

The effects of compounds **13**, TRB, and AmB applied at the same concentration as the MIC determined in liquid FMM on the morphology of representative isolates of *F. solani*, *F. keratoplasticum*, *F. oxysporum*, and *F. verticillioides* were examined by optical microscopy after 72 h (Table 4). While in the untreated controls, a regular morphology of the mycelium with abundant presence of microconidia as well as normal spore germination were observed, in the presence of 250 µM of compound **13**, degradation of the hyphae and vacuolisation of the cytoplasm were evident, along with an alteration of the rarely formed conidia, showing a compromised germination. The release of cell constituents was also noticeable, which can explain the progressive increase in the absorbance signal over time in liquid FMM (Figures S4–S9).

After 72 h of exposure to 256 µM TRB, a marked reduction on mycelium formation was observed, and fungal hyphae and spores showed a distorted morphology with tortuous growth (Table 4). This concentration of TRB could not totally inhibit the mycelium growth or the spore production but caused an evident swelling of conidia. The effects of TRB were

more noticeable compared with those induced by AmB at the MIC of 135 µM, causing only some mycelial distortion and fewer microconidia compared with the control (Table 4).

Table 4. Optical microscopy (100×) images of the fungal growth of *Fusarium* spp. after 72 h on liquid Fusarium minimal medium (FMM) amended with 250, 256, or 135 µM of *p*-coumaric acid 3,3′-dimethyl allyl ester **13**, terbinafine (TRB), or amphotericin B (AmB), respectively, in comparison with the untreated control.

Species	Control 0 (µM)	Ester 13 MIC (250 µM)	TRB MIC (256 µM)	AmB MIC (135 µM)
F. keratoplasticum				
F. solani				
F. oxysporum				
F. verticillioides				

3. Discussion

By introduction of a short, medium, or long prenylated chain at one of the hydroxyl functionalities of *trans*-cinnamic acids **1–3**, a series of prenylated cinnamic derivatives **4–13** was prepared to test the hypothesis that differences in the chemical and physical proprieties would influence the antifungal activity of the compounds against clinical *Fusarium* spp. The presence of a conjugated double bond confers particular conformational and electronic characteristic to these compounds strongly influenced by the phenol-OH group in para position. In order to observe the effect of the hydroxylated aromatic ring on the fungicide activity, a set of cinnamic esters **10–12** was prepared by functionalisation of the corresponding carboxylic acid with citronellol, while a set of allyl cinnamic ethers **4–6** was prepared with the aim to evaluate the influence of the phenolic-OH group in para position to the alpha, beta-unsaturated carboxylic chain. No significant differences were observed in the synthesis and yields of each set of compounds with different aromatic rings, whereas lower yields in esters in comparison with ethers were achieved, evidencing the higher reactivity of the phenolic-OH group.

The Food and Drug Administration (FDA) considers *trans*-cinnamic acids **1–3** as “generally recognized as safe” (GRAS), enabling their use in the field of food additives [40]. Compounds **1–3** are commercially available at an affordable price and can been obtained by direct extraction from plants biomass where the compounds are the main components [41] or by chemical and biotechnological processes [42]. Besides cinnamic acids **1–3**, prenylated cinnamic ester **13** and ethers **7** and **9** are plant components, whose extracts were studied for their remarkable biological properties [43]. In particular, compounds **9** and **13** are present in propolis, a source of valuable compounds with antioxidant and antimicrobial activity [44]. Compound **9** is not toxic to human cells and presents antitumoral and anti-inflammatory activities, in addition to acting as an inhibitor of biofilm formation by oral pathogenic bacteria [45]. While 4′-geranyloxy ferulic acid **9** is generally extracted from citrus fruit, quinoa seeds, and several vegetable oils, compound **13** was only detected in propolis extract. Propolis, produced by honeybees, is a very complex mixture composed of 50% resin, 30% wax, 10% essential oil, and 5% of polyphenols as flavonoids, terpenes, fatty acids, stilbenes, β-steroids, cinnamic acids, and their prenylated derivatives [46,47]. Change in chemical composition of propolis is frequently observed [44]. A few studies have been conducted on the antifungal and antibiofilm activity of propolis against onychomycosis caused by *Fusarium* spp. [48,49], but no studies aimed to identify the active component of the propolis extract against these fungi.

In a previous article reporting the activity of natural phenols against clinical *Fusarium* spp., we observed that the percentage of growth inhibition measured in liquid medium (Vogel’s) and solid (PDA) was comparable [50]. To achieve full solubilisation of compounds **1–13** at 0.5 mM, the preliminary screening was carried out with a sustainable solid medium based on gellan/water, where each compound was solubilized in a 0.1% water/gellan solution. In the preliminary in vitro screening of compounds **1–13** against *Fusarium* spp., cinnamic acids **1–3** were generally ineffective, whereas significant growth inhibition was achieved by prenylated derivatives **4–13**, evidencing ester **13** as the most active. Among the *Fusarium* spp. investigated, *F. solani* was the most resistant to compounds **1–13**, whereas *F. verticillioides* was the most sensitive, in accordance with data present in the literature for these species. In fact, *Fusarium* spp. are increasingly reported as resistant to many antifungal compounds in vitro; among them, *F. solani* is considered as the most resistant taxon, albeit some reports pointed out that the resistance may be species- and even isolate-dependent [51].

The antifungal activity of compounds **1–13** may be explained by the key role played by some moieties of their structure. The prenylated chain present in compounds **4–13** has the ability to penetrate and to accumulate within the fungal cell membrane, resulting, according to the size, in the disruption of its integrity as generally acknowledged for prenylated phenols [36–38]. The position and size of the prenylated chain in the studied compounds appear crucial for their antifungal activity. Although we did not perform a

proper bioavailability assay, we observed a detrimental effect on the fungal membrane when treated with compound **13** (Table 4).

Esters were more active than ethers as inhibitors of all tested *Fusarium* spp. Ester **13** was definitely more active than the corresponding ether **7** containing the same 3,3′-dimethyl allyl moiety, even though an identical lipophilicity was measured for both compounds (LogP 3.04). In the esters series, the alcoholic unit represented by a 3,3′-dimethyl allyl chain (compound **13**) was more active than the citronellyl one (compound **10**), a substituent that significantly increases the lipophilicity of the molecule (LogP 4.9).

We suppose that different prenylated chains may change the bioavailability of the compound influencing the hydrolytic degradation of the prenylated esters within the fungal cell. In fact, hydrolytic degradation, mediated by fungal enzymes, of the esters in the parent cinnamic acid and the corresponding alcohol cannot be ruled out. In esters **11** and **10**, a too long prenylated chain could be partially metabolized by the fungus at the first stages of contact, whereas in ester **13**, the hydrolysis would take a longer time, allowing it to reach sensitive compartments of the fungal cell where the prenyl alcohol may exert its antifungal activity. A similar effect has been reported by farnesol on *F. keratoplasticum*, which is associated with biofilm formation in hospital water systems and internal pipelines: this prenylated alcohol has a remarkable anti-biofilm activity; causes the destruction of hyphae and of the extracellular matrix; and prevents the adhesion of conidia, filamentation, and the formation of biofilm [52].

Compounds **4**–**13** contain an α,β-unsaturated Michael acceptor pharmacophore effective in interacting with nucleophiles present in the fungal cell; nevertheless, this feature is not exhaustive for the antifungal activity. The presence of a free phenol-OH in para position would play a key role in the radical scavenging and stabilisation of the radical by electronic delocalisation along the structure. In general, we observed that compounds with a catechol and guaiacyl ring favouring an intramolecular hydrogen bond and hampering the availability of the H donor to scavenge radicals were less active as antimycotic (compounds **5**, **8**, and **12**).

The antifungal activity of compound **13** was compared with that of TRB and AmB, two of the most effective conventional fungicides for clinical use [53]. TRB and AmB were applied at clinical dosage ranging between 2–256 μM and 1–135 μM, respectively, whereas compounds **13** was amended at concentrations comprised between 7.8 and 500 μM. Both TRB and AmB interact at the level of fungal cell membrane, the first one by inhibiting squalene epoxidase, a key step along the ergosterol biosynthesis pathway, and the second one by a complex interaction with phospholipid bilayers [54]. Our results demonstrate that compound **13** presents MIC and LD_{50} values against *F. verticillioides* 115 and *F. oxysporum* 89 that are consistent with those reported for AmB. Similarly, while AmB was indeed the most effective compound in terms of MIC and LD_{50} against *F. keratoplasticum* 93 and *F. solani* 96, the antifungal efficacy of compound **13** against these members of the FSSC was comparable to that of terbinafine. Besides its remarkable biological activity, ester **13** presents some attractive advantages; that is, it is as natural compound with a simple structure, a straightforward synthesis, low production cost with easy recovery of the starting materials. Considering the increasing frequency of multi-drug resistance patterns in opportunistic *Fusarium* spp. [55], the development of compound **13** as an effective antifungal drug represents a valuable alternative to the conventional therapeutic agents in onychomycosis treatment.

The results of this study provide useful insights to the optimal design of the structure of cinnamic esters with improved antifungal properties. Although cinnamic acids and their derivatives have been studied on some plant pathogenic fungi [56], to the best of our knowledge, no investigation was conducted on prenylated cinnamic esters and ethers on clinical *Fusarium* spp., thereby offering an intriguing opportunity in drug repositioning strategy.

4. Materials and Methods

4.1. Chemical Synthesis

4.1.1. General

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without further purification. Melting points were determined on a Büchi 530 apparatus and are uncorrected. All ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ (if not otherwise indicated) solution at 399.94 MHz and 75.42 MHz, respectively, with a Varian VXR 5000 spectrometer (Varian, Palo Alto, CA USA). Chemical shifts are given in ppm (δ); multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or dd (doublet of doublets). Elemental analyses were performed using an elemental analyser model 240 C (Perkin-Elmer, Walthan, MA USA). Acetone was freshly distilled from $CaCl_2$. Flash chromatography was carried out with silica gel 60 (230–400 mesh; VWR; Radnor, AF, USA) eluting with appropriate solution in the stated v:v proportions. Analytical thin-layer chromatography (TLC) was performed with 0.25 mm thick silica gel plates (Sigma Aldrich, Munich, Germany). All reactions were monitored by TLC performed on 0.2 mm thick silica gel plates (60 F254 Merck). Microwave reactions were carried out on a MW instrument (CEM-Discover SP MW, Matthews, NC, USA). Melting points were determined on a 530 apparatus (Büchi, Flawil, Switzerland) and are uncorrected. The purity of all new compounds was judged to be >98% by ^{1}H-NMR spectral determination. Lipase from *Candida antarctica* (Novozym 435 CAL-B) is immobilized on a macroporous polyacrylic resin beads (recombinant, expressed in *Aspergillus niger*, activity ≥ 5000 PLU/g (propyl laurate units/g) and purchased from Merck (Milan, Italy). Compound **8** was prepared according to the literature [57].

Lipophilicity of the compounds was estimated using the logarithm of the partition coefficient for *n*-octanol/water (log P), which was calculated using 403 ChemBioDraw Ultra 13.0.

4.1.2. General Procedure for the Synthesis of Compounds **10–13**

Ethyl chloroformate (2 eq for **10**, **12**, and **13** or 3 eq for **11**) and triethylamine (2 eq for **10**, **12**, and **13** or 3 eq for **11**) were added to a suspension of appropriate cinnamic acid (1 eq) in dichloromethane (10 mL) and stirred for 1 h at −30 °C until all of the starting material disappeared, as determined by TLC. Appropriate alcohol (1 eq) and 4-dimethylaminopyridine (0.2 eq) were then added, and the mixture was stirred at room temperature for 6 h. The reaction mixture was acidified with hydrochloridric acid (10% solution) and extracted with dichloromethane (3 × 50 mL), and the organic phases were combined and dried over anhydrous sodium sulphate. The product was then concentrated under reduced pressure and filtered on a pad of silica gel using dichloromethane as eluent to give a yellow oil. The oil was diluted in dichloromethane (15 mL) and piperidine (30 eq) was added at 0 °C. The reaction mixture was stirred at room temperature for 3 h, acidified with hydrochloridric acid (10% solution), and extracted with dichloromethane (3 × 50 mL), and the organic phases were combined and dried over anhydrous sodium sulphate. The crude product was concentrated under reduced pressure and purified by flash chromatography using a 1:1 mixture of petroleum ether/ethyl acetate as eluent to give the pure ester.

(E)-3,7-Dimethyloct-6-en-1-yl 3-(4-hydroxyphenyl)acrylate **10**: oil; 44%; $[\alpha]_D^{20}$ 0.5 (c = 0.9, $CHCl_3$); ^{1}H-NMR δ 0.94 (d, *J* = 6.4 Hz, 3H), 1.22 (m, 1H), 1.38 (m, 1H), 1.51 (m, 1H), 1.60 (m, 1H), 1.61 (s, 3H), 1.67 (s, 3H), 1.76 (m, 1H), 1.99 (m, 2H), 4.26 (m, 2H), 5.11 (m, 1H), 6.28 (d, *J* = 16.0 Hz, 1H), 6.81 (m, Ar, 2H), 7.42 (m, Ar, 2H), 7.63 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 17.66, 19.43, 25.40, 25.73, 29.54, 35.48, 36.99, 63.40, 114.97, 115.99, 124.57, 126.63, 130.07, 131.37, 145.12, 158.51, 168.44; Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67; Found: C, 75.44; H, 8.60.

(E)-3,7-Dimethyloct-6-en-1-yl 3-(3,4-dihydrophenyl)acrylate **11**: brown solid; 47%; mp 100–101 °C; $[\alpha]_D^{20}$ 2.9 (c = 0.4, $CHCl_3$); ^{1}H-NMR δ 0.93 (d, *J* = 6.4 Hz, 3H), 1.21 (m, 1H), 1.36 (m, 1H), 1.52 (m, 1H), 1.59 (m, 1H), 1.60 (s, 3H), 1.67 (s, 3H), 1.74 (m, 1H), 1.99 (m, 2H),

4.30 (m, 2H), 5.09 (m, 1H), 6.28 (d, *J* = 15.6 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, Ar, 1H), 6.99 (dd, *J* = 2.1, 8.4 Hz, Ar, 1H), 7.12 (d, *J* = 2.1 Hz, Ar, 1H), 7.59 (d, *J* = 15.6 Hz, 1H); ^{13}C-NMR δ 17.66, 19.42, 25.37, 25.72, 29.51, 35.42, 36.97, 63.61, 114.44, 115.14, 115.46, 122.46, 124.52, 127.17, 131.42, 144.03, 145.56, 146.78, 168.69; Anal. Calcd. for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23; Found: C, 71.60; H, 8.26.

(E)-3,7-Dimethyloct-6-en-1-yl 3-(4-hydroxy-3-methoxyphenyl)acrylate **12**: oil; 37%; $[\alpha]_D^{20}$ 0.6 (c = 0.4, $CHCl_3$); ^{1}H-NMR δ 0.93 (d, *J* = 6.8 Hz, 3H), 1.22 (m, 1H), 1.37 (m, 1H), 1.52 (m, 1H), 1.60 (m, 1H), 1.61 (s, 3H), 1.67 (s, 3H), 1.73 (m, 1H), 2.01 (m, 2H), 3.99 (s, 3H), 4.23 (m, 2H), 5.09 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H), 6.89 (d, *J* = 8.4 Hz, Ar, 1H), 7.02 (d, *J* = 2.0 Hz, Ar, 1H), 7.06 (dd, *J* = 2.0, 8.4 Hz, Ar, 1H), 7.59 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 17.64, 19.44, 25.39, 25.71, 29.54, 35.55, 37.01, 55.90, 62.94, 109.32, 114.74, 115.61, 123.03, 124.59, 127.01, 131.31, 144.67, 146.78, 147.30, 168.41; Anal. Calcd. for $C_{20}H_{28}O_4$: C, 72.26; H, 8.49; Found: C, 72.34; H, 8.40.

(E)-3-Methylbut-2-en-1-yl 3-(4-hydroxyphenyl) acrylate **13**: oil; 45%; ^{1}H-NMR δ 1.73 (s, 3H), 1.77 (s, 3H), 4.71 (d, *J* = 7.2 Hz, 2H), 5.41 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H), 6.87 (m, Ar, 2H), 7.38 (m, Ar, 2H), 7.64 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 18.08, 25.82, 61.73, 114.91, 115.99, 118.42, 126.69, 130.07, 139.50, 145.19, 158.42, 168.36; Anal. Calcd. for $C_{14}H_{16}O_3$: C, 72.39; H, 6.94; Found: C, 72.45; H, 6.96.

4.1.3. General Procedure for the Synthesis of Compounds **14**–**17**

In a 30 mL glass pressure microwave tube, equipped with a magnetic stirrer bar, a few drops of concentrated sulphuric acid were added to a solution of hydroxycinnamic acid (*p*-coumaric acid or caffeic acid or ferulic acid) (1 eq) in methanol (for **14**–**16**) or ethanol (for **17**) (10 mL). The mixture was subjected to microwave irradiation (power: 150 W; temperature: 80 °C for **14**–**16** and 98 °C for **17**) for 15 min, basified with aqueous sodium bicarbonate (5% solution), and extracted with dichloromethane (3 × 5 mL). The collected organic phases were dried over anhydrous sodium sulphate and evaporated to dryness to give the pure ester.

(E)-Methyl 3-(4-hydroxyphenyl)acrylate **14**: white solid; 82%; mp 125–127 °C ([58] 132–134 °C); ^{1}H-NMR δ 3.79 (s, 3H), 5.37 (bs, 1H), 6.28 (d, *J* = 16.0 Hz, 1H), 6.85 (m, Ar, 2H), 7.42 (m, Ar, 2H), 7.63 (d, *J* = 16.0, 1H); ^{13}C-NMR δ 51.75, 114.52, 115.91, 125.98, 129.89, 144.79, 158.12, 167.65. Anal. Calcd. for $C_{10}H_{10}O_3$ C, 67.41; H, 5.66; Found: C, 67.53; H, 5.56.

(E)-Methyl 3-(3,4-dihydroxyphenyl)acrylate **15**: brown solid; 88%; mp 155–156 °C ([59] 160 °C); ^{1}H-NMR δ 3.80 (s, 3H), 6.25 (d, *J* = 16.0 Hz, 1H), 6.87 (d, *J* = 8.4 Hz, Ar, 1H), 7.01 (dd, *J* = 2.0, 8.4 Hz, Ar, 1H), 7.07 (d, *J* = 2.0 Hz, Ar, 1H), 7.58 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 51.78, 114.33, 115.27, 115.49, 122.46, 127.52, 143.74, 145.03, 146.28, 168.18. Anal. Calcd. for $C_{10}H_{10}O_4$; C, 61.85; H, 5.19; Found: C, 62.05; H, 5.78.

(E)-Methyl 3-(4-hydroxy-3-methoxyphenyl)acrylate **16**: brown solid; 92%; mp 62–64 °C ([60] 65 °C); ^{1}H-NMR δ 3.75 (s, 3H), 3.83 (s, 3H), 6.14 (bs, 1H), 6.25 (d, *J* = 16.0 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, Ar, 1H), 6.96 (d, *J* = 2.0 Hz, Ar, 1H), 6.96 (dd, *J* = 2.0, 8.0 Hz, Ar, 1H), 7.58 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 51.64, 55.84, 109.52, 114.72, 114.81, 122.98, 126.78, 145.12, 146.92, 148.12, 167.92. Anal. Calcd. for $C_{11}H_{12}O_4$ C, 63.45; H, 5.81; Found: C, 63.51; H, 5.76.

(E)-Ethyl 3-(4-hydroxyphenyl)acrylate **17**: brown solid; 87%; mp 70–72 °C ([61] 73–74 °C); ^{1}H-NMR δ 1.32 (t, *J* = 7.1 Hz, 3H), 4.26 (q, *J* = 7.1 Hz, 2H), 6.39 (d, *J* = 16.0 Hz, 1H), 7.35 (m, Ar, 2H), 7.47 (m, Ar, 2H), 7.58 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 14.43, 60.74, 119.14, 124.58, 129.54, 132.22, 133.52, 143.29, 166.78. Anal. Calcd. for $C_{11}H_{12}O_3$: C, 68.74; H, 6.29; Found: C, 68.81; H, 6.34.

4.1.4. Procedure for the Synthesis of Compound **13** with Lipase

To a solution of **17** (1 eq) in cyclohexane (2.5 mL), 3,3-dimethylallyl alcohol (2 eq) was added. The reaction mixture was stirred at 60 °C for 15 min at a speed of 300 rpm. The reaction was initiated by adding a known fixed quantity of lipase (100 mg). The progress of the reaction was monitored by TLC using a 1:1 mixture of petroleum ether/ethyl acetate as eluent. After three days, the starting material was still present and another aliquot of

lipase (100 mg) was added, and the mixture was left stirring at 60 °C for two additional days. The reaction mixture was filtered over Buchner funnel, solvent concentrated under reduced pressure, and purified by flash chromatography using a 1:1 mixture of petroleum ether/ethyl acetate as eluent to obtain compound **13** (0.19 g, 80% yield).

4.1.5. General Procedure for the Synthesis of Compounds **4**, **6**, **7**, and **9**

Compound **14** or **15** or **16** (1 eq) was dissolved in dry acetone (15 mL) and then anhydrous potassium carbonate (1 eq) and appropriated alkenyl bromide (1 eq) were added. The resulting mixture was stirred at 50 °C for 12 h, then sodium hydroxide 2 N (15 mL) was added and the reaction mixture was stirred at 90 °C for an additional 3 h. The cooled solution was acidified to pH 2 with hydrochloridric acid (10% solution) and extracted with dichloromethane (3 × 50 mL). The collected organic phases were dried over anhydrous sodium sulphate and evaporated to dryness to give the pure ether.

(E)-3-(4-(Allyloxy)phenyl)acrylic acid **4**: white solid; 85%; mp 161–162 °C ([62] 160 °C); ^{1}H-NMR δ 4.56 (d, *J* = 5.2 Hz, 2H), 5.29–5.44 (series of m, 2H), 6.03 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H), 6.91 (m, Ar, 2H), 7.49 (m, Ar, 2H), 7.73 (d, *J* = 16 Hz, 1H); ^{13}C-NMR δ 68.85, 114.72, 115.13, 118.08, 126.91, 130.07, 132.66, 146.67, 160.73, 172.55. Anal. Calcd. for $C_{12}H_{12}O_3$ C, 70.57; H, 5.92; Found C, 70.78; H, 5.87.

(E)-3-(4-(Allyloxy)-3-methoxyphenyl)acrylic acid **6**: white solid; 53%; mp 152–154 °C ([63] 151–153 °C); ^{1}H-NMR δ 3.91 (s, 3H), 4.66 (m, 2H), 5.30–5.44 (series of m, 2H), 6.08 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H); 6.86 (d, *J* = 8.4 Hz, Ar, 1H), 7.08 (dd, *J* = 2.0, 8.4 Hz, Ar, 1H), 7.09 (d, *J* = 2.0 Hz, Ar, 1H), 7.71 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 55.92, 69.70, 110.08, 112.71, 114.90, 118.51, 122.92, 127.14, 132.61, 147.01, 149.52, 150.46, 172.72. Anal. Calcd. for $C_{13}H_{14}O_4$ C, 66.66; H, 6.02; Found: C, 66.87; H, 6.12.

(E)-3-(4-((3-Methylbut-2-en-1-yl)oxy)phenyl)acrylic acid **7**: white solid; 69%; mp 146–147 °C ([16] 148–150 °C); ^{1}H-NMR δ 1.73 (s, 3H), 1.79 (s, 3H), 4.52 (d, *J* = 6.4 Hz, 2H), 5.47 (s, 1H), 6.28 (d, *J* = 16 Hz, 1H), 6.89 (d, *J* = 8.8 Hz, Ar, 2H), 7.47 (d, *J* = 8.4 Hz, Ar, 2H), 7.73 (d, *J* = 16 Hz, 1H); ^{13}C-NMR δ 25.81, 29.19, 64.91, 114.62, 115.04, 119.11, 126.67, 130.04, 138.73, 146.60, 161.04, 171.36. Anal. Calcd. for $C_{14}H_{16}O_3$ C, 72.39; H, 6.94; Found C, 72.59; H, 6.03.

(E)-3-(4-(((E)-3,7-Dimethylocta-2,6-dien-1-yl)oxy)-3-methoxyphenyl)acrylic acid **9**: white solid; 75%; mp 59–60 °C ([64] 60–61 °C); ^{1}H-NMR δ 1.63 (s, 3H), 1.70 (s, 3H), 1.77 (s, 3H), 2.01–2.24 (series of m, 4H), 3.95 (s, 3H), 4.62 (m, 2H), 5.11 (m, 1H), 5.44 (m, 1H), 6.33 (d, *J* = 16.0 Hz, 1H), 6.86 (d, *J* = 7.6 Hz, Ar, 1H), 7.01–7.15 (series of m, Ar, 2H), 7.37 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 16.70, 17.70, 25.70, 26.20, 39.53, 55.91, 65.82, 109.91, 112.52, 114.71, 119.11, 123.01, 123.71, 126.81, 131.84, 141.22, 147.01, 149.52, 150.83, 172.11. Anal. Calcd. for $C_{20}H_{26}O_4$ C, 72.70; H, 7.93; Found: C, 72.80; H, 7.92.

4.1.6. Synthesis of Compound **5**

(E)-3-(4-(Allyloxy)-3-hydroxyphenyl)acrylic acid **5**: Compound **15** (0.5 g, 2.57 mmol) was dissolved in dry acetone (15 mL) and then anhydrous potassium carbonate (0.35 g, 2.57 mmol) and allyl bromide (0.31 g, 2.57 mmol) were added. The resulting mixture was stirred at 50 °C for 12 h. The cooled solution was acidified to pH 2 with hydrochloridric acid (10% solution) and extracted with dichloromethane (3 × 50 mL). The collected organic phases were dried over anhydrous sodium sulphate and evaporated. The crude product was purified by flash chromatography using a mixture of 3:1 petroleum ether/acetone as eluent to give compound **18** as a white solid (0.47 g, 78%).

(E)-Methyl 3-(4-(allyloxy)-3-hydroxyphenyl)acrylate **18**: mp 95–96 °C ([65] 94–95 °C); ^{1}H-NMR δ 3.78 (s, 3H), 4.65 (d, *J* = 5.6 Hz, 2H), 5.33–5.43 (series of m, 2H), 5.69 (s, 1H), 6.08 m, 1H), 6.30 (d, *J* = 15.6 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, Ar, 1H), 6.99 (dd, *J* = 2.0 8.4 Hz, Ar, 1H), 7.14 (d, *J* = 2.0 Hz, Ar, 1H), 7.61 (d, *J* = 15.6 Hz, 1H); ^{13}C-NMR δ 51.59, 69.80, 11.84, 113.21, 115.94, 118.80, 121.64, 123.13, 128.20, 132.26, 144.60, 146.02, 147.39, 167.68. Anal. Calcd. for $C_{14}H_{16}O_4$ C, 67.73; H, 6.50; Found: C, 67.52; H, 6.42. To compound **18** (0.47 g, 2.00 mmol) in a 3:1 solution of MeOH:H_2O, sodium hydroxide 2 N (15 mL) was added and the mixture was stirred at 90 °C for 3 h. The cooled solution was acidified to pH 2 with

hydrochloridric acid (10% solution) and extracted with dichloromethane (3 × 50 mL). The collected organic phases were dried over anhydrous sodium sulphate and evaporated to dryness to give the pure **5** as white solid (0.33 g, 75%); mp 184–185 °C; ^{1}H-NMR δ (acetone d$_6$) 4.66 (m, 2H), 5.26 (m, 1H), 5.46 (m, 1H), 6.07 (m, 1H), 6.32 (d, *J* = 16 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, Ar, 1H), 7.09 (dd, *J* = 2.4, 8.4 Hz, Ar, 1H), 7.19 (d, *J* = 2.4, Hz, Ar, 1H), 7.56 (d, *J* = 16 Hz, 1H); ^{13}C-NMR δ 64.41, 112.87, 113.95, 115.94, 117.05, 121.08, 128.03, 133.44, 144.61, 147.08, 148.52, 167.09. Anal. Calcd. for $C_{12}H_{12}O_4$ C, 65.45; H, 5.49; Found: C, 65.52; H, 5.32.

4.2. Fungal Strains and Culture

Two monosporic isolates collected from human samples from an Italian hospital (Table 2) were selected as representative of each *Fusarium oxysporum*, *F. solani*, and *F. fujikuroi* species complexes. Conidial suspensions of each strain were pre-cultured in a carboxymethyl cellulose medium (CMC; [66]) for 5 days on a rotary shaker at 24 °C and 180 rpm. Cultures were filtered through four layers of sterile cheesecloth, and spores were collected by centrifugation, adjusted to 1 × 10^6 colony-forming units (CFU)/mL in sterile water, and used as inoculum.

4.3. Evaluation of the Antifungal Activity of Compounds **1–13** in FMM Solid Medium

A total of 13 phenolic compounds (Table 1) were tested for their antifungal activity against the six *Fusarium* spp. isolates (Table 2) in Fusarium minimal medium (FMM) [67]. Each phenolic compound was resuspended in H_2O/gellan 0.1 % solution and sonicated at room temperature for 1 h at 80 Hz (Elmasonic P 180 H, Elma Schmidbauer GmbH, Germany). Solid FMM with nitrate sodium $NaNO_3$ as nitrogen source was distributed into Ø90 mm Petri dishes (15 mL/Petri dish) and amended with each compound at a final concentration of 0.5 mM at a temperature of 45 °C. Ten microliters of the conidial suspension of each strain were spotted onto the center of the Petri dish amended FMM. Antifungal activity of each compound was measured after 5 d of growth at 25 °C in the dark and expressed as the colony diameter (percentage relative to control). Three replicates were prepared for each isolate/inhibitor combination and the experiment was repeated once.

4.4. Evaluation of the Antifungal Activity of p-Coumaric Acid 3,3′-Dimethyl Allyl Ester (**13**) in FMM Liquid Medium

The conventional antifungal drugs used in the study were AmB and TRB. AmB was purchased by Sigma Aldrich (A2942, Germany) as a standard solution. TRB was extracted from Terbinafine Hexal 250 mg tablets by fine crushing and dissolution in a solution 1:2 (v:v) dichloromethane and water. The emulsion was stirred at room temperature until two phases clearly appeared. The organic phase was extracted and dried on Na_2SO_4 and the TRB was recovered in neat form after evaporation of the solvent under vacuum. NMR spectra of the solid extract confirmed the presence of TRB with a purity ≅98%. TRB was dissolved in 60% ethanol/H_2O (*v*/*v*), while AmB was diluted with water to reach the desired concentration and was frozen in aliquots at −20 °C. AmB and TRB concentrations were selected according to clinical dosage and standard experimental procedures with some modifications [68].

The minimal inhibitory concentration (MIC) and the lethal dose 50 (LD_{50}) of each strain were assayed by a standardized micro-dilution method in the 96-well plate. Two-fold serial dilutions of each antifungal agent in liquid FMM in a total volume of 200 µL were tested. Further, 10 µL of fungal spore suspension (4 × 10^6 CFU/mL) was added. A blank control with water was used for each treatment. The optical density mOD of each microplate well was measured at 2 h intervals during 72 h of incubation with a microplate spectrophotometer SpectrostarNano (Euroclone, Germany) at a 595 nm wavelength. The inhibitory activity of each compound was expressed as MIC, representing the lowest concentration of active ingredient (µM) that is sufficient to inhibit the absorbance signal, whereas the LD_{50} of each compound was calculated as the concentration of active ingredient

(μM) able to reduce by 50% the mOD_{595} signal. The experiments were repeated at least two times in quadruplicate.

4.5. Optical Microscopy Examination

A drop (15 μL) of the total volume present on the wells corresponding to the MIC of each isolate/compound combination was pipetted after 72 h of incubation onto a glass slide. A clean glass cover slip was placed on the sample prepared with emulsion oil. Each slide was examined at 100× for the presence of mycelium, branched hyphae, fialides, microconidia, and germinating spores, using an optical microscope (LEICA ICC50) at a scale of 20 μm.

4.6. Data Acquisition and Analysis

In the first screening, an analysis of variance (one-way ANOVA) followed by multiple comparisons by Tukey HSD test at the significance level $p < 0.05$ using Minitab for Windows, release 17 was performed.

In the second screening, data were recorded and analysed with Mars Data Analysis Software, BMG Labtech, and exported to Microsoft Excel for generation of the graphs. Graphs for the determination of the MIC and LD_{50} (Figures S4–S9) were generated for an incubation time between 0 h and 48 h because, after this interval time, the drug free-test (control) curve started to reach the stationary phase for almost all strains investigated. Optical microscopy images were captured and treated with LAS V4.13 Leica application software.

5. Conclusions

The design of cinnamic derivatives **4**–**13** was focused on both electronic and steric modification of the parent compounds **1**–**3** by esterification and etherification reaction with bioactive prenylated chains, with the aim to enhance the antifungal activity of the final compound. Compounds **1**–**3** are commercially available at a reasonable price and offer a successful example of repositioning of natural compounds. In this study, we provided data that may contribute to increasing the knowledge about the promotion of the importance of antifungal susceptibility testing. *p*-Coumaric acid 3,3′-dimethyl allyl ester **13**, a component of propolis, showed good antifungal activities against *Fusarium* spp., causing onychomycosis, and identifies prenylated hydroxy cinnamic acids as interesting pharmacophore for developing new drugs effective against this pathology. The activity of this compound will be investigated over a larger number of isolates belonging to different species complexes and haplotypes. Furthermore, the mechanism of action of compound **13** needs to be fully characterized and possibly tested in combination with other bioactive molecules that may be enabled to reach their target within the fungal cell.

Noteworthy, this study cannot be adopted as a clinical guideline, and the MIC values obtained must be tested in an appropriately designed clinical study.

Supplementary Materials: The following are available online. Figures S1–S3: Antifungal activity of compounds **1**–**13** against FOSC, FFSC, and FFSC, respectively. Figures S4–S9: MIC and LD_{50} ranges expressed as absorbance (milliOD) at 595 nm at 48 h of ester **13** (A), TRB (B), and AmB (C) against FOSC, FSSC, and FFSC strains, respectively.

Author Contributions: Conceptualization, G.D. and Q.M.; methodology, S.O., V.B., M.A.D., and D.F.; software, S.O.; validation, S.O., P.C., and D.F.; formal analysis, S.O., V.B., S.C., W.C., P.C., and D.F.; resources, Q.M. and D.F.; data curation, S.O., S.C., and P.C.; writing—original draft preparation, G.D., M.A.D., S.O., and S.C.; writing—review and editing, G.D. and Q.M.; funding acquisition, Q.M. and D.F. All authors have read and agreed to the published version of the manuscript.

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J86C16000070009 SSPIT: Selection of sustainable phenolic inhibitors of trichothecene biosynthesis: in vitro studies and computational insight into the structure- activity relationship".

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Ethics Statement: This article does not contain any studies with human participants or animals performed by any of the authors.

Sample Availability: Samples of the compounds **1–13** are available from the authors.

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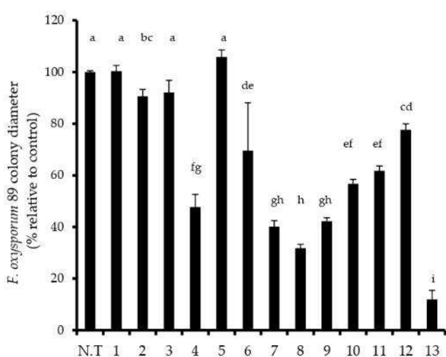
Supplementary materials



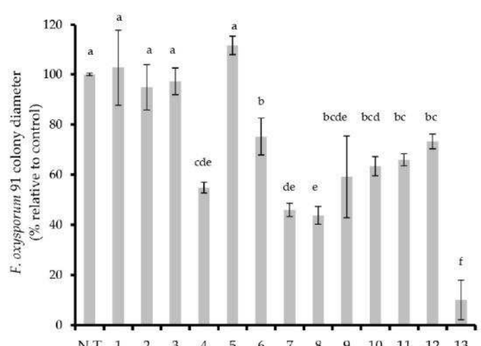
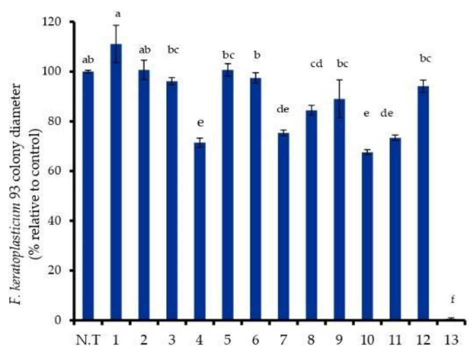


Figure S1. Antifungal activity of compounds **1-13** against FOSC.

*N.T: Not treated (drug-free test).
Compounds that id not share the same letter are statistically different at $P < 0.05$.



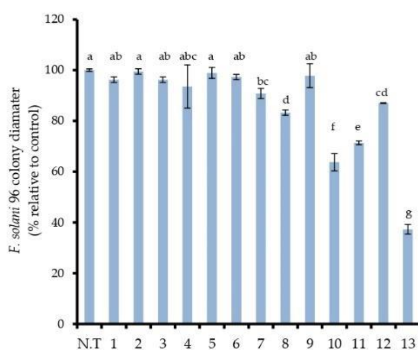
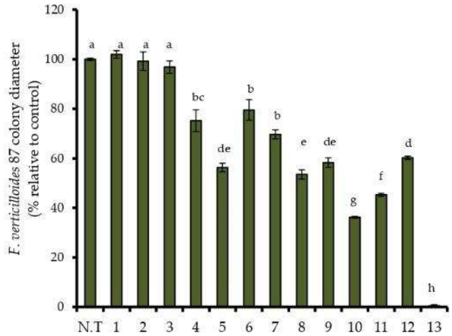


Figure S2. Antifungal activity of compounds **1-13** against FSSC.

*N.T: Not treated (drug-free test).
Compounds that id not share the same letter are statistically different at $P < 0.05$.



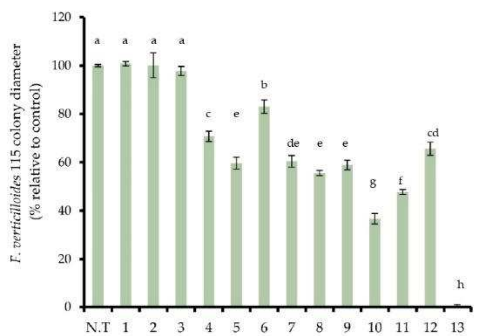


Figure S3. Antifungal activity of compounds **1-13** against FFSC.

*N.T: Not treated (drug-free test).
Compounds that id not share the same letter are statistically different at $P < 0.05$.

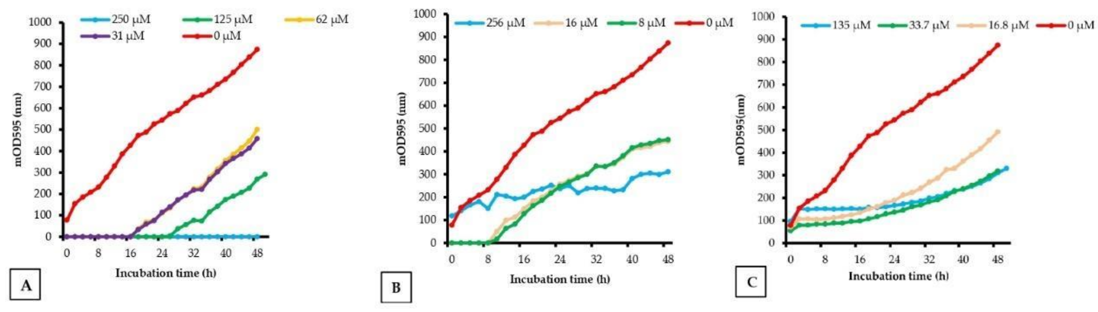


Figure S4. MIC and LD_{50} ranges expressed as absorbance (milliOD) at 595 nm at 48 h of Ester **13** (A), TRB (B) and AmB (C) against *F. oxysporum* 89.

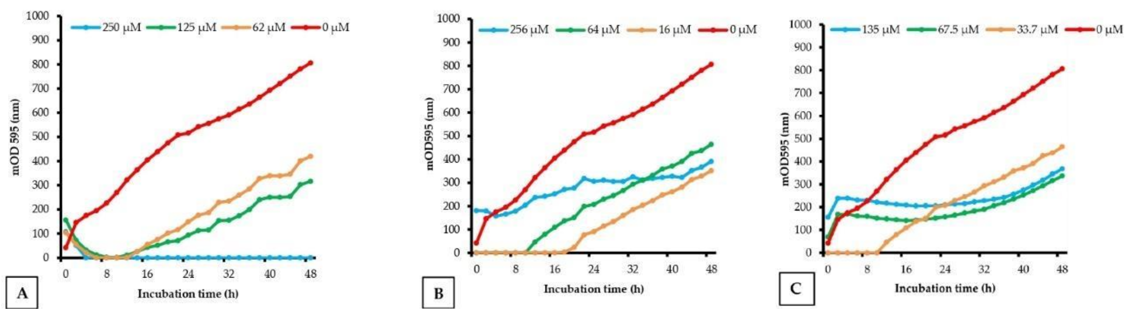


Figure S5. MIC and LD_{50} ranges expressed as absorbance (milliOD) at 595 nm at 48 h of Ester **13** (A), TRB (B) and AmB (C) against *F. oxysporum* 91.

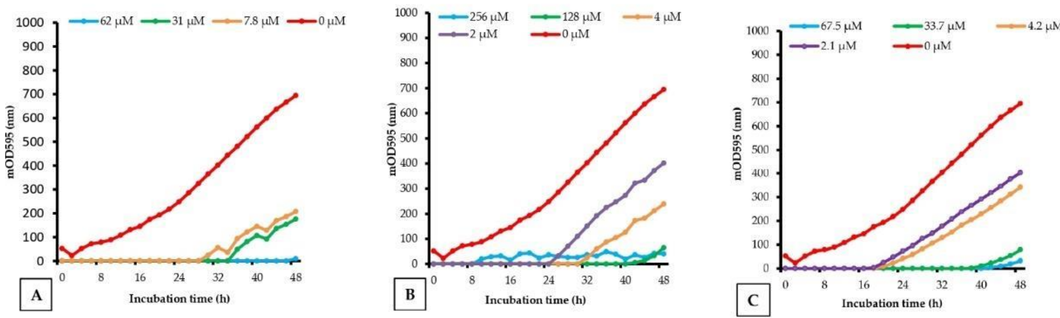


Figure S6. MIC and LD_{50} ranges expressed as absorbance (milliOD) at 595 nm at 48 h of Ester **13** (A), TRB (B) and AmB (C) against *F. keratoplasticum* 93.

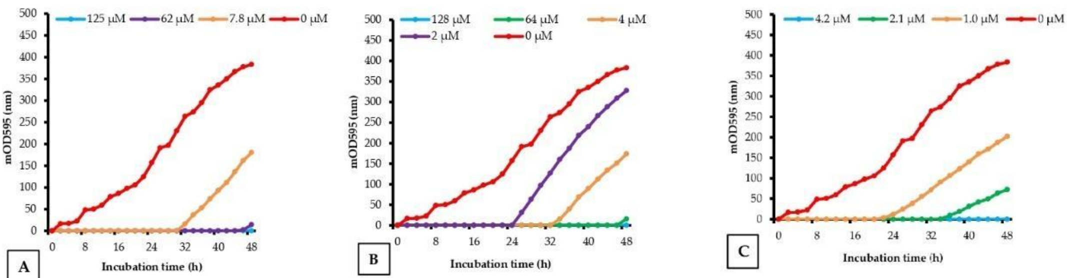


Figure S7. MIC and LD_{50} ranges expressed as absorbance (milliOD) at 595 nm at 48 h of Ester **13** (A), TRB (B) and AmB (C) against *F. solani* 96.

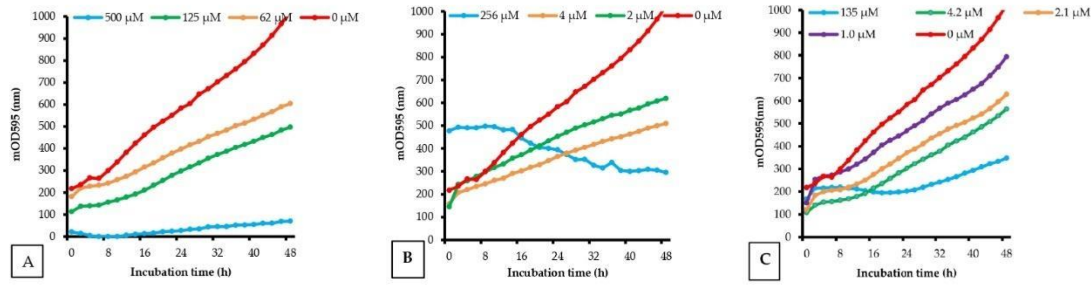


Figure S8. MIC and LD_{50} ranges expressed as absorbance (milliOD) at 595 nm at 48 h of Ester **13** (A), TRB (B) and AmB (C) against *F. verticillioides* 87.

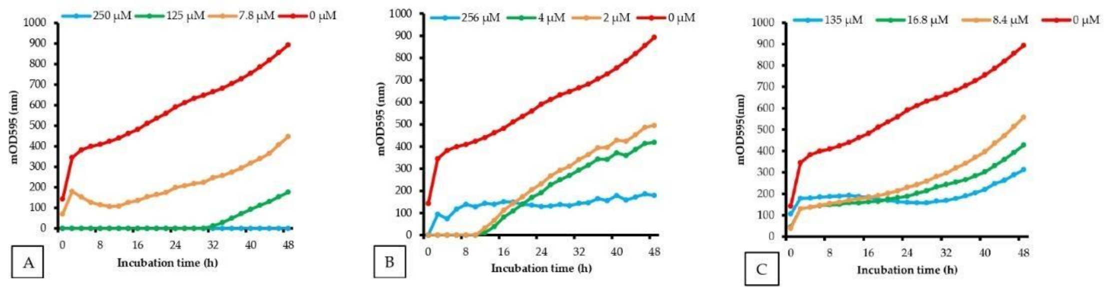


Figure S9. MIC and LD_{50} ranges expressed as absorbance (milliOD) at 595 nm at 48 h of Ester **13** (A), TRB (B) and AmB (C) against *F. verticillioides* 115.

Chapter 4

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Summary

Natural plant extracts can provide an alternative to synthetic fungicides to prevent infection of grapes by *Aspergillus carbonarius* and reduce ochratoxin A (OTA) contamination. The present study investigates the antifungal and anti-mycotoxigenic properties of aqueous extracts of chestnut flower, cistus, eucalyptus, and orange peel at 10 mg/mL. Cistus and orange peel extracts appeared promising in their inhibition of vegetative growth. All extracts were effective against OTA after ten days of grape storage, with inhibition ratios ranging from 7.6% to 82.3%. Eucalyptus extract was particularly efficient in inhibiting OTA production by up to 80%, but had no effect on fungal growth. Thus, it maintained the natural microbial balance on grapes. This study suggests that plant extracts may be useful sources of bioactive chemicals for the prevention of grape infection and OTA production. However, further work is required in order to evaluate their organoleptic effects on grapes before these extracts can be used in practical applications.

Plant extracts as biocontrol agents against *Aspergillus carbonarius* growth and ochratoxin A production in grapes

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Highlights

- Cistus and orange peel extracts inhibit *Aspergillus carbonarius* growth.
- Chestnut flower, cistus, eucalyptus, and orange peel extracts reduce ochratoxin A produced by *Aspergillus carbonarius* in grape berries.
- Eucalyptus extract inhibits ochratoxin A synthesis of *Aspergillus carbonarius* by up to 85.75% in grapes.

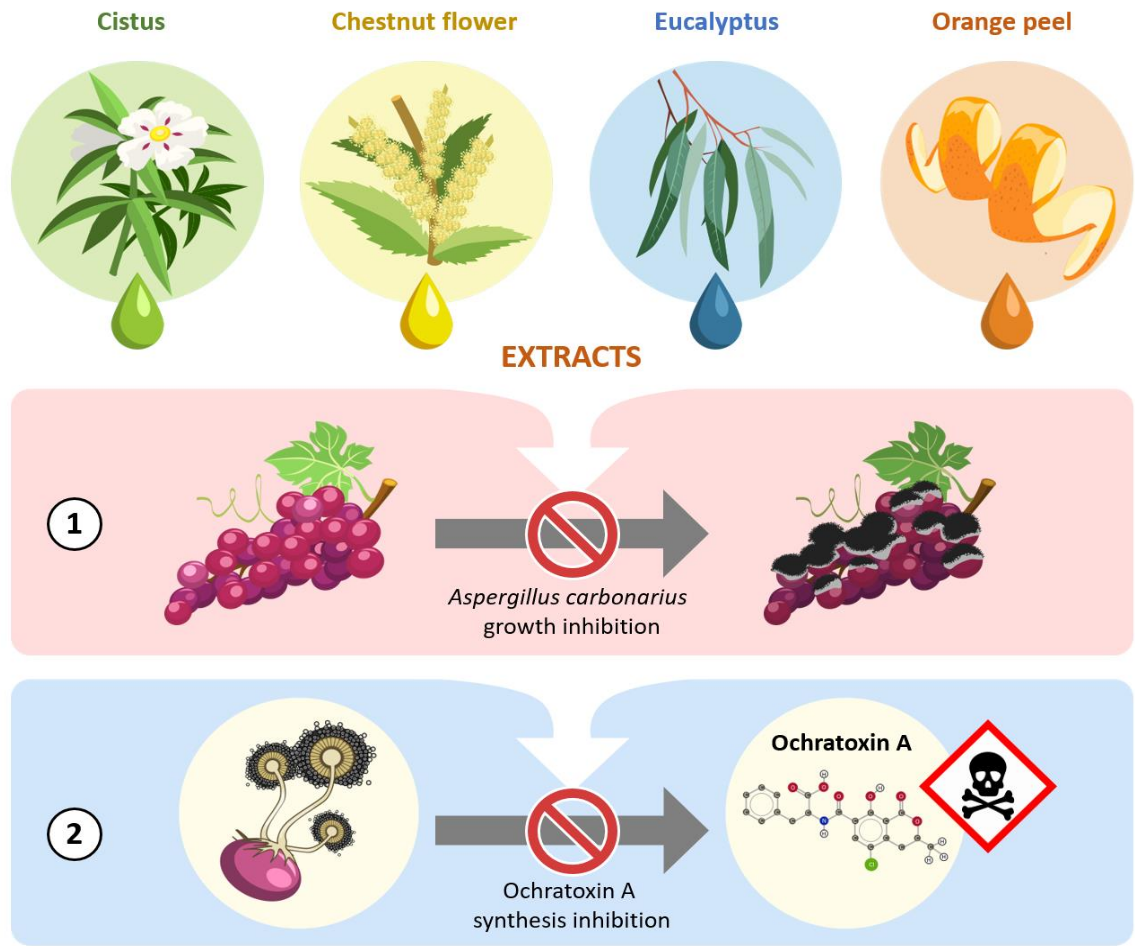
Abstract

Aspergillus carbonarius (Bainier) Thom. is an important pathogen and ochratoxin A (OTA) producer in grapes that can be controlled by adopting sustainable approaches. Here we evaluate the application of natural plant extracts as an alternative to synthetic fungicides to reduce OTA contamination and to prevent infection of grapes by two isolates of *A. carbonarius*. In a preliminary screening, natural extracts of chestnut flower, cistus, eucalyptus, fennel, and orange peel were evaluated for their antifungal and anti-mycotoxigenic efficiency in a grape-based medium at concentrations of 10 and 20 mg/mL. Cistus and orange peel extracts demonstrated the best antifungal activity at both concentrations. Although the eucalyptus extract demonstrated no significant effect on *Aspergillus* vegetative growth, it significantly reduced OTA by up to 85.75% at 10 mg/mL compared to the control. Chestnut flower, cistus, eucalyptus, and orange peel extracts were then tested at the lowest concentration (10 mg/mL) for their antifungal activity in artificially inoculated grape berries. The cistus and orange peel

extracts demonstrated the greatest antifungal activity and significantly reduced mold symptoms in grapes. Moreover, all tested natural extracts were able to reduce OTA content in grape berries (17.7 ± 8.3% - 82.3 ± 3.85% inhibition), although not always significantly. Eucalyptus extract was particularly efficient, inhibiting OTA production by both strains of *A. carbonarius* by up to > 80% with no effects on fungal growth. The use of natural eucalyptus extract represents a feasible strategy to reduce OTA formation without disrupting fungal growth, while cistus and orange peel extracts appear promising as inhibitors of *A. carbonarius* mycelial growth. Our findings suggest that plant extracts may be useful sources of bioactive chemicals for preventing *A. carbonarius* contamination and OTA production. Nonetheless, it will be necessary to evaluate their effect on the organoleptic properties of the grapes.

Keywords: OTA; mycotoxins; biofungicides; food safety; bioactivity

Graphical Abstract



1. Introduction

Ochratoxin A (OTA) is ranked among the five most common and harmful mycotoxins in agriculture (Malir et al., 2016). *Aspergillus* and *Penicillium* species are the main producers of OTA (Wang et al., 2016) and contaminate a number of foodstuffs including grapes and their derivatives (Gil-Serna et al., 2018; Mondani et al., 2020; Ortiz-Villeda et al., 2021; Zimmerli & Dick, 1996). Grapes contribute significantly to human nutrition and are valued for their sensory properties as well as for the vitamins and bioactive compounds (e.g., flavonoids) they contain (FAO-OIV, 2016; Sabra et al., 2021). However, wine and grape juice are ranked after cereals as the second greatest sources of dietary exposure to OTA (Kizis et al., 2021; Li et al., 2021).

Mycotoxin contamination of grapes typically begins in the vineyard (Tini et al., 2020). *Aspergillus carbonarius* (Bainier) Thom. (and *Aspergillus niger* Tiegh., which produces less OTA) is a major source of OTA contamination in grapes grown in Mediterranean countries due to the ability to grow effectively and to produce significant quantities of toxins at high temperatures (Battilani et al., 2006; Bellí et al., 2006; Cabañes et al., 2002; Lasram et al., 2007). In the current context of climate change, it is predicted that OTA contamination in grapes will increase due to the interaction between temperature and high levels of atmospheric CO_2 (Cervini et al., 2021).

OTA exposure is a major health concern (Bui-Klimke & Wu, 2015; Stoev, 2022). Data from the European Food Safety Authority (EFSA) most recent risk assessment indicated OTA as a cause of kidney cancer and damage to DNA (EFSA et al., 2020). Based on toxicological and exposure data, the European commission has set maximum thresholds for the presence of OTA

in wine, fruit wine, grape juice, nectar, and must for human consumption (2 µg/kg), and in dried vine fruits (10 µg/kg) (European commission, 2006).

Fungicides have long been used to reduce fungal proliferation and mycotoxin production, and viticulture is one of the agricultural systems that most frequently uses spraying (Bouagga et al., 2019; Komárek et al., 2010). However, the European Commission is increasing the restrictions on the number of pesticide applications (European commission, 2009), and on the maximum amount of copper-based fungicides in organic farming (European commission, 2018). In this context, there is an urgent need for sustainable approaches to manage toxigenic fungi at pre- and postharvest phases that can replace or supplement synthetic fungicides (Ponsone et al., 2012).

Plant extracts are a promising tool for controlling fungal contamination in food commodities (Chen et al., 2019; Makhuvele et al., 2020). In addition to being naturally abundant, they are easily biodegradable and have no negative environmental impacts (da Cruz Cabral et al., 2013). Unlike pure molecules, plant extracts include a wide variety of phenolic compounds and terpenes with distinct modes of action that may trigger a synergistic or additive effect (Badr et al., 2022; Chtioui et al., 2022). Another advantage of plant extracts is that a blend of active compounds with diverse physiological targets can prevent fungal resistance to treatment (Fuentefria et al., 2018; Vaou et al., 2021).

Mediterranean flora provides a rich source of secondary metabolites, especially terpenoids and phenylpropanoids with antibacterial and antioxidant characteristics (Alonso-Esteban et al., 2022; Barros et al., 2009; Xavier et al., 2021; Zalegh et al., 2021). However, their antifungal and anti-mycotoxigenic activities have not yet been fully characterized.

The goal of this study was to explore the properties of aqueous extracts from a range of Mediterranean plants when used as bio-fungicides and OTA inhibitors. Extracts of chestnut flowers, cistus, eucalyptus, fennel, and orange peel were prepared at ambient temperature using an inexpensive and eco-friendly extraction procedure. This work was strongly motivated by concerns related to the environment and circular economy.

2. Methodology

2.1. Biological material

2.1.1. Plant material and preparation of extracts

This study used five natural organic sources from wild and farmed plants, namely: male flowers of European chestnut (*Castanea sativa* Miller.), rockflower (*Cistus ladanifer* L.), orange (*Citrus aurantium* var. *sinensis* L.) peel, eucalyptus (*Eucalyptus globulus* Labill.), and fennel (*Foeniculum vulgare* Mill.) The origin and botanical data of the plant species used are summarized in **Figure 1** and **Table 1.**

Figure 1. Sampling area of the plants used in the study: 1) Braganca (chestnut flower, cistus, eucalyptus, and orange); 2) Vila nova de Gaia (Fennel)

Table 1. Plant material used

Scientific name (Family)	English common name	Source	Plant organ
Castanea sativa Mill. (cv. Judia) (Fagaceae)	Sweet chestnut or European chestnut	Samil, Bragança	Male flowers
Cistus ladanifer L. (Cistaceae)	Gum rockrose, laudanum, labdanum	Orchards, Bragança	Leaves
Citrus aurantium var. *sinensis* L. (Rutaceae)	Orange	Local supermarket, Bragança	Peel
Eucalyptus globulus Labill. (Myrtaceae)	Eucalyptus or Tasmanian blue gum	Campus of the Polytechnic Institute of Bragança	Leaves
Foeniculum vulgare Mill.	Fennel	Company "Cantinho das Aromáticas", Vila Nova de Gaia	Leaves

Aqueous extracts were prepared from the various organic plant materials. Plants were collected or purchased fresh, and shade-dried at room temperature. They were powdered using a kitchen mill (Moulinex). The powder from each plant material (1 g) was extracted by swirling with 30 mL of distilled water at 150 rpm for 1 hour at room temperature. Subsequently, it was filtered through Whatman no. 4 filter paper. Then, 30 mL of water was added to re-extract the residue. The final extracts were frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA; collector chamber at −50 ºC and 0.012 torr).

Aqueous extracts were prepared at concentrations of 10 mg/mL and 20 mg/mL for subsequent assays by dissolving the lyophilized extracts in 5% of dimethyl sulfoxide (DMSO) (Merck KGaA, Germany), followed by dilution in water to obtain the final concentration. Test concentrations were established after a preliminary microdilution susceptibility test (Svobodova et al., 2017) against *A. carbonarius* MUM 04.46 and MUM 04.52 (see below). *A. fumigatus* Fresen. (ATCC 204305) and *A. brasiliensis* Varga, Frisvad & Samson (ATCC 16404) were used as reference fungi (data not shown).

2.1.2. Fungal isolates

Two strains of *A. carbonarius*, MUM 04.46 and MUM 04.52, respectively coded Ac46 and Ac52 in this study, were provided by the fungal culture collection "Micoteca da Universidade

do Minho (MUM)", Braga, Portugal. These were originally isolated from Portuguese grapes and confirmed as OTA producers (Serra et al., 2003). The fungi were kept at -20 °C in 20% glycerol and cultivated on potato dextrose agar (PDA; Biolife, Italy). Whenever needed, the isolates were cultivated in PDA for 5 to 7 d at 25 ºC in the dark. Immediately before the assays, spore suspensions of each strain were obtained by scraping the top of a 5- to 7-day-old fungal culture, then diluted in 3 mL of sterile water containing 0.05% Tween 80. The spore concentrations were adjusted as needed using a Neubauer counting chamber.

2.2. Screening the *in vitro* effect of the extracts on *A. carbonarius* growth and OTA production in a grape-based medium

2.2.1. Preparation of the grape-based medium

To simulate the grape matrix, a grape-based medium was prepared from cv. Touriga Franca (red wine cultivar) berries from an orchard in Vila Real, northern Portugal. The medium was created by rinsing berries with 1% sodium hypochlorite for 1 min, washing them twice with distilled water, and then blending at a low speed to obtain a homogeneous juice. Next, the pH was adjusted to 3.5 with 10% tartaric acid to ensure proper solidification, and agar was added to achieve a final concentration of 2%. The medium was autoclaved at 121 °C for 15 minutes. The cooled medium was then combined with an equal volume of the autoclaved (10 minutes, 110 °C) aqueous plant extracts (final grape concentration: 50%, v/v) to obtain two final concentrations: 10 mg/mL and 20 mg/mL. Grape juice amended with sterile water was used as a negative control. Additionally, a fungicide control consisted of a mixture containing the commercial formulate Teldor® (active substance fenhexamid, 50% a.i. (w/w); Bayer CropScience, Portugal), which is generally applied as a field fungicide to grapevine at the recommended concentration of 1.5 g/L (fenhexamid 0.75 g/L). The fungicide was added to the

medium at the concentration of 0.75 mg/mL of fenhexamid, to reproduce the concentrations recommended in field applications.

The mixtures were then homogenized, and 3 mL of each mixture was pipetted into 12-well plates, in triplicate.

2.2.2. Inoculation and incubation

Spore suspensions of Ac46 and Ac52 at 10^5 spores/mL were used as inoculum. Two μL of inoculum were pipetted into the center of each well containing the different media, and the plates were then incubated in the dark at 25 °C. The fungal colonies were examined after 5 and 10 d of growth to assess the efficiency of each treatment. Three replicates of each treatment were evaluated.

2.2.3. Observation of growth

This assay evaluated fungal growth qualitatively according to inhibition activity (++= high inhibiting activity, += inhibiting activity, -= no inhibiting activity) after incubation for 5 and 10 d, compared with the negative control (grape medium without plant extracts).

2.2.4. OTA analysis

After 10 d of incubation, OTA extraction was performed on all cultures. Fungal mycelium and grape medium from the three replications of each treatment were removed from the wells and weighed. OTA was extracted with methanol for 60 minutes in the dark by mixing every 15 minutes. The extract was cleaned using an OTA Immunoaffinity column (IAC; Ochratest™, VICAM, Milford, USA) following the manufacturer's instructions, and filtered with a 0.22 µm

polytetra-fluorethylene (PTFE) membrane (FiltraTech, Saint Jean de Braye, France). OTA was quantified using the HPLC methodology described below.

2.3. Effect of selected extracts on fungal growth in 9 cm Petri dishes

Quantitative growth assessment assays were carried out in 9 cm Petri dishes containing 20 mL of grape-based medium prepared as described above. For this assay, only the four extracts and extract concentrations that showed the best results in the *in vitro* screening assay were selected and tested. Thus, the plant extracts of chestnut flower, eucalyptus, cistus, and orange peel were tested at a concentration of 10 mg/mL. Similarly, fenhexamid (0.75 mg/mL) was used as a positive control, while Petri dishes containing a water-added medium were used as a negative control. Subsequently, 2 µL of 10^5 spores/mL of conidial suspension of Ac46 and Ac52 was deposited in the center of each Petri dish and incubated in the dark at 25 °C. All tests were run in triplicate. The colony diameter was measured daily in two perpendicular directions to determine the maximum growth rate (μm, in cm of radius/day), which was obtained as the slope of the line of the linear regression of colony radii plotted against the incubation time.

2.4. Effects of the selected extracts on fungal growth and OTA production in grapes

2.4.1. Preparation of grape berries

Healthy mature grapes (cv. Touriga Franca) of similar size and showing no signs of mechanical or fungal damage were selected. Berries were surface disinfected with 1% sodium hypochlorite for 1 min, rinsed twice with sterile distilled water, and air-dried on a laminar flow bench. Subsequently, a single wound (3 mm deep) was made using a sterile needle in the equatorial region of each berry. Thereafter, berries were immersed for 3 minutes in the selected extracts: chestnut flower, cistus, eucalyptus, and orange peel extracts (10 mg/mL), and in fenhexamid

(0.75 mg/mL) as a control. The four extracts and the concentration used in this assay were selected based on the best results obtained in the *in vitro* screening.

After 2 h, the wound on each berry was inoculated with 10 µL of spore suspension (10^5 spores/mL) of each *A. carbonarius* strain. Wounded berries immersed in sterile water and inoculated after 2 h with a conidial suspension of *A. carbonarius* were used as negative controls. Each treatment consisted of 10 berries, and each experiment was repeated three times. Berries were air-dried and then placed in plastic holders (60 cm × 40 cm × 15 cm, one layer), wrapped in transparent polyethylene foil to avoid evaporation, and incubated for 10 days at 25 °C in the dark.

2.4.2. *Aspergillus* rot severity symptoms in grape berries

Following incubation, fungal growth, and fruit rot were evaluated for each berry by estimating the percentage of its surface area presenting signs of fungus growth and symptoms of spoilage. A score from 0 to 4 was assigned to the percentage of berry area presenting symptoms: 0 = 0% with symptoms, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, and 4 = 76-100%. Subsequently, the infection severity index (McKinney's index), which incorporates the incidence and the severity of the disease, was calculated according to the following formula:

$$I = [\Sigma (d \times f)/(N \times D)] \times 100$$

d is the category of the disease intensity scored for the grape bunches, f is the disease frequency, N is the total number of berries examined, and D is the highest category of disease intensity that occurred on the empirical scale (McKinney, 1923).

2.4.3. Quantification of OTA production in grape berries

OTA was extracted from grapes according to Serra et al. (2004). After 10 days of fungal growth, 10 g of previously homogenized berry tissue from each treatment was transferred into a 50 mL Falcon tube and brought up to 30 mL using a solution of 5% $NaHCO_3$ and 1% PEG 8000. The mixture was vortexed every 15 min for 1 h and then centrifuged at 8,500 rpm for 10 min at 4°C. The supernatant was filtered through a glass microfiber filter (1.5 µm pore size, Whatman), and 10 mL of this filtrate was passed through the Ochratest IAC for cleaning. OTA was then eluted with methanol and passed through a 0.22 µm PTFE syringe filter before HPLC analysis.

2.5. OTA analysis by HPLC

OTA was analyzed using a High-Performance Liquid Chromatography (HPLC) Smartline Pump 1000 (Knauer, Berlin, Germany) coupled with a fluorescence detector FP-2020 (Jasco, USA). A C18 reverse-phase column PLRP-S 300 Å (250 x 4.6 mm, 8 µm, Polymer Laboratories, Church Stretton, UK) was used at 35 °C. The mobile phase consisted of water: acetonitrile: acetic acid (29.5:70:0.5), and was pumped in an isocratic mode at 0.8 mL/min. The injection volume was 20 µL. OTA was detected at 330 nm (excitation) and 463 nm (emission), with a run time of 15 min. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as follows:

$$LOD = 3 \times (sa/b)$$

$$LOQ = 10 \times (sa/b)$$

sa is the standard deviation of the regression line obtained from the calibration curve and *b* is the slope of the line (Taverniers et al., 2004). The LOD and LOQ were 3 and 9 ng/mL, respectively.

2.6. Statistical analysis

Data were analyzed using R software (R Core Team, 2020). The investigated dependent variables (fungal growth, OTA production, McKinney index) were analyzed using the linear regression model below:

$$Yij = \mu + Ti + Fj + eij$$

μ is the overall mean, Ti is the fixed effect of the treatment, Fj is the fixed effect of the fungi and eij is the random residual/error.

The results obtained from OTA production, fungal growth and the McKinney index were tested for normality using the Shapiro test. Since all dependent variables were not normally distributed, a non-parametric Kruskal- Wallis and a post hoc Dunn test were also performed. Statistical significance was declared when $p \leq 0.05$.

3. Results

3.1. Screening the *in vitro* effect of plant extracts on *A. carbonarius* growth and OTA production in the grape-based medium

The five natural extracts (i.e., chestnut male flower, cistus, eucalyptus, fennel, and orange peel) were tested at concentrations of 10 and 20 mg/mL for their antifungal activity in a grape-based medium. **Figure** 2 shows fungal growth for Ac52 as affected by the fungicidal preparations.

After 5-10 d of incubation, neither chestnut flower nor eucalyptus or fennel inhibited mycelial growth for either *A. carbonarius* strain when compared to the untreated control. On the other hand, orange peel and cistus extracts at both 10 and 20 mg/mL inhibited mycelial growth compared to the untreated control. When the extract concentration was increased, little to no variability was observed in rates of mycelial growth inhibition. Fenhexamid (0.75 mg/mL) also inhibited fungal growth after 5 days of growth, but not at day 10.

The amendment of the grape-based medium with the different extracts caused a macroscopic change in the *A. carbonarius* morphology when compared to the control. The extracts that inhibited radial growth also resulted in lower sporulation. Moreover, a difference in mycelial pattern was observed between Ac46 and Ac52 for the chestnut flower, cistus, eucalyptus, and fennel extracts.

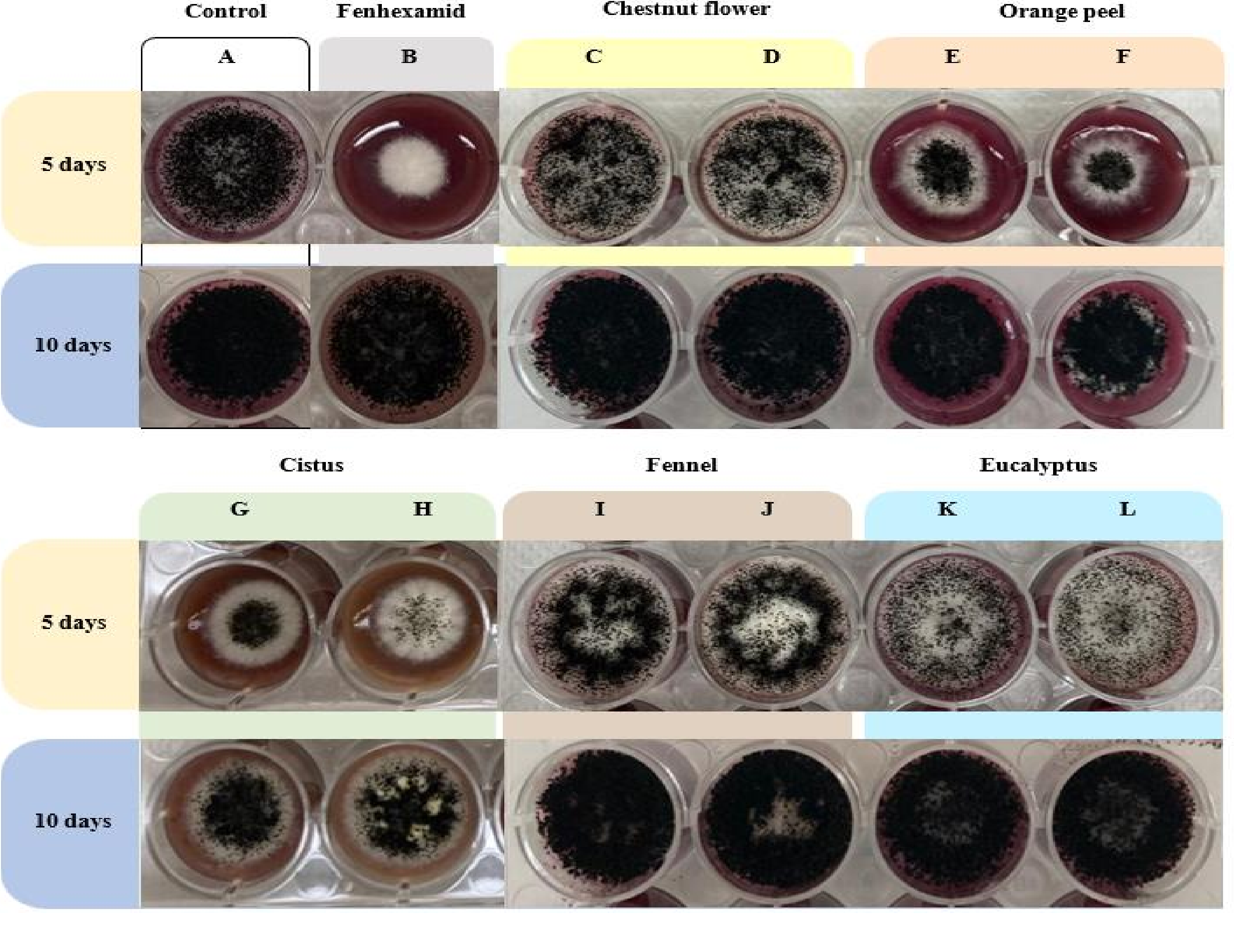


Figure 2. Morphological aspect of *A. carbonarius* MUM 04.52 in the grape-based medium at 25°C after 5 (top) and 10 (bottom) days of growth: (A) control; (B) fenhexamid (0.75 mg/mL), (C) male chestnut flower, 10 mg/mL; (D) male chestnut flower, 20 mg/mL; (E) orange peel, 10 mg/mL; (F) orange peel, 20 mg/mL; (G) cistus, 10 mg/mL; (H) cistus, 20 mg/mL; (I) fennel extract, 10 mg/mL; (J) fennel, 20 mg/mL; (K) eucalyptus, 10 mg/mL; (L) eucalyptus, 20 mg/mL.

3.2. Effects of selected extracts on OTA production in the grape-based medium

The two tested *A. carbonarius* strains differed significantly in their ability to produce OTA (Table 2). For Ac46, OTA was reduced significantly to 10.3 ± 2.7 ng/mL (85.7% reduction) when the grape medium was amended with eucalyptus at 10 mg/mL and to 27 ± 21.2 ng/mL (79.5% reduction) when the grape medium was amended with eucalyptus at 20 mg/mL. Similarly, amendment of the medium with 10 mg/mL of chestnut flower significantly reduced OTA to 13 ± 3.8 ng/mL (82% reduction). In contrast, the addition of cistus and orange peel extracts at 20 mg/mL activated OTA accumulation for Ac46 compared to the non-treated control (**Table 2**).

Regarding Ac52, of the natural plant extracts, cistus and eucalyptus reduced the OTA rate by up to 57.58% when used at 10 mg/mL and up to 62% at 20 mg/mL. However, the reduction in OTA was not significant in either case. In contrast, fennel (10 mg/mL and 20 mg/mL) treatments and orange peel (20 mg/mL) significantly activated OTA accumulation in the grape medium.

Table 2. Ochratoxin A production by *A. carbonarius* MUM 04.46 and *A. carbonarius* MUM 04.52 in grape medium supplemented with natural extracts (chestnut flower, cistus, eucalyptus, fennel, and orange peel) and fenhexamid, expressed as a percentage compared with the control (mean ± standard deviation, n = 3).

	Percentage of OTA	
Treatments	*A. carbonarius* MUM 04.46	*A. carbonarius* MUM 04.52
Chestnut flower (10 mg/mL)	-82% ± 5.3%**	+9.1% ± 9.3%
Chestnut flower (20 mg/mL)	-54% ± 45.3%	+33.6% ± 15.5%
Cistus (10 mg/mL)	+7.8% ± 9.6%	-29.6% ± 12.7%
Cistus (20 mg/mL)	+96.2% ± 18.7%	-62% ± 1.8%
Eucalyptus (10 mg/mL)	-85.7% ± 3.8%**	-40.7% ± 21.4%
Eucalyptus (20 mg/mL)	-79.5% ± 1.4%*	-16.1% ± 14%
Fennel (10 mg/mL)	+165.1% ± 8.4%	+141.1% ± 23.5%*
Fennel (20 mg/ml)	+1220.2% ± 123.3%	+154.8% ± 51.5%*
Orange peel (10 mg/mL)	-10.7% ± 3.4%	+56.6% ± 38.1%
Orange peel (20 mg/mL)	+5430.2% ± 453.6%	+206.3% ± 44.7%*
Fenhexamid (0.75 mg/mL)	-78.9% ± 1.6%	-95.4% ± 3%

Significance (Kruskal-Wallis + post hoc Dunn test), significant differences with respect to the control were declared as following *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

3.3. Growth assessment

Figure 3 shows the effect of the different treatments on Ac46 and Ac52 with regard to growth assessment. Concerning the µm, the natural extracts of cistus, and orange peel significantly slowed both Ac46 and Ac52. Similarly, fenhexamid significantly reduced µm, whereas no significant effects on fungal growth were observed for chestnut flower and eucalyptus extracts.

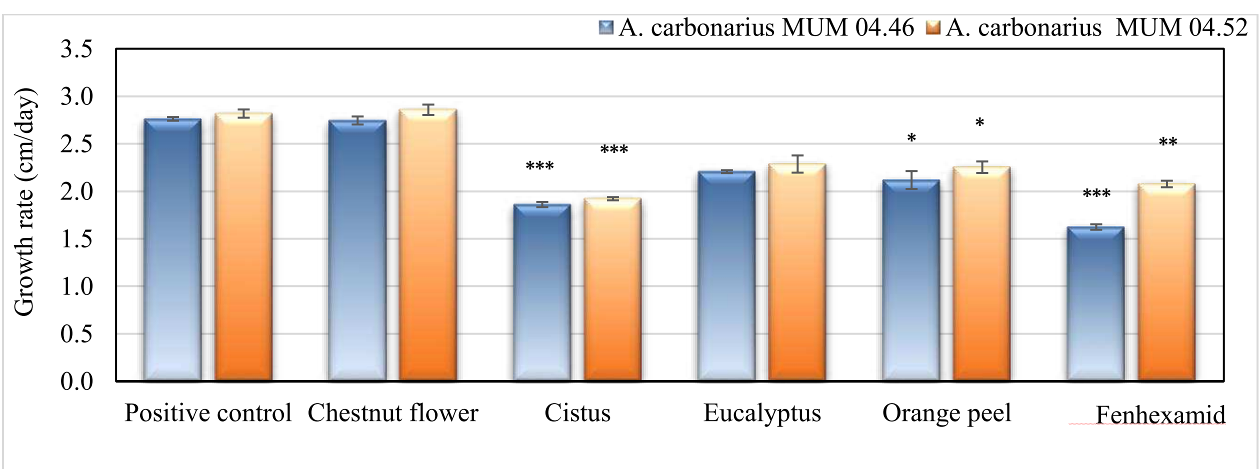


Figure 3. Maximum growth rate (µm, cm/day) of *A. carbonarius* MUM 04.46 and *A. carbonarius* MUM 04.52 grown for 6 days at 25 °C in a grape-based agar medium with the addition of different natural extracts (chestnut

flower, cistus, eucalyptus, and orange peel) and a commercial antifungal preparation (fenhexamid). Statistical differences between the non-treated control and treatments are indicated as following *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

3.4. Effect of plant extracts on *Aspergillus* rot severity in grapes

Based on the *in vitro* results, the natural extracts of chestnut flower, cistus, eucalyptus, and orange peel were selected for evaluation of their ability to modulate *Aspergillus* symptoms in grape berries at 10 mg/mL.

The relative levels of rot severity of the berries treated with the different plant extracts and with the fungicide, as determined by the McKinney index, are shown in **Figure 4**, and expressed as percentages in comparison to the non-treated control. The McKinney index of the negative control was 69% ± 1% for Ac46 and 68% ± 9% for Ac52. No significant difference was detected between the two MUM strains. The orange peel and cistus treatments significantly reduced rot symptoms for Ac46 and Ac52 compared to the untreated grapes, with inhibition levels that ranged between 24% and 33%. Chestnut flower extract reduced *Aspergillus* in grape berries significantly (by 33%) when applied for Ac52 but had no significant effect on Ac46. Eucalyptus extract had no significant effect on berry infection by Ac46 or Ac52 (**Figure 4**).

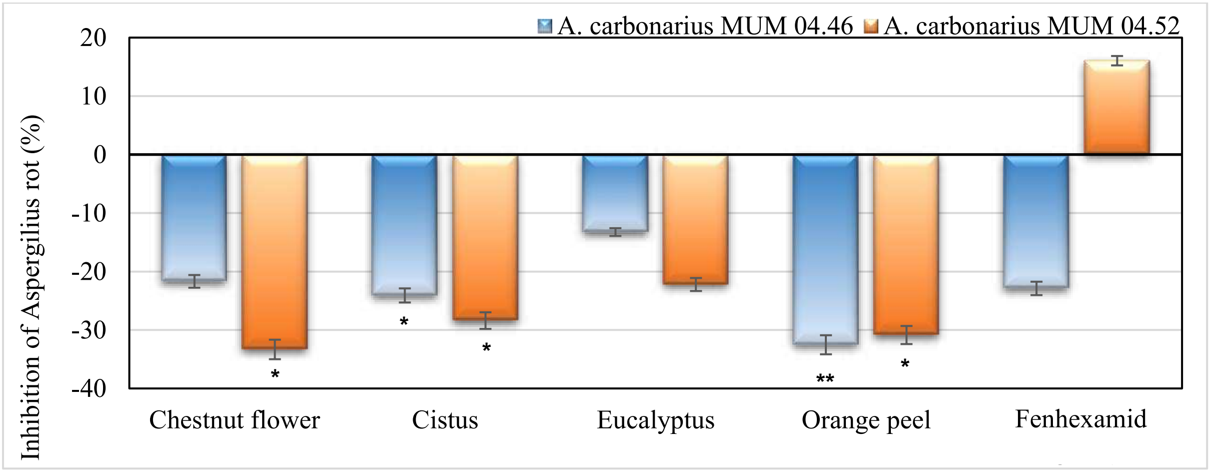


Figure 4. Rot severity caused by *Aspergillus carbonarius* MUM 04.46 and *A. carbonarius* MUM 04.52 in grape berries (cv. Touriga Franca) immersed in natural extracts (chestnut flower, cistus, eucalyptus, and orange peel) at 10 mg/mL and in a synthetic fungicide (fenhexamid 0.75 mg/mL), expressed as a percentage compared to the control (mean of three replicas, error bars represent standard deviation). Statistical differences between the non-treated control and treatments are indicated as following *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

3.5. Effect of plant extracts on OTA production in grape berries

After 10 days of incubation, no significant difference was found between the two *A. carbonarius* strains grown on artificially inoculated berries ($p = 0.1$) and OTA contamination observed on the non-treated grape berries (117.3 ± 60.4 ng/g for Ac46 and 63.5 ± 19.5 ng/g for Ac52).

All treatments reduced the OTA rate for both *Aspergillus* strains. Eucalyptus, orange peel, and cistus extracts caused a significant decrease in OTA for Ac46, with inhibition percentages that ranged from 67.7% to 82.3%. In contrast, no natural treatment had a statistically significant effect on OTA production by Ac52, and no significant difference was found between treatments ($p = 0.3$). Eucalyptus was found to give the highest inhibition rate for both *Aspergillus* strains (**Figure 5**).

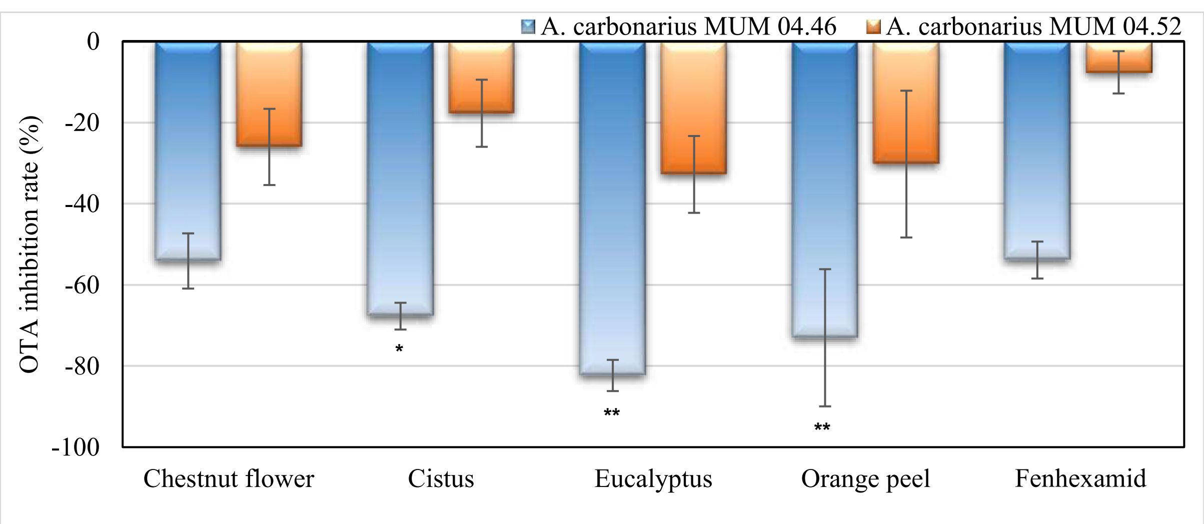


Figure 5. OTA production by *Aspergillus carbonarius* MUM 04.46 and *A. carbonarius* MUM 04.52 in grape berries (cv. Touriga Franca) immersed in natural extracts (chestnut flower, cistus, eucalyptus, and orange peel) at 10 mg/mL and in a synthetic fungicide (fenhexamid 0.75 mg/mL), expressed as a percentage compared to the control (mean of three replicas, error bars represent standard deviation). Statistical differences between the non-treated control and treatments are indicated as following *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

4. Discussion

Plant extracts, essential oils, and phenolic compounds are of interest to researchers as possible control agents against a variety of fungi. The aim of this study was to evaluate the potential of natural plant extracts to reduce OTA contamination and *Aspergillus* infection in grapes using an environmentally friendly extraction procedure.

A grape-based culture medium was used for the *in vitro* screening assay to simulate the growth and OTA production of two strains of *A. carbonarius* in grapes. Culture media prepared from food matrices have been reported as good model systems for the *in vitro* evaluation of fungal growth and mycotoxin production (Pardo et al., 2005), and they have been frequently used in studies involving major OTA-producing species, such as *A. niger* (Astoreca et al., 2009), *A. carbonarius* (Cervini et al., 2021), *Aspergillus ochraceus* G. Wilh. (Pardo et al., 2005), *Aspergillus westerdijkiae* Frisvad & Samson (Álvarez et al., 2023; Meftah et al., 2018; Vipotnik et al., 2017), and *Penicillium nordicum* Dragoni & Marino (Meftah et al., 2018; Vipotnik et al., 2017).

Both orange peel and cistus extracts showed an antifungal effect on *A. carbonarius*. *In vivo*, orange peel was the most promising extract for its capacity to inhibit *A. carbonarius* growth. The antifungal or antibacterial activity of extracts obtained from citrus plants has been frequently highlighted. Viuda-Martos et al. (2008) found that lemon, orange, mandarin, and grapefruit essential oils, obtained by cold-pressing the peel, reduced the growth of *A. niger* and *A. flavus* Link. Of these, orange essential oil was found to be the most effective inhibitor of *A. niger*, and mandarin was the most effective inhibitor of *A. flavus*. Here we report for the first time the antifungal activity of orange peel extract against *A. carbonarius*. Velázquez-Nuñez et al. (2013) attributed the antifungal properties of *Citrus sinensis* peel essential oil against *A.*

flavus to the presence of limonene as the most important compound (96.62%) of orange peel, followed by other molecules such as *β*-pinene, *β*-myrcene, *α*-pinene, and citral (*Z* and *E*). Limonene in the monoterpene form was also observed to have antifungal activities against *A. niger* (Jing et al., 2014). The antifungal activity of orange polyphenolic extracts was also reported on *Monilinia fructicola* (G. Winter) Honey, *Botrytis cinerea* Pers. and *Alternaria alternata* (Fr.) Keissl. At a concentration of 1.5 mg/mL, orange extracts totally inhibited the mycelial growth and conidial germination of these fungi (Hernández et al., 2021).

Of the extracts studied in the present work, cistus was highly effective *in vitro* and also reduced fungal growth *in vivo*. Our results agree well with the existing literature on cistus. Kalli et al. (2018) reported that the hydro-methanolic extract of cistus inhibited the growth of *A. parasiticus* Speare. by 46% when applied at a concentration of 0.2 mg/mL. Barros et al. (2013) have described the antifungal activity of cistus phenolic extract against *Candida* species when used at 0.625 mg/mL; they attributed this activity to the presence of phenolic acids and derivatives, ellagic acid derivatives, and flavonoids, specially catechins, flavonols, and flavones.

Overall, aqueous plant extracts can be powerful antifungals against several molds, as reported in literature. For example, aqueous extracts prepared from chestnut flowers have been studied for their capacity to inhibit the growth of *A. parasiticus* in a nutraceutical formulation, due to the presence of phenolic compounds, namely hydrolisable tanins (Fernandes et al., 2020). The same was recorded for methanolic extracts of fennel seeds, observed to inhibit *Candida albicans* (C.P. Robin) Berkhout. and *Aspergillus clavatus* Desm. at 25 µg/mL (Agarwal et al., 2017). Eucalyptus hydromethanolic extracts were described as being powerful antifungal agents against *Candida* species at minimum inhibitory concentrations (MICs) ranging from MIC_{50} = 0.1875 mg/mL to 1.5 mg/mL (Martins et al., 2015). However, plant extracts can have

a variable effect, depending on the matrix, fungal species and the extraction solvent (Akullo et al., 2022; García-Díaz et al., 2020; Lira-De León et al., 2014). It is also important to note that factors such as harvesting time, storage, and modification processes, among others, may have a substantial impact on the phytochemical content of plant extracts and, as a result, on their antifungal activity (Ali et al., 2018; ElNaker et al., 2021; Mandim et al., 2021; Shao et al., 2022).

With regard to OTA production, while conditions were the same for both *A. carbonarius* strains, Ac52 produced higher levels of mycotoxin *in vitro* than Ac46. The difference in mycotoxin production between different strains of the same fungal species grown in similar conditions is inherent to *Aspergillus* species and has been observed by other studies (Astoreca et al., 2009; Freire et al., 2018; Vipotnik et al., 2017).

Furthermore, the natural extracts had a strain-dependent effect on OTA production: eucalyptus was highly effective in controlling OTA for Ac46, followed in order of decreasing effectiveness by chestnut flower, at both tested concentrations, whereas the most effective extracts for Ac52 were cistus, followed by eucalyptus.

The capacity of plant extracts to modulate mycotoxin synthesis *in vitro* has long been documented. Ahmed et al. (2015) found that fenugreek seed extract significantly inhibited OTA production in grapes by *A. carbonarius*. Similarly, EL Khoury et al. (2017) used *Salvia officinalis* L. and *Melissa officinalis* L. at 5 µL/mL (essential oil) against *A.carbonarius* in a grape-based medium, and observed OTA reductions of 25% and 80%, respectively. However, despite evidence of the potential effectiveness of plant extracts in reducing OTA rate, the *in vitro* effect of natural extracts on mycotoxin accumulation appears to be largely dependent on the fungal strain, even within the same species.

For example, cistus, fennel, and orange peel extracts activated OTA production by Ac46 in our *in vitro* experiments, while chestnut flower, fennel, and orange peel extracts activated OTA production by Ac52. Several studies and review papers have reported that, under certain conditions and at specific concentrations, phenolic compounds (Boonmee et al., 2020; Chtioui et al., 2022; Etzerodt et al., 2015; Gauthier et al., 2016; Ponts et al., 2011), essential oils (Dammak et al., 2019; Lorán et al., 2022; Prakash et al., 2010) or even plant extracts (Garcia et al., 2011) can activate mycotoxin production.

However, in our study this was observed only in the *in vitro* trial, while all the extracts reduced mycotoxin production in grape berries, regardless of the strain. Although synthetic media are considered a good representation of food matrices (Pardo et al., 2005), they present a uniform distribution of nutrients that makes them easily accessible to fungi. Consequently, the interaction of the fungus with the synthetic media may differ from its behavior in grape berries. The differences in nutrient distribution and water availability between *in vivo* and *in vitro* conditions may also cause differences in mycotoxin production by *Aspergillus* (Maor et al., 2021). What may be an even more important factor is that fresh fruits continue their physiological activity after harvest, so post-harvest treatments can still activate fruit defense mechanisms to combat infection, namely by increasing defense-related enzymes and metabolites (Li et al., 2019; Li et al., 2022; Zixun et al., 2020). This might mean that *in vitro* models are less representative of the *in vivo* conditions of fresh fruits than of other types of food products, especially processed products.

Our findings are that all extracts reduced OTA production in grapes, with eucalyptus being particularly efficient. The reduction of OTA by orange peel extract may be correlated to the reduction of mycelial growth in grape berries. On the contrary, the eucalyptus extracts reduced OTA production by up to 82.3% without significantly affecting fungal growth, which suggests

that the mechanism of OTA inhibition could be distinct from that of mycelial inhibition. Similar results were reported by Bluma et al. (2008), who found that eucalyptus essential oil had no effects on mycelial growth or on spore germination in *Aspergillus* section *Flavi,* although it significantly reduced aflatoxin production. Our results are also in line with the study of Vilela et al. (2009), who reported an anti-aflatoxigenic effect of eucalyptus on *A. flavus* and *A. parasiticus*. However, to the best of our knowledge, ours is the first report demonstrating the anti-OTA activity of *E. globulus* extract on *A. carbonarius* in grapes and in a grape-based medium.

Teixeira et al. (2019) characterized the phenolic composition of eucalyptus aqueous extract collected from the same sampling area in Bragança, Portugal. They identified seven flavonoids (quercetin, isorhamnetin, and myricetin derivatives), three phenolic acids (chlorogenic acid and ellagic acid derivatives), and eight gallotannin derivatives. While quercetin is a powerful antimicrobial reported to alleviate OTA toxicity (Yang et al., 2020), chlorogenic acid is involved in the mechanism of cereal resistance to *Fusarium* and its deoxynivalenol detoxification (Atanasova-Penichon et al., 2012; Gauthier et al., 2016), and ellagic acid, together with ascorbic acid and α-tocopherol, is regarded as a major antioxidant molecule in plants (Ratnam et al., 2006; Sharifi-Rad et al., 2022). However, regardless of the plant extract and phenolic compound, the precise mechanisms behind ochratoxin inhibition are not fully unveiled. One hypothesis is that inhibition is linked to the disruption of the fungal membrane via a modification in its charge, hydrophobicity or porosity. Another hypothesis is that the antioxidant activity of the extract and its phenolic compounds reduces oxidative stress and therefore OTA production. Finally, plant extract and phenolic compounds might induce a downregulation in the key genes involved in OTA biosynthesis (Boonmee et al., 2020).

Our main findings are that orange peel and cistus can be efficient antifungals against *A. carbonarius* strains. Their ability to modulate fungal growth is of environmental interest. Orange peel is considered an agricultural waste (Farhat et al., 2011) and cistus is a Mediterranean shrub highly tolerant to drought and to poor soils (Zalegh et al., 2021). Their reuse as a fungicide could promote a circular economy and sustainability.

On the other hand, eucalyptus is an efficient botanical extract to use for OTA reduction. This is extremely important for preventing OTA contamination in vineyards and in harvested grapes. Therefore, the use of eucalyptus extract could provide a low-cost and environmentally friendly means of limiting OTA formation without disrupting fungal growth.

It is, however, possible that using eucalyptus on wine grapes may affect their organoleptic properties. To this regard, a winery-scale study by González-Rompinelli et al. (2013) tested eucalyptus as an alternative to sulfur dioxide for use during the aging of white wines in oak barrels, and found that the addition of eucalyptus phenolic extracts had no effect on the wine's organoleptic properties. Also, the use of chestnut flowers has been patented as a natural substitute of sulfites in wines (Patent No. WO2017212351A1). According to the results of their study, chestnut flowers had no negative effect on the wine's organoleptic properties, but actually increased the wine's flavor, which is usually affected by the presence of sulfites (Ferreira et al., 2016).

Nonetheless, further research will be required to investigate whether the selected plant extracts have any effect on the organoleptic properties of grapes.

5. Conclusions

The current study represents progress toward the production of environmentally acceptable plant-based fungicides to control *A. carbonarius* and OTA contamination in vineyards, and of marketable botanical formulations. However, further research is required to elucidate the chemical identity of the active compounds responsible for the reported antifungal and anti-mycotoxin effects of these extracts and to gain a better understanding of their mode of action. This should enable enhancement of the plant extracts' efficacy.

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Chapter 5

Summary
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Summary

Fusarium control should also consider living microorganisms that produce metabolites with antifungal activity. The potential of four *Bacillus* strains (AngB1, BV, F33, 54) to protect wheat from *Fusarium culmorum* (Wm.G. Smith) Sacc. infection and deoxynivalenol (DON) contamination was examined in this study. The bacteria was first used as a seed coat and resulted in an inhibition rate between 77 and 97% of *Fusarium* symptoms compared to the control. Particularly, *Bacillus* sp. BV induced a marked inhibition of up to 90%. *Bacillus* sp. BV was selected for further studies in field conditions. Used as a spray on wheat spikes, *Bacillus* sp. BV reduced Fusarium head blight by 67% and DON contamination on wheat caryopsis by 68% compared to the control. This microorganism provides an alternative biocontrol tool for the management of *Fusarium* and DON contamination.

Bacillus sp. strains as biocontrol agents against *Fusarium culmorum* on wheat

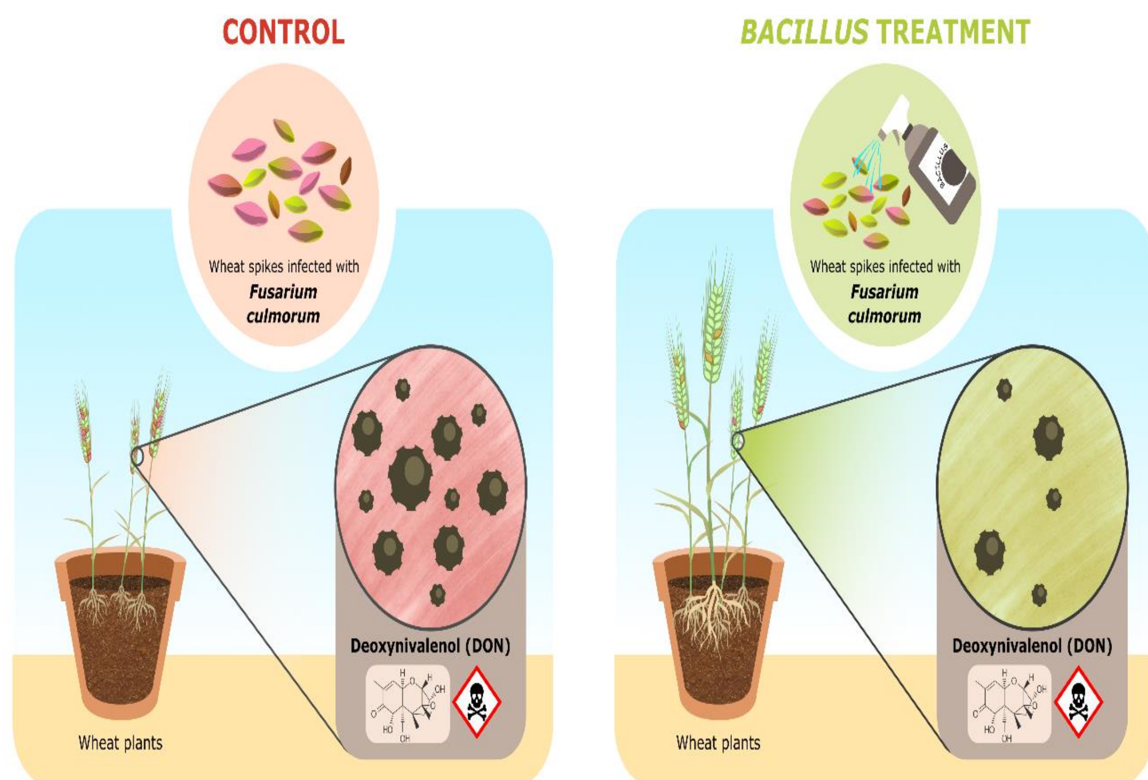
Abstract

Fusarium culmorum (Wm.G. Smith) Sacc. is the causal agent of both Fusarium head blight (FHB) and foot and root rot (FRR), which cause significant yield and quality losses in wheat crops. The use of chemical fungicides has placed a burden on the environment and has increased the risk of drug resistance, therefore biological control using microbial antagonists shows promise as an alternative approach for *Fusarium* management. The present study aimed to investigate four *Bacillus* sp. strains, coded AngB1, BV, F33, and 54, for potential application to protect wheat seedlings against *F. culmorum* infection and deoxynivalenol (DON) contamination as a consequence of FHB. Dual culture assays showed that all *Bacillus* sp. strains significantly inhibited the mycelial growth of the pathogen. Subsequently, their biocontrol and plant growth-promoting activity was evaluated *in planta* via seed coating. *Bacillus* F33 significantly increased the fresh weight of diseased wheat plants over non-inoculated plants grown in *Fusarium*-infested soil. Additionally, all *Bacillus* strains reduced disease severity (by 77% to 97%) in comparison to the control. The strain with the greatest biocontrol efficiency was BV, which had a significant effect on *Fusarium*. Therefore, *Bacillus* BV was selected for further investigation and tested for its ability to reduce FHB and DON when sprayed on wheat spikes. *Bacillus* BV application on wheat spikes was seen to reduce the disease index by 67%, although the high variability of data meant that this effect was not significant. However, *Bacillus* BV significantly reduced DON production by 68%. In conclusion, *Bacillus* BV demonstrated antagonistic activity against *F. culmorum* and reduced DON, while F33 proved capable of promoting plant growth traits. Most importantly, these strains have the potential for

use in eco-friendly, cost-effective, and sustainable management practices of FHB and FRR. Nonetheless, further investigations are still required to elucidate the mechanisms underlying the antifungal effect.

Keywords: *Bacillus* spp.; *Fusarium culmorum*; biocontrol; wheat; deoxynivalenol; biofungicides

Graphical Abstract



1. Introduction

Wheat (*Triticum aestivum*) is one of the world's most widely grown cereals, and an important source of nutrients such as proteins, carbohydrates, minerals, B-group vitamins, and dietary fiber (FAO, 2022; Shewry & Hey, 2015). It is the world's most important staple food and has great economic value. World wheat output has increased over time and amounted to over 776.6 million tons in 2022 (FAO, 2022).

However, around 20% of cereal crop production is lost each year to plant diseases, including fusariosis (Asaturova et al., 2022; Azizbekyan, 2019). *F. culmorum* (Wm.G. Smith) Sacc. is a soil-borne fungus that causes foot and root rot (FRR) and Fusarium head blight (FHB) on small-grain cereals such as wheat and barley (Scherm et al., 2013). *F. culmorum* produces mycotoxins, which can lodge in the grains. Deoxynivalenol (DON) and T-2 toxin are the main mycotoxins found in wheat. Mycotoxins are extremely stable and remain in grains until they are processed for human or livestock consumption; they are toxic for humans and animals, and can be fatal if consumed in large quantities (Perincherry et al., 2019).

To date, the use of fungicides has been the principal means of control for plant pathogens. However, long-term exposure to synthetic fungicides has frequently led to the emergence of resistant mutants among pathogenic fungi, and fungicide resistance is now regarded as one of the most serious challenges facing modern agriculture. The emergence of cross-resistance to azole has been observed in several fungi, including *Aspergillus fumigatus* Fresen. (Snelders et al., 2008), *Candida albicans* (C.P. Robin) Berkhout (Pfaller & Diekema, 2004), and *Cryptococcus neoformans* var. *grubii* Franzot, Salkin & Casadevall (Deising et al., 2008; Lucas et al., 2015), and this has had a negative impact on the clinical management of human fungal infections. Accordingly, rapid adaptation to resistance requires the continuous development of

variants of existing fungicides or the development of novel chemicals to prevent resistance accumulation (Fisher et al., 2018; Steinberg et al., 2020). The emergence of resistance to fungicides, together with their negative impact on the environment and on human health, will probably lead to major restrictions on the use of synthetic fungicides in agriculture in the future (Johns et al., 2022; Miller et al., 2022). In this context, the use of biological control agents can be a viable option for preventing fungal and mycotoxin contamination (Liu et al., 2013; Nunes, 2012).

The range of biological control strategies includes the use of antagonistic bacteria such as *Pseudomonas* and *Bacillus* spp. Described as biofactories, *Bacillus* spp. produce a plethora of biologically active molecules capable of inhibiting pathogenic fungi (Caulier et al., 2019; Wang et al., 2022). These antimicrobial compounds include cyclic lipopeptides, enzymes, bacteriocins, polyketides, and volatile compounds, which have important applications in the control of pathogenic fungi and thus give *Bacillus* strains advantages in a range of ecological niches (Miljaković et al., 2020; Tran et al., 2022). To date, several *Bacillus*-based biocontrol agents have been patented and are commercially available as biofungicides and biofertilizers in the European market: Serenade® (*Bacillus subtilis* QST 713) (EFSA et al., 2021), TAEGRO® (*Bacillus amyloliquefaciens* FZB24) (EFSA, 2016), Amylo-X® (*Bacillus amyloliquefaciens* D747) (EFSA, 2014), Quantum-400® (*Bacillus subtilis* GB03) (Radhakrishnan et al., 2017), and YIB® (*Bacillus* spp.)(Radhakrishnan et al., 2017). However, the market still requires specimens adapted to the local environment that display potential biocontrol capacities against *F. culmorum*.

With the aim of developing biological control against *F. culmorum* and DON contamination using antagonistic microorganisms, autochthonous *Bacillus* strains from Sardinia were isolated from several food matrices. The objectives of the present work were as follows: (i) to explore

the antifungal potential and control efficiency of the four *Bacillus* sp. strains (BV, F33, 54, Ang B1) against *F. culmorum;* (ii) to determine the possible growth-promoting effect of *Bacillus* sp. strains on wheat seedlings; (iii) to determine the effect of *Bacillus* sp. strains on FHB disease.

2. Methodology

2.1. Microorganisms and culture media

Four bacterial strains AngB1, BV, F33, and 54 were isolated, respectively, from the following food matrices in Sardinia: brewers' spent grain, Vermentino grape must, fruit pulp, and olive oil. The isolates were deposited at the microbial culture collection of the University of Sassari (UNISSMMC) and identified as belonging to the *Bacillus amyloliquefaciens* operational group and *Bacillus subtilis* complex, based on analysis of *16S rRNA, recA,* and *RpoB* sequences. Cultures were grown overnight at 30°C on yeast extract peptone dextrose (YEPD) when required. They were preserved at 4°C for short-term storage and in 30% (v/v) glycerol at -80 °C for long-term storage.

This study used *F. culmorum* (UK99) from the culture collection of the Department of Plant Pathology at the University of Sassari (Urban et al., 2016). The fungus was regularly cultured on potato dextrose agar (PDA; Sigma-Aldrich, St. Louis, MO, USA). For long-term storage, mycelial plugs were transferred to 50% (v/v) glycerol and preserved at −80 °C.

2.2. Screening for salt, temperature, and pH tolerance

Starter cultures were prepared by transferring a single colony of each *Bacillus* sp. into 50 mL of liquid YEPD medium, followed by incubation at 30 °C with 150 rpm for 18h. Subsequently, 5 µL of a cell suspension from the preculture containing 10^9 CFU ml^{-1} was then spotted onto

(i) YEPD supplemented with various NaCl concentrations (0.5 M, 1 M, 1.5 M and 2 M) and incubated for 24 h at 30 °C to evaluate NaCl tolerance; (ii) YEPD incubated at 5 °C, 10 °C, 25 °C, 30 °C, 37 °C, and 45 °C for the evaluation of optimal growth temperature; (iii) YEPD pH = 3, 5, 7 and 9 at 30 °C for evaluation of the optimal pH. Bacterial growth was observed after 24, 48, and 120 hours.

2.3. Bacterial effect on fungal growth *in vitro*

2.3.2. *In vitro* antifungal activity assay

Antagonistic tests were performed in YEPD plates using a dual culture assay to investigate the biocontrol effect of *Bacillus* sp. strains AngB1, BV, F33, and 54 against *F. culmorum*. A 5-mm diam. A plug of agar colonized by *F. culmorum* was placed at the center of the YEPD plate and a loopful of fresh overnight bacterial cells was streaked 1.5 cm from the fungal block on both sides. Other plates containing only the fungus were used as the control. Treatments were performed in duplicate, and the plates were incubated in static conditions at 25 °C for 7 days. Growth was recorded on a daily basis, and the growth rate was calculated as the slope of the line of the linear regression of colony radii plotted against the incubation time and compared to the control.

2.4. *In vivo* antagonistic assay

2.4.1. Preparation of bacteria and pathogen spore suspensions

Bacillus sp. strains were grown in 250 mL baffled flasks with 150 mL liquid YEPD medium (1% inoculum from a 24 h starter culture) for 72 h at 30 °C in a rotary shaker (150 rpm). Biomass was harvested by centrifugation at 3500 rpm for 8 min. The cell pellet was

resuspended in 50 mL of gellan gum water solution (0.1% w/v) for seed coating (final cell density 10^9 CFU mL^{-1}).

F. culmorum UK99 was grown in a 250 mL flask with 100 mL of carboxymethyl cellulose medium on a rotary shaker (200 rpm) at 25 °C for 3 to 5 days to produce conidia. A conidial suspension was prepared by filtering the culture through sterile filter paper to remove mycelia. The concentration of the suspension was adjusted to 10^4 conidia/mL for the following experiments using a Neubauer chamber.

2.4.2. Seed coating

Wheat seeds were surface disinfected by immersion in 1% sodium hypochlorite for 1 min and then rinsed three times with sterile water. Coating treatment consisted of soaking seeds for 30 min in the gellan gum bacterial suspensions. The bacterial cell concentration for the treatment was approximately 10^9 CFU mL^{-1}. The control treatment consisted of seeds soaked in sterile water. After coating, wheat seeds were left to dry under laminar flow.

2.4.3. Pot test

This experiment used plastic pots (140 x 110 x 50 mm) containing 140 g of commercial soil (Vigor Plant®, Italy). Firstly, coated or uncoated wheat seeds were sown in the soil at a depth of 2 cm. Subsequently, 250 µL of the conidial suspension of *F. culmorum* (10^4 CFU mL^{-1}) was inoculated into the soil in contact with the wheat seed in order to induce disease. The tests were conducted at 20–25 °C with 12 h photoperiod for 15 days. Each treatment was performed in triplicate, with nine seeds in each pot. Pots were arranged in a randomized design. After 15 days, plants were uprooted in order to evaluate symptoms, and the severity of *F. culmorum* was recorded on a 0–4 visual scale where 0 = no symptoms; 1 = light yellowing of leaves, light or

moderate rot on taproot and secondary roots, and crown rot; 2 = moderate yellowing of leaves with or without wilting, stunting, moderate rot on taproot and secondary roots, crown rot with or without hypocotyls rot, and vascular discoloration in the stem; 3 = severe yellowing of leaves with or without wilting, stunting, severe rot on taproot and secondary roots, crown rot with or without hypocotyls rot, and vascular discoloration in the stem; 4 = dead seedlings(McKinney index; (McKinney, 1923).

The disease severity percentage was determined using the following formula:

(∑scale × number of plants infected) / (highest scale × total number of plants) × 100

The fresh weight of the harvested wheat plants was recorded, and dry weights of samples were also measured after wheat seedlings were oven-dried at 45 °C for 24 h.

2.6. Greenhouse head infection assay

A greenhouse assay using the *Bacillus* BV strain was performed at the Center for Conservation and Valorization of Plant Biodiversity (Alghero, Italy) of the University of Sassari, using durum wheat (*Triticum durum)* cv. “Saragolla”, which is susceptible to *F. culmorum*. Six wheat spikes were sprayed with a water suspension of *Bacillus* BV at 10^9 CFU mL^{-1} and a treatment with water only was used as a control. A total of 2 mL was sprayed onto each spike. The treated spikes were spray-inoculated 24 h later with 250 µL of 10^4 CFU mL^{-1} of *F. culmorum* UK99 inoculum, and then covered with plastic bags for 48 hours to create an adequate moisture level for infection. Three replicates were performed for each treatment, and the experiment was repeated three times, at 1-week intervals. The results of this experiment were used to calculate the disease index (McKinney, 1923) and total DON in wheat caryopsis.

2.7. Analytical method, instruments, and equipment for the detection of DON

2.7.1. Sample preparation

Wheat caryopsis (5.0 g) were finely ground and transferred to a 50 mL centrifuge tube with 10 mL of water; 10 mL of 10% (v/v) HOAc in ACN was added and vortexed for 1 minute at high speed. Phenomenex extraction kit KSO-8909 (Agilent Technologies, Santa Clara, CA, USA) was added and hand-shaken for 1 minute (4.0 g MgSO4, 1.0 g NaCl, 1.0 g sodium citrate tribasic dehydrate SCTD, and 0.5 g sodium citrate dibasic sesquihydrate SCDS). After centrifuging the mixture at 4000 rpm for 5 minutes, the supernatant was transferred to a 15 mL centrifuge tube containing the KSO-8924 purification kit (900 mg MgSO4, 150 mg PSA). The mixture was then shaken over a vortex mixer (3000 rpm for 30 seconds) and centrifuged for 5 minutes at 4000 rpm. An aliquot of the supernatant (2 mL) was evaporated to dryness under a nitrogen gas stream and reconstituted in 1 mL of LC-MS mobile phase (A: 5 mM ammonium acetate in methanol with 0.5% acetic acid, B: 5 mM ammonium acetate in methanol with 0.5% acetic acid). Prior to LC-MS analysis, the extracts were filtered through 0.22 m PTFE syringe filters.

2.7.2. Q-Orbitrap-HRMS analyses

Mycotoxins were separated using an Agilent 1200 LC (Agilent Technologies, Santa Clara, CA, USA). Eluent A was 5 mM ammonium acetate, 0.5% acetic acid, and Eluent B was 5 mM ammonium acetate in methanol, 0.5% acetic acid. The gradient elution was carried out at a flow rate of 0.4 mL min−1 and for 11 minutes. Time program = 0 min, 95% A/B; 2 min, 95% A/B; 5 min, 70% A/B; 10 min, 70% A/B. Injection volume: 5 µL. The column was then washed for 3 minutes with 50% B before being reconditioned for 3 minutes with the initial mobile phase composition. The column compartment was kept at 37◦C. The HPLC system was linked to a Thermo Fisher Scientific Q ExactiveTM Hybrid Quadrupole-Orbitrap High Resolution

Mass Spectrometer HRMS (San Jose, CA, USA). HRMS used a heated electrospray ionization source (HESI-II) and ran in full scan mode (m/z 100-600) with a resolving power of 140,000 FWHM. Spray voltage, 3.3 kV; sheath gas flow rate (N2) 35 units; capillary temperature 300 C; S-lens RF level 50; heater temperature 350°C were the HESI parameters conditions. The maximum inject time was 0.1 s, and the automatic gain control (AGC) was set to 1×10^{-6}. Nitrogen was used as both a collision and damping gas (nitrogen generator Zefiro; Clantecno-logica, Seville, Spain). PierceTM negative/positive ion calibration solution (Thermo Fisher Scientific, Rockford, IL, USA) was used for external calibration every three days to maintain a working mass accuracy of less than/equal to 5 ppm. For LC-MS control and data processing, Thermo Fisher Scientific's XCalibur 2.2 and Trace Finder 3.0 (San Jose, CA, USA) were used. The limit of detection (LOD) was calculated by injecting scalar dilutions of analyte standards in triplicate and analyzing the response standard deviation and slope. LOD was calculated as LOD = 3.3σ /S, where = standard deviation of the response, calculated as σ = the standard deviation of the regression line's y-intercepts, and S = slope of the calibration curve. The LOQ is the lowest assessed concentration that can reliably produce an analyte response that is both accurate (100 % recovery) and precise (relative standard deviation RSD of 20%).

2.8. Statistical analysis

Data were analyzed by ANOVA and mean values were compared using Tukey’s test at the 5% significance level, while R software was used for statistical analysis (R Core Team, 2020).

3. Results

3.1. Characterization of *Bacillus* strains

All the *Bacillus* colonies grew well in a temperature range between 25 °C and 45 °C after 24h of incubation. They were also able to grow in a pH range between 5 and 9 after 48h. With regard to salinity, *Bacillus* sp. BV, F33, and 54 were able to grow in YEPD media with a salinity concentration between 0.5 and 2 M after 48h; *Bacillus* sp. AngB1 was less tolerant to salinity and was able to grow between 0.5 M and 1.5 M after 120h of incubation **(Table 1)**.

Table 1. Bacterial growth in YEPD plates at different temperatures (°C), pHs, and levels of salinity (M) after 24, 48, and 120 hours of incubation (Bacterial concentration of 10^9 CFU/ml).

Bacillus sp.	Tested parameter											
	Temperature (°C)	**Hours**			**pH**	**Hours**			**Salinity (M)**	**Hours**		
		24	**48**	**120**		**24**	**48**	**120**		**24**	**48**	**120**
AngB1	5	-	-	-								
	10	-	-	-	3	-	-	-	0.5	+	+	+
	25	+	+	+	5	-	+	+	1	+	+	+
	30	+	+	+	7	+	+	+	1.5	-	-	+
	37	+	+	+	9	+	+	+	2	-	-	-
	45	+	+	+								
BV	5	-	-	-								
	10	-	-	-	3	-	-	-	0.5	+	+	+
	25	+	+	+	5	-	+	+	1	+	+	+
	30	+	+	+	7	+	+	+	1.5	-	+	+
	37	+	+	+	9	+	+	+	2	-	+	+
	45	+	+	+								
F33	5	-	-	-								
	10	-	-	-	3	-	-	-	0.5	+	+	+
	25	+	+	+	5	-	+	+	1	+	+	+
	30	+	+	+	7	+	+	+	1.5	-	+	+
	37	+	+	+	9	+	+	+	2	-	+	+
	45	+	+	+								
54	5	-	-	-								
	10	-	-	-	3	-	-	-	0.5	+	+	+
	25	+	+	+	5	-	+	+	1	+	+	+
	30	+	+	+	7	+	+	+	1.5	-	+	+
	37	+	+	+	9	+	+	+	2	-	+	+
	45	+	+	+								

3.2 *Bacillus* sp. antifungal activity *in vitro*

Dual culture assays showed that the four *Bacillus* sp. strains significantly decreased the growth rate of *F. culmorum,* and that *Bacillus* F33 had the most effective antifungal activity **(Figure1)**. The inhibition rate ranged between 61 and 78% compared with the control. The confrontation test on YEPD plates also showed that *Bacillus* strains exerted a fungistatic effect against the phytopathogenic fungus *F. culmorum*.

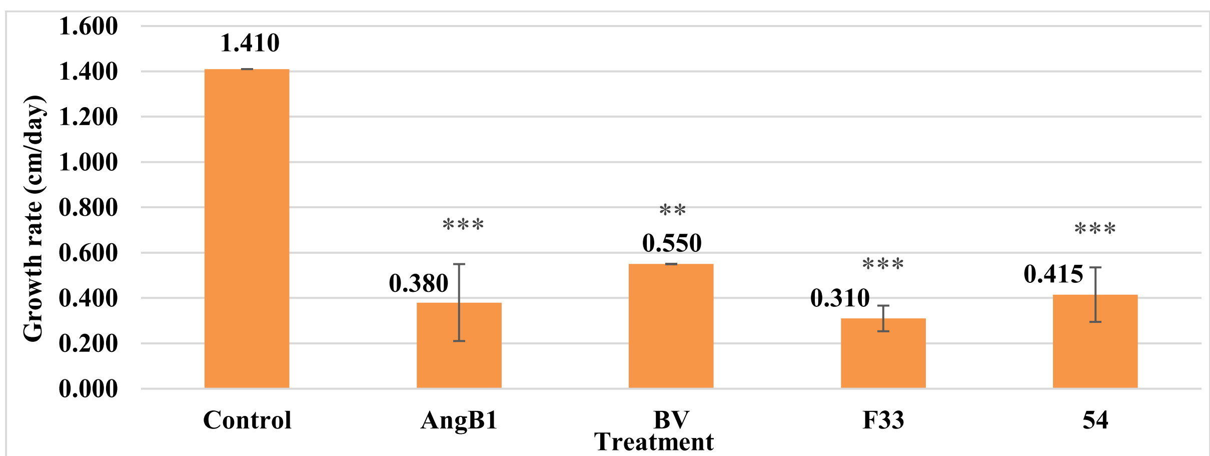


Figure 1. Effect of *Bacillus* strains (AngB1, BV, F33, and 54) on the growth rate of *F. culmorum* over seven days. Data represent mean (n = 2) ±, standard deviation. The level of significance was expressed in comparison to the control as follows: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3. Effect of *Bacillus* strains on *F. culmorum* in the field

3.3.1. Seed biocontrol assessment *in planta*

Pot experiment data were collected 15 days after inoculation, and the results of the assay are shown in **Figure 2.** The average disease severity on the wheat seedlings infected by *F. culmorum* but without *Bacillus* treatment was 54 ± 11%. Although no significant protection against *F. culmorum* was observed on seedlings treated with *Bacillus* AngB1, F33, and 54, these strains reduced FRR by 77-87%. In contrast, *Bacillus* BV was observed to be the most effective treatment, and had a significant effect on reducing *Fusarium* severity; it reduced FRR

by 97% (p = 0.04). Therefore, BV was selected for further research on disease and DON inhibition in wheat spikes **(Figure 2)**.

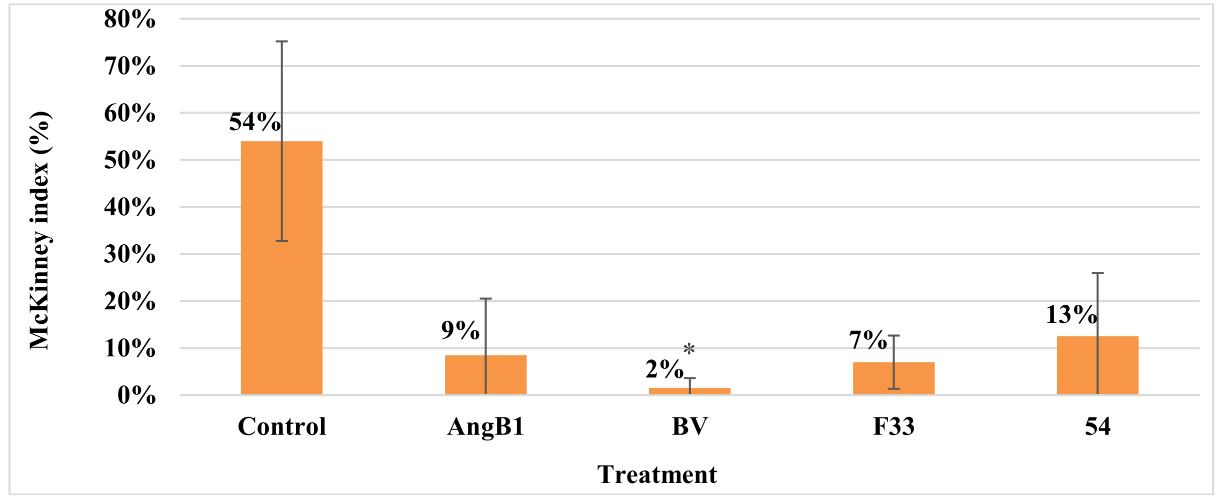


Figure 2. Effect of *Bacillus* strains (AngB1, BV, F33, and 54) on FRR on wheat seedlings 15 days after treatment. Data represent the mean of replicates (n = 3) ± standard deviation. The level of significance was expressed as follows in comparison to the control: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3.2. *Bacillus* strains improved the biomass of wheat plants infected by *F. culmorum*

In the absence of *Fusarium*, the fresh and dry weights of wheat plants inoculated with *Bacillus* were no different from the control **(Table 2)**. However, in the presence of *Fusarium*, a significant (p = 0.03) effect of seed coating with *Bacillus* sp. was observed on the fresh of the infected plants. In particular, the fresh weights of the diseased plants treated with F33 were statistically higher than those of the diseased control plants **(Table 3)**.

The effect on growth promotion of fresh weight was of borderline significance for diseased plants seed-coated with *Bacillus* BV (p = 0.062) and *Bacillus* 54 (p = 0.067) compared with the diseased control plants.

Table 2. ANOVA statistical analysis performed with data about the fresh weight of wheat plants infected with *Fusarium* without *Bacillus* (control) and with *Bacillus* treatment (AngB1, F33, BV, 54)

	P values of the tested treatments				
	Control	AngB1	F33	BV	54
Control	-	0.241	0.041*	0.062	0.067

AngB1	0.241	-	0.475	0.690	0.722
F33	0.041	0.475	-	0.991	0.983
BV	0.062	0.690	0.991	-	0.999
54	0.067	0.722	0.983	0.999	-

*Indicates statistical differences ($p \leq 0.05$)

Table 3. ANOVA statistical analysis performed with data about the dry weight of wheat plants infected with *Fusarium* without *Bacillus* (control) and with *Bacillus* treatments (AngB1, F33, BV, 54)

	P values of the tested treatments				
	Control	AngB1	F33	BV	54
Control	-	0.959	0.881	0.889	0.898
AngB1	0.959	-	0.999	0.999	0.999
F33	0.881	0.999	-	0.999	0.999
BV	0.889	0.999	0.999	-	0.999
54	0.898	0.999	0.999	0.999	-

*Indicates statistical differences ($p \leq 0.05$)

3.4. Greenhouse evaluation

3.4.1. Effect of BV strain on *F. culmorum* sprayed on spikes in the greenhouse

In order to test its effectiveness under greenhouse conditions, BV was further evaluated for its ability to protect wheat spikes against *F. culmorum* via spraying. Pathogen inoculation in the untreated control resulted in 50% disease severity. Although treatment with BV did not result in a significant reduction in symptoms in comparison to the control, due to the high variability of data, it reduced FHB by 67% **(Figure 3)**.

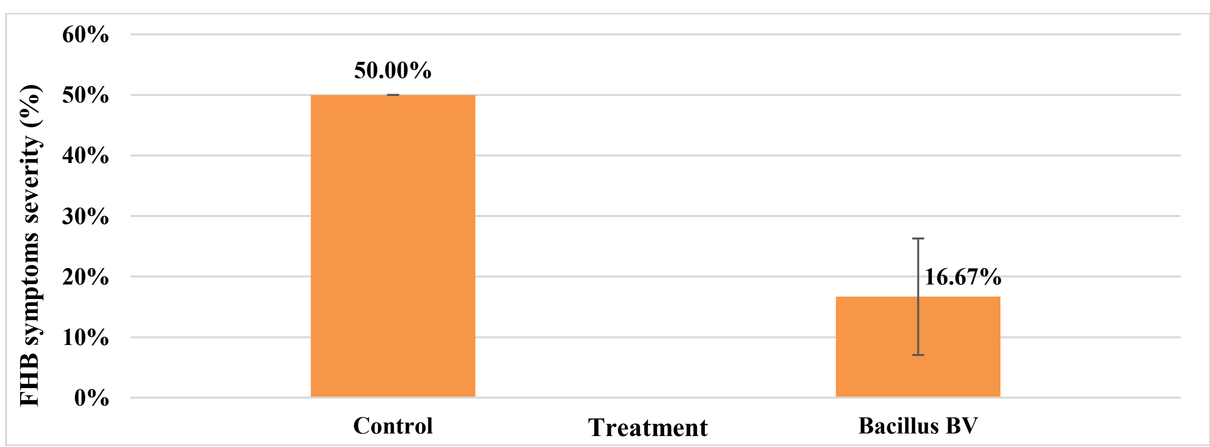


Figure 3. Effect of *Bacillus* BV on FHB after 15 days' growth. The values represent means of replicates (n = 3) ± standard deviation. No significant effect was observed between the control and *Bacillus* sp. BV.

3.4.2. Effect of *Bacillus* strain BV on DON production by *F. culmorum* on wheat caryopsis

Bacillus strain BV was selected for assessment of its impact on DON production on wheat caryopsis **(Figure 4)**. The level of DON production for *F. culmorum* not exposed to bacteria, was 6000 ng/g. Treating wheat spikes with *Bacillus* strain BV significantly reduced DON synthesis by 68% ($p = 0.001$).

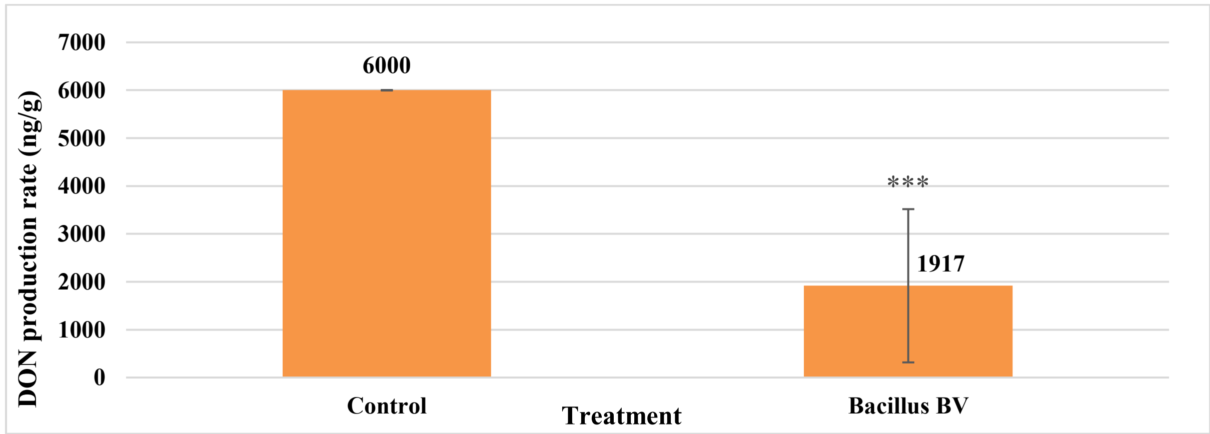


Figure 4. Effect of *Bacillus* BV on DON production by *F. culmorum*. The values represent means of replicates (n = 3) ± standard deviation. The level of significance was expressed as follows in comparison to the control: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

4. Discussion

F. culmorum reduces crop yields and the phytosanitary quality of wheat through the accumulation of mycotoxins in contaminated products. Microorganisms such as *Bacillus* spp. are attractive candidates for biological control of wheat diseases (Zalila-Kolsi et al., 2016). The present study used two complementary approaches, *in vitro* and *in planta* assays (at the seedling and anthesis stages), to evaluate the potential of four autochthonous *Bacillus* strains from Sardinia against *F. culmorum*. All *Bacillus* isolates showed promising antifungal activity *in vitro*. Coating wheat seeds with *Bacillus* was found to provide efficient protection against *Fusarium* seedling infection and to reduce FRR. The strong antagonistic capacities of the *Bacillus* strains observed in this work agree with a number of reports on the biocontrol

capabilities of *Bacillus* spp. such as *B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis* against various *Fusarium* spp. (Khan et al., 2018; Kim et al., 2022; Li et al., 2022; Zhang et al., 2022)

The antagonistic effect of *Bacillus* has been linked to the synthesis of a broad spectrum of chemical compounds (Dame et al., 2021). Among the antimicrobial and antifungal secondary metabolites produced by members of *Bacillus* species are lipopeptides (Rani et al., 2021). These include surfactin, iturin, and fengycin (Jasim et al., 2016). Surfactin's primary antifungal mechanism is to destroy and lyse the lipid membrane of pathogenic fungi (Lima et al., 2018). Iturin and fengycin, on the other hand, induce changes in the surface tension of the fungal cell membrane, resulting in the formation of micropores and the leakage of K+ and other important ions, thus causing cell death (Banerjee et al., 2022; Lei et al., 2019). When tested for seed coating, *B. subtilis* was found to gradually release iturin A in the soil and to reduce infection by pathogenic fungi such as *Rhizoctonia solani* (Zohora et al., 2016). Fengycin derived from *B. amyloliquefaciens* induced deformation in fungal hyphae of *F. graminearum* (Hanif et al., 2019). Furthermore, *Bacillus* strains secrete lytic enzymes such as glucanase, chitinase, and cellulase, which can play a key role in defending plants against fungal pathogens. Lytic enzymes produced by *B. velezensis* were shown to damage the cell wall of *F. pseudograminearum* and to cause apoptosis in the pathogen’s cells, thereby reducing the disease index in wheat by 66% (Zhang et al., 2022).

Bacillus strains also produce different types of diffusible and volatile compounds (VOCs) involved in the inhibition of pathogenic fungi growth and spore germination. VOCs produced by *B. amyloliquefaciens* strain NJN-6 were seen to exert antifungal activity against *F. oxysporum* f. sp. *cubense in vitro* and antagonize this pathogen in the soil (Yuan et al., 2012). Further investigations are needed to ascertain whether the antifungal effect of *Bacillus* strains

on *F. culmorum* observed in our study could be the result of one or more of the reported antifungal compounds.

In addition to biocontrol activity, seed coating with *Bacillus* sp. F33 significantly increased the fresh weight of wheat seedlings infected by *Fusarium*. Treated plants presented a significantly enhanced biomass compared with the non-inoculated control. These results agree with the growth promotion attributes of *Bacillus* spp. reported in the literature. *Bacillus* strains have been seen to influence and enhance the growth of several cereal species, including wheat and rice (Chithrashree et al., 2011; Rajer et al., 2022; Wang et al., 2013; Yasmin et al., 2016). These results could be explained by the ability of the *Bacillus* group to produce growth regulators, such as auxin, which are important in regulating plant growth via cell differentiation and expansion. Auxin is a key signal for root development, increasing the surface area of root systems in contact with soil and resulting in improved nutrient and water uptake, which ultimately improves yields. Likewise, Park et al. (2017) demonstrated that auxin produced by *B. licheniformis* MH48 can increase the dry weights of *Camellia japonica* seedling leaves and roots by 2.6 and 2.2-fold, respectively, compared to the control. Moon et al. (2021) reported that treatment using *B. velezensis* CE 100 bacterial inoculation significantly increased seedling growth and biomass production of *C. obtusa* seedlings. Tu et al. (2016) observed that seed coating with *B. subtilis* increased not only the fresh and dry weight of cotton seedlings but also their height and root length.

Anthesis is a critical stage for wheat infection, due to the high level of expression of stimulants like choline and betaine, which can stimulate *Fusarium* and contribute to pathogen infection of the ears. When it was sprayed at anthesis, *Bacillus* sp. BV reduced FHB severity by 38% in our study. Similar results were obtained by Zhao et al. (2014), who found that *B. subtilis* had a strong antifungal effect when applied in the field, and significantly reduced the FHB index. In

the same study, ultrastructure examination showed that the antifungal effect of *B. subtilis* is linked to the production of chitinase, fengycin, and surfactins, which damage the cell wall of *Fusarium* hyphae (Zhao et al., 2014).

Finally, the selection of antagonists that not only inhibit pathogen growth and sporulation but also reduce DON is critical to the biocontrol of *Fusarium* (Zhao et al., 2014). In our study, the BV strain reduced DON production by *F. culmorum*. This result agrees with the effect of *Bacillus* strains on DON reported in the literature. He et al. (2009) tested nine *Bacillus* isolates and reported that these reduced DON significantly (by 32-100%) in wheat spikes. According to Kim et al. (2017). *B. amyloliquefaciens* reduces trichothecene production and could be used as a chemosensitizer to chemical fungicides. Interestingly, some studies have focused on single groups of lipopeptides produced by *Bacillus* spp. on mycotoxins. For instance, purified fengycins from *B. amyloliquefaciens* extract were able to reduce DON, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, and zearalenone production in infected wheat grains (Hanif et al., 2019). In addition, fengycin inhibited patulin production and the gene expression of patulin in *Penicillium expansum* (Fu et al., 2022). Iturin A has been shown to inhibit the production of ochratoxin A by *Aspergillus carbonarius*, while a mixture of surfactins and fengycins produced by *B. mojavensis* inhibited T-2 and HT-2 toxin production by *F. oxysporum* (Galitskaya et al., 2022). Moreover, some *Bacillus* spp. have been shown to degrade mycotoxins, and it has been reported that *B. licheniformis* and *B. subtilis* degrade zeralenone (Fu et al., 2016; Zhang et al., 2021).

5. Conclusion

Autochthonous strains of *Bacillus* originating from Sardinia displayed capacities to control the phytopathogenic fungus *F. culmorum* on wheat. The results of this work suggest that *Bacillus*

sp. BV examined here is a promising candidate for use as a biocontrol agent against *F. culmorum,* due to its capacity to reduce fungal growth parameters and DON levels in wheat. Future studies will focus on the action mechanisms facilitating biological control by seeking to identify the bioactive compounds responsible for these antifungal effects. In addition, sequencing of the entire genome of the biocontrol candidate would contribute to the identification and understanding of the genetic and physiological mechanisms involved in its controlling effects on *F. culmorum*.

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Chapter 6

Discussion
Conclusions and Perspective
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Discussion

Fungi of the genus *Aspergillus* and *Fusarium* constitute a threat in both agriculture and medicine. Not only do they cause economic and food losses due to mycotoxin contamination, but they are also responsible for serious diseases in humans and animals, which range from allergic syndromes to life-threatening invasive fungal diseases and affect over one billion people worldwide (Fisher et al., 2022). Moreover, the resistance of these two genera to synthetic antifungals has now become a major global problem. In fact, the resistance of *Aspergillus* and *Fusarium* spp. is not limited to azole fungicides, since progressive loss of echinocandin activity has also been reported in clinical isolates. Furthermore, the continued use of fungicides also drives the development of resistance to all major classes of fungicides, including benzimidazoles, anilinopyrimidines, strobilurins, succinate dehydrogenase inhibitors, and the sterol demethylation inhibitors (Johns et al., 2022). One of the challenges involved in the management of these fungi and the containment of drug resistance in both agriculture and medicine consists of the limited pool of antifungal drug classes (Fisher et al., 2022). In this context, the present PhD project aimed to explore various approaches with the intention of contributing to the identification and synthesis of naturally-derived eco-friendly fungicides for possible use in the management of *Aspergillus* and *Fusarium* pathogens.

In summary, the main findings from this thesis were as follows: i) Phenolic compounds can be esterified and etherified to increase their antifungal activity against *Fusarium*. In particular, *p*-coumaric acid 3,3′-dimethyl allyl ester (13) demonstrated an antifungal efficacy that was comparable to synthetic antifungal (amphotericin B) and may thus provide a promising alternative to amphotericin B against *Fusarium*. ii) Aqueous extracts of cistus and orange peel, at 10mg/mL, significantly reduced the vegetative growth of *A. carbonarius* on grape berries *in vitro* and *in vivo*, while eucalyptus extracts were effective in reducing OTA biosynthesis in

grape berries. iii) *Bacillus* seed coating on wheat reduced FRR symptoms and increased fresh and dry weights (*Bacillus* sp. F33). In addition, *Bacillus* sp. BV was found to reduce FHB and DON in wheat grain when sprayed at anthesis. These findings suggest that biofungicides based on phytochemicals or microorganisms (alone or in combination) could offer a viable alternative to synthetic fungicides for use in agriculture.

The natural fungicides of plant origin proposed in this study were chosen as being eco-friendly, cost effective, and naturally occurring (Chtioui et al., 2022; da Cruz Cabral et al., 2013). The microorganism-derived fungicides, on the other hand, were chosen for their adaptability to local climatic conditions, their ability to reproduce exponentially, and their ability to act both as biostimulants and as biofungicides (Shafi et al., 2017; Tsotetsi et al., 2022). In fact, *Bacillus* spp. and *Trichoderma* spp. can produce spores or conidia capable of surviving at high temperatures for long periods of time (Checinska et al., 2015; Harman et al., 1991).

Apart from the agricultural and economic impacts of mycotoxigenic fungi on plant production and the food supply chain, the primary reason for studying *F. culmorum* is that it is an important wheat pathogen and DON producer. Furthermore, it has received less attention than other *Fusarium* species like *F. graminearum*, which have been the focus of ample research. *A. carbonarius*, on the other hand, was investigated in the current study because it is the principal producer of OTA in grape and wine in the Mediterranean region.

In addition to the known activity of *Fusarium* and *Aspergillus* in as producers of DON and OTA, respectively, it is noteworthy that other genera such as *Alternaria* (Aichinger et al., 2021), and other species within the *Aspergillus* genera, including *A. niger* (Gil-Serna et al., 2019) and *A. flavus* (Cary et al., 2018), can produce infections of the utmost importance in agriculture.

Wheat is the world's third largest crop after maize and rice (FAO, 2022) and it contributes to human nutrition by providing around 20% of energy and protein requirements worldwide.

However, in recent years, fungal pathogens have caused yield losses of up to 21.48% (Shewry & Hey, 2015). When *Bacillus* was used on wheat as a seed coat, we found that FRR decreased by 77% to 97% in comparison with the control. In addition, when *Bacillu*s sp. BV was sprayed on wheat caryopsis, we observed that FHB decreased by 68% and DON by 69%. These results suggest that *Bacillus* biofungicides could be viable substitutes for synthetic fungicides in the control of wheat diseases caused by *F. culmorum*.

This study investigated grapes because they are one of the Mediterranean area's most important fruit crops, valued for their sensory properties and the vitamins and bioactive compounds (e.g., flavonoids) they contain (FAO-OIV, 2016; Sabra et al., 2021). Since OTA in grapes has been linked to adverse health impacts, and since the continued use of synthetic fungicides is problematic, there is a pressing need for alternative biofungicides with comparable or better efficacy. Chapter 3 of the current study describes how four plant-based extracts (i.e., chestnut flower, cistus, eucalyptus, and orange peel) demonstrated their potential as suitable alternatives to synthetic fungicides in reducing OTA production in grapes. The most effective of the extracts was eucalyptus, which reduced OTA by 82% in comparison with the control (i.e., no extract). A similar study conducted by Koteswara Rao et al. (2015) has demonstrated comparable efficacy of eucalyptus against OTA production by *P. verrucosum* and *Penicillium Nordicum* Dragoni & Marino 1979.

In the current study, *Aspergillus* mycelial growth was targeted to study the fungicidal efficiency of the plant extracts and to monitor OTA produced. However, measurement of the growth response (colony diameter, colony area, biomass, biomass dry weight, and colony density) of *A.carbonarius* and its correlation to OTA production have been inconsistent in literature. While Marín et al. (2008) used model development to demonstrate a correlation between colony diameter changes and toxin production in a solid medium, Camardo Leggieri et al. (2020) found that *P. nordicum* and *P. verrucosum* have a variable mycotoxin production pattern that depends

on the growth media and environmental conditions and which could not be correlated to their growth. Similarly, the current study found no association between the radial growth of *A. carbonarius* and the OTA produced. This explains to a reasonable extent the decision to investigate the mycelial growth of *A.carbonarius*, as it could be a potential indicator of OTA production.

Although current knowledge of the mechanisms driving the relationship between fungal growth and OTA production remains rudimentary and limited, several studies have attempted to support an increase or changes in the diameter of the fungi as an indicator of the toxin produced (Garcia et al., 2013; Marín et al., 2008). In any case, continued research is necessary to evaluate these speculations. Nonetheless, since measurement of radial growth is probably the simplest and most direct measure of fungal growth, this was the approach adopted in the current study. It is important to note that other target modes of action such as gene expression could be potential parameters to quantify OTA produced from *A.carbonarius*. However, this was beyond the scope of the present study.

This study was subject to some limitations. Although the plant extracts investigated in this study have significant antifungal properties, commercial use of these biosolutions is not without constraints. The widespread practical applicability of these alternative plant protection products is still limited by farmers' reluctance to accept natural products (Lengai et al., 2019; Rojht et al., 2012). Additional limitations to the use of biosolutions developed in this study are the need for (1) development of effective stabilization processes (e.g., microencapsulation); (2) simplification of the complex and expensive authorizations required for the use of natural plant protection products; and (3) optimization of plant growth conditions and extraction processes to achieve a homogeneous chemical composition (Pavela & Benelli, 2016).

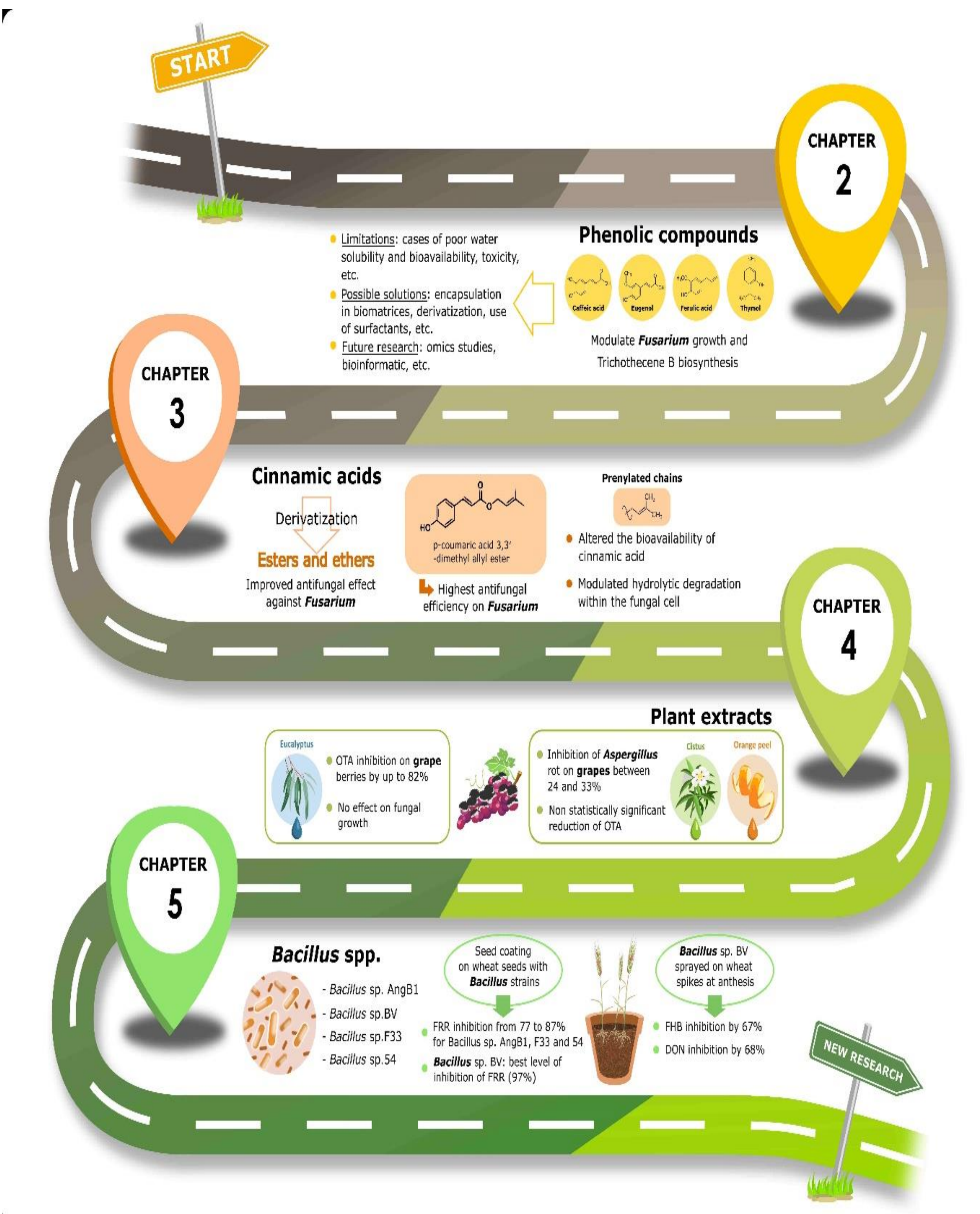
In the case of microbial biofungicides, the presence of the active compound at the time of pathogen inoculation is directly related to efficacy. When microbial biocontrol agents are

exposed to a harsh environment and compete with resident microflora, their survival is jeopardized, and their persistence on plants is often very limited. Furthermore, rain can easily wash hydrosoluble metabolites away. As a result, treatments with microorganisms that act through direct antibiosis should be applied frequently and immediately before pathogen infection to have the greatest effect.

Conclusions and Perspective

This thesis work has enabled the identification of biofungicides that are active against toxigenic fungi of the genera *Aspergillus* and *Fusarium,* and we report that derivatizing cinnamic acid can improve its antifungal properties against *Fusarium*. These findings open up new perspectives for research into tailored phenolic compounds and into the use of prenylated cinnamic acid esters and ethers in the development of drugs against *Fusarium*. Our study also opens up perspectives regarding the use of plant extracts as environmentally acceptable plant-based fungicides for the management of *A. carbonarius* and OTA contamination in vineyards. We suggest the use of *Bacillus* strains as a biocontrol tool against *F. culmorum* and DON. Overall, the field of research on natural antifungals is wide and there are still possibilities to explore. We believe that further studies are required to broaden the knowledge in this area. Therefore, we recommend that the interaction between phytochemicals (phenolic compounds and plant extracts) and the microbiological component should be further investigated for use in integrated pest management programs.

Graphical Abstract



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