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Exploring *Streptomyces-Fusarium* Interaction to Hamper Wheat Head Blight, Crown Rot and Deoxynivalenol Production

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Abstract

Fusarium head blight (FHB), root rot (FRR) and foot rot (FFR) cause important yield losses in wheat. The harvested product is often contaminated with mycotoxins, belonging to the group of trichothecenes. The main causal agents are *Fusarium graminearum*, *F. culmorum* and *F. pseudograminearum*. The biocontrol approach is a feasible option in order to reduce disease severity, as well as trichothecene contamination in grains.

Streptomyces spp. are Gram-positive bacteria, ubiquitous in soil and endophytes of inner tissues of plant roots. They produce a wide range of secondary metabolites able to limit pathogen development and disease severity *in planta*, as well as to enhance plant growth.

This PhD project aimed to select *Streptomyces* strains active within the wheat-*Fusarium* spp. pathosystem.

To achieve this, a detailed literature and patents analysis focused on biocontrol of toxigenic *Fusarium* spp. was carried out (Chapter 1) and new methodological approaches for antagonist screening have been developed (Chapter 2). Furthermore, the biocontrol efficacy of a selected subset of strains obtained from the culture collection maintained at the Plant Pathology laboratory (DeFENS, University of Milan, Italy) was evaluated in different conditions (Chapter 3) and bioactive metabolites were isolated (Chapter 4).

The influence of growth media and *Fusarium* strain diversity on streptomycete antifungal activity was assessed in dual culture assays. All the factors influenced the level of antifungal activity. The media commonly used for *in vitro* screening reduced the inhibitory activity of streptomycetes. Overall, results from dual culture assays and level of disease protection observed *in planta* did not correlate, except for those recorded on a medium based on wheat grains. Indeed, it was the most effective in eliciting antifungal activity and showed the highest correlation ($r = 0.5$) with FRR inhibition. Subsequently, being *TRI5* the first and essential gene involved in trichothecene biosynthetic pathway in *Fusarium* spp., a microplate bioassay using a *TRI5::GFP* transformed *F. graminearum* strain was developed and

validated in order to screen the effect of natural products on GFP fluorescence and consequently on trichothecene production. Surprisingly, culture filtrate from DEF39 strain completely suppressed deoxynivalenol (DON) production without affecting fungal growth.

The most promising isolates (*N* = 21) were further characterized for their potential plant growth promotion ability, as well as for their activity against FRR and FFR in wheat seedlings. None of them was able to increase plant growth. However, DEF09 strain exhibited consistent efficacy to limit FRR-FFR symptom severity (protection level > 40%) in soil and soilless conditions. Therefore, a field trial was performed to test its ability to reduce FHB severity, obtaining up to 60% protection.

Based on the activity observed from the previous screenings, four promising streptomycetes (DEF09, DEF20, DEF39, DEF48) were applied on sterilized wheat grains (microsilage) at two timepoints of application, in order to evaluate their ability to suppress fungal growth and DON production. Moreover, the fitness of streptomycetes in microsilage conditions was assessed by qPCR analysis. Streptomycetes were able to efficiently colonize the substrate, which resulted in reducing fungal biomass and DON accumulation only when co-inoculated with the pathogen.

A pool of promising biocontrol agents has been selected against fungal development and/or DON production. This research highlighted the complexity of finding an efficient screening procedure due to multiple interactions occurring in wheat-*Fusarium* spp. pathosystem. Further studies will be needed to confirm the activity of the strains *in planta.* The identification of the mechanisms of action and the molecules involved in the bioactivity of the strains will possibly allow to develop effective treatments limiting trichothecene accumulation in wheat.

Riassunto

La fusariosi della spiga e il marciume al colletto in frumento sono malattie causate da *Fusarium graminearum*, *F. culmorum* e *F. pseudograminearum*. Essi determinano ingenti perdite di raccolto oltre a contaminare il prodotto con micotossine appartenenti alla famiglia dei tricoteceni.

I batteri Gram-positivi appartenenti al genere *Streptomyces* sono ubiquitari nel suolo ed endofiti dei tessuti interni delle radici. Essi producono una vasta gamma di metaboliti secondari con proprietà antimicrobiche e possono essere utilizzati come agenti promotori della crescita delle piante, limitando lo sviluppo dei patogeni.

L'obiettivo del presente dottorato di ricerca è stato quello di selezionare ceppi di streptomiceti attivi contro *Fusarium* spp. in grano.

La prima fase dello studio ha permesso di conoscere lo stato dell'arte sull'utilizzo di streptomiceti contro specie micotossigene appartenenti al genere *Fusarium* (Chapter 1). Successivamente sono state sviluppate strategie innovative per la selezione degli stessi (Chapter 2) e l'efficacia dei ceppi più promettenti è stata poi saggiata in diverse condizioni (Chapter 3). Inoltre, i metaboliti secondari responsabili dell'attività antifungina sono stati caratterizzati (Chapter 4).

L'influenza della variabilità dei ceppi di *Fusarium* spp. e dei substrati colturali sull'attività di biocontrollo è stata valutata tramite saggi di antibiosi. Questi fattori hanno avuto un'influenza significativa nel determinare il livello di attività *in vitro*. I mezzi standard utilizzati in laboratorio hanno diminuito infatti tale parametro. Inoltre è stata riscontrata un'assenza di correlazione con il livello di biocontrollo ottenuto in pianta. Unica eccezione è per i risultati ottenuti utilizzando un terreno a base di frumento, che ha permesso di osservare un valore di correlazione più elevato con il livello di biocontrollo riscontrato contro marciume radicale in frumento ($r = 0.5$).

Successivamente, al fine di saggiare metaboliti limitanti la produzione di tricoteceni, è stato sviluppato un sistema in micropiastra che misura la fluorescenza emessa da un ceppo di *F. graminearum* trasformato con il

costrutto *TRI5::GFP*. *TRI5* è il primo gene essenziale coinvolto nella via metabolica di produzione di tricoteceni. Da questa prova si è potuto selezionare il ceppo di streptomicete DEF39 che riduce significativamente la produzione di DON.

Le potenziali attività di promozione della crescita e di biocontrollo di una selezione dei ceppi più promettenti (*N* = 21) sono state saggiate in pianta. Gli streptomiceti testati non hanno esibito la capacità di aumentare i parametri di sviluppo dei germinelli di frumento, ma il ceppo DEF09 ha ridotto significativamente il marciume al colletto e la fusariosi della spiga ottenendo livelli di protezioni sopra al 40% e del 60% rispettivamente.

Basandosi sui risultati ottenuti, quattro ceppi (DEF09, DEF20, DEF39, DEF48) sono stati applicati su grano sterilizzato testando due tempistiche di trattamento per osservarne la capacità di riduzione della biomassa fungina e della produzione di DON. Inoltre tramite qPCR si è osservata la fitness degli agenti di biocontrollo nelle condizioni testate. Gli streptomiceti, abili colonizzatori del substrato testato, sono stati efficaci nel ridurre la produzione di micotossine, limitando -quando co-inoculati con il patogeno- lo sviluppo dello stesso.

I ceppi selezionati agiscono perciò sia contro lo sviluppo fugino e/o contro la produzione di DON. Ulteriori studi saranno necessari per confermarne l'attività in pianta, così come per permettere lo sviluppo di formulati efficaci per limitare la contaminazione da tricoteceni.

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Introduction

1. *Fusarium* diseases in wheat

Wheat is one of the most important cereal worldwide, with a global production of 759 million tons reached in 2017/2018 (http://www.fao.org/worldfoodsituation/csdb/en/). Improvement in the yield potential of wheat will be necessary in order to satisfy the consumption of a rising global population and the demand for renewable fuel source (Curtis and Halford, 2014). One of the key aspects to meet this challenge is the management of fungal diseases, which can reduce its annual production by 15-20% (Figueroa et al., 2018). Severe grain losses occur due to the development of diseases caused by a group of *Fusarium* spp. Indeed, Fusarium head blight (FHB) and Fusarium crown rot (FCR) are associated to the presence in field of *Fusarium graminearum*, *F. culmorum* and *F. pseudograminearum* (GoswamI and Kistler, 2004; Chakraborty et al., 2006). Both diseases are linked by etiology, pathogen biology and epidemiology. *F. graminearum*, the main responsible of FHB, was found in rotten crown tissues and *F. culmorum* and *F. pseudograminearum* have been isolated from bleached wheat heads (Burgess et al., 1987; Backhouse et al., 2004; Balmas et al., 2015). In 1990s FHB losses have been estimated to be close to $3 billion in the United States alone (Windels, 2000). A yield reduction of 1 Mt/ha is predicted to occur at 19% of FHB (Salgado et al., 2015). The presence of FCR in commercial field in the Pacific North-West resulted in yield losses up to 35% (Smiley et al., 2005).

A generalized life cycle of *F. graminearum*, the main causal agent of FHB, is reported in Figure 1. Briefly, the fungus overwinters as saprophytic mycelia in crop residues left from the previous cultivation. In spring, during moist weather conditions, conidia and perithecia carring ascospores develop and infects flowering wheat spikelets. Indeed, the conidia or ascospores germinate and enter through natural openings of the spikelets like stomata and other susceptible sites (Trail, 2009). The fungus develops particular morphological structures similar to appressoria and infection cushions during this initial stage

of infection (Boenisch and Schäfer, 2011). A true biotrophic growth cannot be identified due to the absence of intercellular growth: indeed, the cytosol of the epicarp cells is invaded rapidly, inducing cell death (Jansen et al., 2005) and in consequence colonized tissues become bleached (Figure 2A). Mycotoxins, belonging to the group of trichothecenes, are produced contemporarily during spikelet colonization. These secondary metabolites help the pathogen to suppress plant defense response and to colonize rapidly the whole spike. Indeed, mutated strains deficient in trichothecene production lack in the ability to pass through the cell wall thickenings in the rachis (Bai et al., 2002; Jansen et al., 2005). DON production is therefore induced in a tissue-specific manner, for example in the developing kernels and the rachis tissues (Ilgen et al., 2009).

Dry weather and water stress increase the susceptibility of the wheat seedlings, favoring FCR epidemics (Backhouse and Burgess, 2002). Necrosis of crown and stem (Figure 2B) and the formation of “whiteheads” (heads without grains) are associated with FCR in field. The soil-borne inoculum, with an infection process similar to the one of FHB, causes root infections (Wang et al., 2015). The fungal colonization usually does not proceed above the third node (Beccari et al., 2018). However, significant DON concentration has been found in heads, suggesting that it plays a role during the root and stem colonization and it can be translocated along the plant (Beccari et al., 2018; Covarelli et al., 2012; Mudge et al., 2006). Therefore, severe FCR infection in field could represent an additional source of mycotoxin contamination.

Figure 1 Life cycle of Fusarium graminearum*, causal agent of Fusarium head blight in wheat (Trail, 2009).*

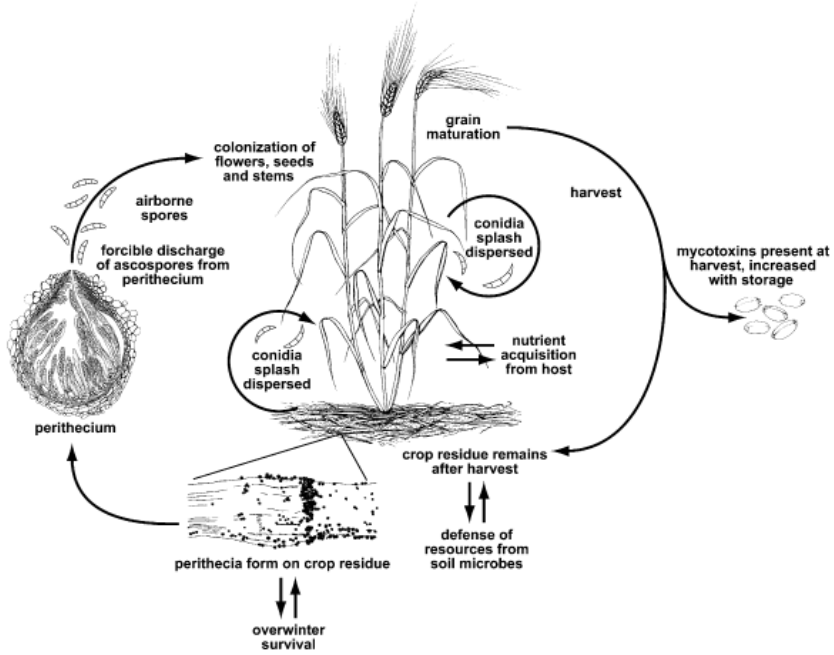


Figure 2 Symptoms of Fusarium head blight (A) and Fusarium crown rot (B).

1.1 *Fusarium* trichothecenes: a threat for feed and food safety

Fusarium spp. infections are directly correlated to a variable accumulation of mycotoxins belonging to the group of type B trichothecenes in grains, including deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives (Foroud and Eudes, 2009). DON occurrence has the highest incidence in analyzed samples (up to 83%) in North and Central America, Europe and China (https://www.biomin.net/en/, accessed on 3/08/2019). The fungal contamination in harvested grains can be managed, but often the mycotoxin incidence in feed and food products increases dramatically during storage periods, becoming a threat for food and feed safety (Beattie et al., 1998; Magan et al., 2010; Yuan et al., 2018). Toxic effects of DON include the alteration of intestinal, nervous and immune systems due to the inhibition of protein synthesis and induction of apoptosis (Maresca, 2013). Acute DON toxicity in the most sensitive species determines abdominal distress, increased salivation, malaise, diarrhea, emesis and anorexia; chronic toxicity is exhibited as feed refusal and altered nutrient uptake (Maresca et al., 2002; Pestka and Smolinski, 2005). The risks for human and animal health forced worldwide organizations to set maximum allowable levels of their occurrence in products for human and animal consumption. In particular, the European Union established the most comprehensive legislation in this sector over the years (Siegel and Babuscio, 2011), fixing maximum limits for DON concentration in different raw and processed foods for human and animal consumption (European Commission, 2006). The trichothecene biosynthetic pathway has been well characterized and studying its regulation is important in order to find strategies able to limit mycotoxin accumulation. The essential genes (*TRI1-TRI16*), required for trichothecene biosynthesis, are organized in three clusters. Indeed, several enzymatic modifications transform a product from primary metabolism (farnesyl diphosphate) to a toxic sesquiterpene epoxoide (McCormick et al., 2011). In addition to *TRI5* -first gene essential to transform farnesyl diphosphate in trichodiene- the main *TRI* cluster includes *TRI8, TRI7, TRI3, TRI4, TRI5, TRI11, and TRI13* genes which encode for

enzymes synthetizing the mycotoxin skeleton, the transcriptional regulators *TRI6* and *TRI10*, a transport protein *TRI12*, and two genes (*TRI9* and *TRI14*) with uncertain function (Proctor et al., 2018). The diversity among the chemical structures is due to diversity in gene functionality within the cluster. Indeed, in NIV-producing *Fusarium* isolates, *TRI13* and *TRI7* are responsible for hydroxylation and acetylation of C-4. In DON-producing isolates, they are not functional, leading to the lack of the hydroxyl group in C-4 (Lee et al., 2002). Modification of the gene responsible for DON esterisation (*TRI8*), which removes the acetyl group from C-3 or C-15 position, results in the production of acetylated derivatives (3ADON or 15ADON) (Alexander et al., 2011). Therefore, different chemotype lineages characterize *Fusarium* isolates, such as 3ADON chemotype (deoxynivalenol, 3 acetyldeoxynivalenol), 15ADON chemotype (deoxynivalenol, 15 acetyldeoxynivalenol), NIV chemotype (nivalenol, 4 acetylnivalenol) (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).

1.2 Disease management

Several strategies have been evaluated in order to control *Fusarium* spp. occurrence in field. The agronomic practices have a direct influence on the survival of the overwintering inoculum. Indeed, previous crop, tillage and also the field size has been reported to affect FHB incidence. In Ontario (Canada), disease intensity was higher when wheat was planted after maize, suggesting that maize represents a good source for inoculum survival. At the same time no-till or minimum tillage combined with maize as a previous crop increased the DON levels (Schaafsma et al., 2005). The same observations were obtained in Minnesota (USA), where disease incidence and severity increased when wheat followed maize in comparison to soybean cultivations (Dill-Macky and Jones, 2000). No-till or minimum-tillage after wheat or maize cultivation contributes to FHB epidemics. In addition, increasing nitrogen input to the soil has been directly correlated to disease severity and DON accumulation in

several reports (Heier et al., 2005; Lemmens et al., 2004). An appropriate fertilization strategy to avoid nutrient stress or modifications in the crop canopy should be evaluated to reduce the plant susceptibility.

Demethylation inhibitor fungicides (DMIs), in particular tebuconazole, have been largely applied at anthesis or near early anthesis for cereal protection (McMullen et al., 2012a). The combined application of different DMIs has been reported to increase their efficacy. Indeed, the application of prothioconazole + tebuconazole was the most efficient fungicide in suppressing FHB (52% protection compared to the non-treated control) (Paul et al., 2008). However, they do not ensure a complete protection. At the same time other type of chemicals, such as strobilurins, did not show strong inhibitory effects *in vitro* (Dubos et al., 2011) and when applied in field, they contribute to increasing the level of DON accumulation (Blandino et al., 2006; Ellner, 2005). Indeed, a reduction in FHB severity after the fungicide application is not positively correlated with a reduction in mycotoxin contamination of grains (Edwards, 2004). In addition, one of the major challenges for growers is their application at the right moment (just before anthesis), often further complicated by the unfavorable weather conditions which can prevent the treatment (Mcmullen et al., 2012b). Moreover, the cost of the treatments as well as the problems in obtaining a complete coverage of heads might discourage their application. The picture is worsened by recent studies which reported sensitivity decline to triazoles in *F. graminearum* isolates in Europe (Klix et al., 2007) and an emergence of resistant isolates in USA and China (Spolti et al., 2013; Yin et al., 2009).

The improvement of host resistance is a cost-effective option. FHB resistance is based on numerous quantitative trait loci (QTLs) which work additively and they are affected by the environmental factors. Some of them have been identified in wheat. In particular, five types of FHB genetic resistance have been reported: 1) resistance to initial infection; 2) resistance to spread in infected tissue; 3) resistance to kernel infection; 4) tolerance; and 5) resistance to accumulation of toxins (Mesterházy, 1995).

However, the process to understand QTLs in a recombinant inbred line population is slow (McCartney et al., 2016). In addition, one of the major challenges is to combine resistance traits with other agronomical features such as yield and quality of the final product (Clark et al., 2016). The wheat variety “Sumai 3” has been extensively used for this purpose, because it combines excellent resistance and agronomical features (Niwa et al., 2014). However, its extensive use could narrow genetic basis, helping the pathogen to overcome the resistance (Ruckenbauer et al., 2001). The continuous breeding efforts for developing resistant varieties resulted in several commercial cultivars expressing moderate resistance to FHB. For instance, in the USA, within a program addressed to fight wheat and barley scab (https://scabusa.org/), newly developed and published cultivars are available for growers (https://scabsmart.org/).

1.3 Biological control

Nowadays, integrated pest/disease management has been adopted at the European level (European Commission, 2009a). In addition, the registration of diverse synthetic active substances has been revoked due to their toxicity for human, animal and environmental health (European Commission, 2009b). Therefore, one of the major challenges is to find strategies to control pests and diseases that are environmentally friendly and safe for human health. One important outcome of the “Joint International Workshop on Biocontrol” in Paris, organized in 2016 is that there is a lack of effective management solutions for certain diseases (Lamichhane et al., 2017). For instance, none of the agricultural practices described above are completely efficient to guarantee an acceptable control of *Fusarium* diseases and associated DON contamination in cereals. Therefore, biocontrol is becoming a feasible option. Despite all the scientific efforts in the biocontrol field, the identification of antagonists active in field conditions and limiting DON accumulation as well as the studies on efficient application strategies are still needed (Lamichhane et al., 2017). Biocontrol agents (BCA) against *Fusarium* pathogens in cereals are characterized by several mechanisms of action.

They can act directly through antibiosis, parasitism, nutrient competition or by eliciting plant defense response.

Trichoderma species have been selected as antagonists against *F. culmorum* and *F. graminearum* (Lutz et al., 2003; Matarese et al., 2012; Sarrocco et al., 2019; Schöneberg et al., 2015). Indeed, they are excellent competitors, parasitizing their prey by the production of cell wall degrading enzymes (Matarese et al., 2012). The same mechanism of action has been observed for *Clonostachys rosea* (Luongo et al., 2005; Xue et al., 2009). Competition for nutrients and space have been evaluated for yeast strains belonging to the genus *Cryptococcus* (Khan et al., 2004; Schisler et al., 2002).

The production of secondary metabolites toxic for other microorganisms (antibiosis) is the most commonly observed mechanism of action of bacterial BCA. Indeed, several bacteria strains have been tested, such as *Bacillus* spp. (Palazzini et al., 2007; Zhao et al., 2014), *Lysobacter enzymogenes* (Jochum et al., 2006), *Pseudomonas* spp. (Khan and Doohan, 2009; Pal et al., 2001) and *Streptomyces* spp. (Jung et al., 2013; Nourozian et al., 2006; Palazzini et al., 2007). The antifungal metabolites produced by *Bacillus* spp. against *Fusarium* pathogens belong to the lipopeptide family, for example surfactins, fengycin and iturin (Dunlap et al., 2011; Zhao et al., 2014). However, the bioactive metabolites include not only antibiotics *sensu stricto*, but also cell wall degrading enzymes and antifungal volatile compounds. FHB resistance in plants through the activation of local and systemic resistances was suggested, but the molecular mechanisms of action were not clearly investigated (Jochum et al., 2006).

The *Fusarium* diseases in cereals are characterized by a complex epidemiology and therefore finding a correct timing of BCA application during wheat cultivation is essential. *Streptomyces* strain RC 87B (Palazzini et al., 2017) or the saprophytic fungus *C. rosea* (Luongo et al., 2005; Xue et al., 2009) have been applied to reduce the inoculum on crop residues in order to delay or inhibit perithecia and ascospore development during the infection period. Seed treatment could be a feasible option and several strains

belonging to the genus *Bacillus*, *Pseudomonas* and *Streptomyces* have been tested to prevent FCR infections (Pal et al., 2001; Yekkour et al., 2012; Colombo et al., 2019). The majority of the studies focused on wheat spike treatment during the most critical step of FHB disease cycle, the anthesis, in order to reduce disease severity and ideally mycotoxin contamination (Comby et al., 2017; Jung et al., 2013; Xue et al., 2009). Eventually, post-harvest treatments to limit fungal growth and DON accumulation in wheat grains have been performed using several strains of *B. amyloliquefaciens* isolated from peanut shells (Shi et al., 2014). The promising antagonists should be easily developed into commercial products to be used in field. However, the majority of the studies did not consider the BCA activity on DON accumulation (Wachowska et al., 2017). Indeed, fungal growth suppression is not directly correlated to a reduction of mycotoxin accumulation, therefore a novel screening strategy must address selection of BCA able to counteract mycotoxin accumulation (He et al., 2009). The lack of appropriate screening procedures, as well as the complex process of registration of biocontrol products resulted in only one commercial product available on the market against FHB (Polyversum® formulation of *Pythium oligandrum* strain ATCC 38472 authorized in France since August 2015).

Microbial degradation by anaerobic and aerobic bacteria has been tested to reduce mycotoxin toxicity during the storage period. Acetylation, de-acetylation, de-epoxidation, epimerization, glucosylation, and oxidation can transform trichothecenes in less toxic molecules (McCormick, 2013). Indeed, DON de-epoxydation activity by mixed bacterial communities isolated from different matrices, such as soil or animal intestines, has been reported in several researches (Islam et al., 2012; Yu et al., 2010). For instance, the contemporary use of bacteria belonging to the genera *Nocardioides* and *Devosia,* which can degrade DON to 3-epi-DON and 3-keto, DON respectively, allowed a complete degradation of DON under detectable limits (Sato et al., 2012). A consortium of Gram-positive bacteria has been reported to de-epoxydize DON in different food and feed matrices (Ahad et al., 2017).

Eubacterium BBSH 797, isolated from bovine rumen fluid, has been formulated in the first commercially available product (Biomin® BBSH 797) for animal feed treatment for its ability to degrade several trichothecenes (Fuchs et al., 2000).

2. Streptomycetes

2.1 Taxonomy

The genus *Streptomyces,* introduced by Waksman and Henrici (1943), belongs to the phylum of Gram-positive Actinobacteria. Their DNA is characterized by high GC content (69–78 mol%) (Korn-Wendishand Kutzner, 1992). After the discovery of their secondary metabolites relevant for human and veterinary medicine in 1940s, their identification was initially conducted based on morphological features. Inadequate descriptions of the species and lack of uniformity in classification criteria led in 1964 to a standardization process through the International *Streptomyces* Project (Shirling and Gottlieb, 1968a, 1968b, 1969, 1972). The main morphological features proposed for species classification were the spore chain morphology, spore surface ornamentation, color of spores, substrate mycelium and soluble pigments, production of melanin pigment and the utilization of a range of carbon sources. In this way, collections of type strains were recognized internationally, as well as about 450 species were newly described. A subsequent attempt to reduce the number of the species was introduced in 1980s with the numerical classification. Briefly, specific algorithms were used in order to cluster the available type strains based on phenotypic traits and carbon utilization (Williams et al., 1983; Langham et al., 1989). In addition, previous classification criteria resulted in incorrect classifications. Therefore, a reduction of the described *Streptomyces* species was achieved: the 1989 edition of Bergey's Manual described 142 species (Williams, 1989). Despite the developed procedures, very often new species and subspecies were introduced, as well as the available reference strains did not match the new isolates.

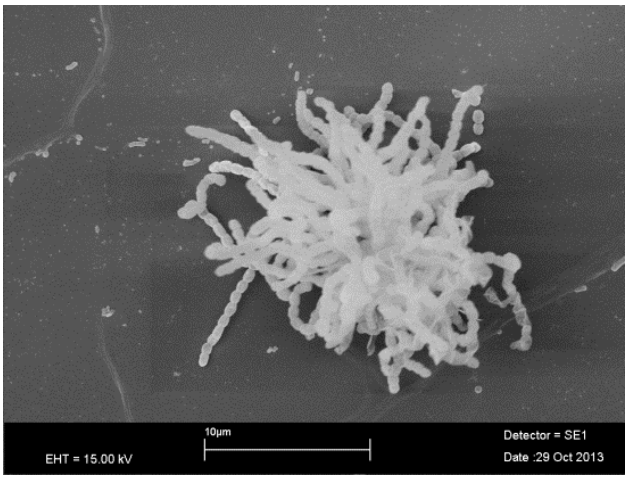
Nowadays, molecular methods have been introduced to better understand species relatedness and support the results obtained with the numerical taxonomic and chemotaxonomic methods (Anderson and Wellington, 2001). Therefore, DNA:DNA hybridization and 16S rRNA gene sequencing were introduced. However, the first technique cannot be used alone to determine the relationships between species (Anderson and Wellington, 2001). The 16S rRNA gene sequencing has been proved to be useful to identify or confirm the novelty of an unknown isolate, but it has not enough taxonomic resolution to provide definitive identification based on similarity with a described species (Labeda et al., 2012). A more recent approach is the MLST (Multilocus Sequence Typing) of house-keeping genes, such as *atpD*, *gyrB*, *recA*, *rpoB*, *trpB* and 16S rRNA which may help to guide *Streptomyces* species discrimination and the classification of novel species (Guo et al., 2008; Labeda et al., 2016). In addition, the advent of cheap and rapid genome sequencing resulted in numerous genomes available, although the quality of the published sequences is often variable (Studholme, 2016). Therefore, comparative genomics increased rapidly, enabling the study of physiological, ecological, and evolutionary attributes of the genus (Chater, 2016).

2.2 Morphology

Many Actinobacteria, similarly to filamentous fungi, grow through a combination of tip extension and branching of hyphae forming a mycelium, and reproduce by spores. However, their cells contain a chromosome organized in a prokaryotic nucleoid and a peptidoglycan cell wall. Aerial and vegetative (substrate) mycelium, spore morphology and chain length, and the presence of melanoid pigments have been extensively studied in order to classify these microorganisms. *Streptomyces* spp. show permanent and highly branched mycelium formed by thin vegetative hyphae (0.5-2 μm in diameter) with a smooth surface. The aerial mycelium with the chains of spores appear floccose, granular, powdery, or velvety (Locci, 1989). Their spore surface observed by electron microscopy appears smooth, warty, spiny, hairy, or rugose (Dietz and Mathews, 1971).

Spore chains have been described as being straight to flexuous (*Rectus-Flexibilis*), open loops *(Retinaculum-Apertum*), open or closed spirals (*Spira*), or verticillate (Pridham et al., 1958). In addition, numerous spore mass colors were identified: blue, gray, green, red, violet, white, and yellow (Shirling and Gottlieb, 1968a).

Figure 3 Streptomyces *aerial mycelium observed at scanning electron microscope.*

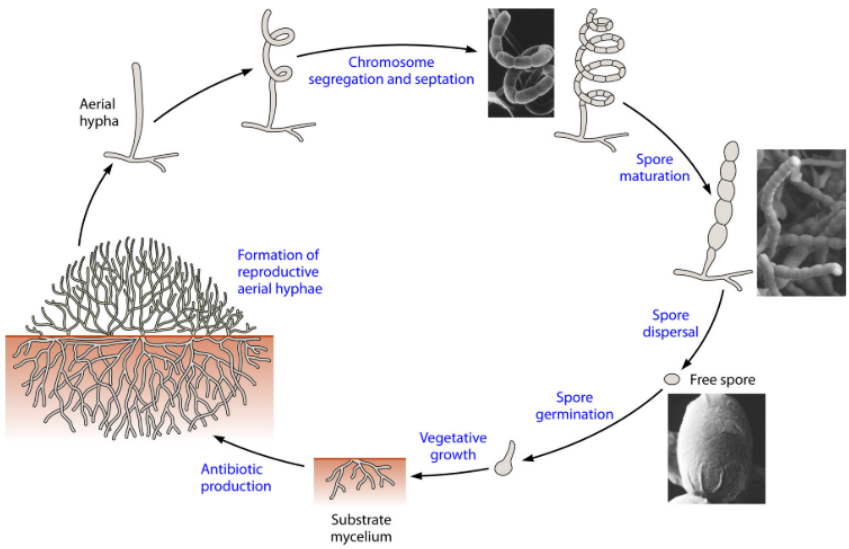


2.3 Life cycle

The *Streptomyces* life cycle has been extensively studied because of its unique features among bacteria (Figure 4). Indeed, the germination of spores leads to the formation of filamentous cells called “vegetative hyphae” which grow by tip extension and branching to form a vegetative (substrate) mycelium firmly attached to the substrate (Chater, 1972). During vegetative growth, septation is rarely observed (Wildermuth and Hopwood, 1970): cross-walls separate hyphae into connected long compartments, each containing multiple copies of the chromosome (Elliot et al., 2008). Under adverse environmental conditions such as nutrient depletion, the vegetative mycelium differentiates to form erected specialized hyphae carrying spores. Indeed, a programmed cell death of the substrate mycelium makes available all the building blocks necessary to support the development of a secondary (aerial) mycelium (Méndez et al., 1985). Septation occurs in these particular structures and

leads to the formation of mature spores: indeed, around 60 unigenomic compartments are developed (Elliot et al., 2008). Interestingly, *bld* genes are required for the formation of aerial hyphae. Therefore, a bald ("hair-less") phenotype is exhibited by mutants lacking the aerial mycelium (Merrick, 1976). These genes and environmental parameters such as carbon source availability have been demonstrated to be the main factors inducing antibiotic production in *Streptomyces* spp. Indeed, during the aerial mycelium development most of the secondary metabolites are produced, suggesting an ecological role for their biosynthesis, such as the protection of the vegetative mycelium and nutrients from other competitors present in the soil (Bibb, 2005).

Figure 4 The life cycle of streptomycetes (Barka et al., 2016).



2.4 Streptomycetes as antibiotic factories

As a result of 70 years of research on natural product, around 10.000 microbial metabolites are known nowadays. During the "golden era" between the 1940s and the beginning of 1950s, almost all groups of important antibacterial antibiotics were discovered (tetracyclines, cephalosporins, aminoglycosides, macrolides) and 70-80% of them were isolated from *Streptomyces* spp. (Bérdy, 2005).

Actinomycin was the first antibiotic discovered in 1940 from *Streptomyces antibioticus* (Waksman and Woodruff, 1940). Streptothricin and streptomycin were then isolated from *Streptomyces lavendulae* and *Streptomyces griseus,* respectively (Schatz and Waksman, 1944; Waksman and Woodruff, 1942).
The biological activity of streptomycete-produced metabolites is commonly referred to as antibacterial and antifungal, but anticancer, antitumor and herbicide compounds have also been isolated (Barka et al., 2016). For seventeen years the number of natural products discovered per year increased, reaching a peak in 1970s, and then dropped due to a decline in screening efforts (Watve et al., 2001). Nevertheless, new challenges need to be addressed nowadays, such as the multi-drug resistance, and the lack of novel defense solutions in the therapy of physiological diseases and in agriculture. Recent technological advances such as genetic engineering and high-throughput screening helped to renew the interest in microbial natural products (Liu et al., 2012). For instance, when the genome of the model organism *Streptomyces coelicolor* was sequenced, a wide range of secondary metabolite biosynthetic gene clusters have been discovered (about 20), suggesting that its metabolite production potential has been underestimated (Bentley et al., 2003). Among them, cryptic polyketide antibiotics have also been identified, which means that novel compounds can potentially be discovered by genome mining, opening a new era of streptomycete natural product research (Pawlik et al., 2007).

2.5 Streptomycetes as plant growth promoting bacteria

Streptomycetes are well known as soil dwelling bacteria, but very often, diverse species colonize the rhizosphere and even plant tissues, establishing symbiotic relationship (Seipke et al., 2012). Therefore, a wide range of secondary metabolites has been evolved against competitors in their niche or to protect the host plant from pathogens. Indeed, it has been proposed that chemically different metabolites act synergistically against different competitors and metabolites with similar bioactivity are produced contingently in order to be recognized singularly by the producing organism and

competitors (Challis and Hopwood, 2003). In addition, they are important microorganisms for the organic matter re-cycle in soil due to their ability to hydrolyze different polysaccharides (Chater et al., 2010). Therefore, these bacteria are able to positively influence plant growth with direct or indirect mechanisms. Nitrogen fixation, siderophore and phytohormone synthesis, as well as phosphate solubilization are the main mechanisms associated to a direct growth promotion carried out by several *Streptomyces* strains (Viaene et al., 2016). However, more assessments *in planta* are still needed to confirm these promising features tested mainly *in vitro*. For the reasons discussed above, streptomycetes have been mainly studied for the antimicrobial metabolite production able to inhibit pathogen development, helping indirectly plants against diseases (Doumbou et al., 2001). Several commercial products for crop protection are available on the market, the most important ones are: Mycostop® based on *S. griseoviridis* strain K61 (Verdera, Finland) and Actinovate®SP based on *S. lydicus* strain WYEC 108 (Natural Industries Inc., Houston, TX).

References

Ahad, R., Zhou, T., Lepp, D., and Pauls, K. P. (2017). Microbial detoxification of eleven food and feed contaminating trichothecene mycotoxins. *BMC Biotechnol.* 17, 30. doi:10.1186/s12896-017-0352-7.

Alexander, N. J., McCormick, S. P., Waalwijk, C., van der Lee, T., and Proctor, R. H. (2011). The genetic basis for 3-ADON and 15-ADON trichothecene chemotypes in *Fusarium*. *Fungal Genet. Biol.* 48, 485–495. doi:10.1016/J.FGB.2011.01.003.

Anderson, A. S., and Wellington, E. M. H. (2001). The taxonomy of *Streptomyces* and related genera. *Int. J. Syst. Evol. Microbiol.* 51, 797–814.

Backhouse, D., Abubakar, A. A., Burgess, L. W., Dennis, J. I., Hollaway, G. J., Wildermuth, G. B., et al. (2004). Survey of *Fusarium* species associated with crown rot of wheat and barley in eastern Australia. *Australas. Plant Pathol.*, 255–261. doi:10.1071/AP04010.

Backhouse, D., and Burgess, L. W. (2002). Climatic analysis of the distribution of *Fusarium graminearum, F. pseudograminearum and F. culmorum* on cereals in Australia. *Australas. Plant Pathol.* 31, 321–327. doi:10.1071/AP02026.

Bai, G.-H., Desjardins, A. E., and Plattner, R. D. (2002). Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. *Mycopathologia* 153, 91–98. doi:10.1023/A:1014419323550.

Balmas, V., Scherm, B., Marcello, A., Beyer, M., Hoffmann, L., Migheli, Q., et al. (2015). *Fusarium* species and chemotypes associated with fusarium head blight and fusarium root rot on wheat in Sardinia. *Plant Pathol.* 64, 972–979. doi:10.1111/ppa.12337.

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., et al. (2016). Taxonomy, physiology, and natural products of Actinobacteria. *Microbiol. Mol. Biol. Rev.* 80, 1–43. doi:10.1128/MMBR.00019-15.

Beattie, S., Schwarz, P. B., Horsley, R., Barr, J., and Casper, H. H. (1998). The effect of grain storage conditions on the viability of *Fusarium* and deoxynivalenol production in infested malting barley. *J. Food Prot.* 61, 103–106.

Beccari, G., Prodi, A., Pisi, A., Nipoti, P., Onofri, A., Nicholson, P., et al. (2018). Development of three fusarium crown rot causal agents and systemic translocation of deoxynivalenol following stem base infection of soft wheat. *Plant Pathol.* 67, 1055–1065. doi:10.1111/ppa.12821.

Bentley, S. D., Chater, K. F., Cerdeño-Tárraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., et al. (2003). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 417, 141–147. doi:10.1038/417141a.

Bérdy, J. (2005). Bioactive microbial metabolites: a personal view. *J. Antibiot. (Tokyo).* 58, 1–26. doi:10.1038/ja.2005.1.

Bibb, M. J. (2005). Regulation of secondary metabolism in streptomycetes. *Curr. Opin. Microbiol.* 8, 208–215. doi:10.1016/j.mib.2005.02.016.

Blandino, M., Minelli, L., and Reyneri, A. (2006). Strategies for the chemical control of Fusarium head blight: effect on yield, alveographic parameters and deoxynivalenol contamination in winter wheat grain. *Eur. J. Agron.* 25, 193–201. doi:10.1016/j.eja.2006.05.001.

Boenisch, M. J., and Schäfer, W. (2011). *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. *BMC Plant Biol.* 11, 110. doi:10.1186/1471-2229-11-110.

Burgess, L. W., Klein, T. A., Bryden, W. L., and Tobin, N. F. (1987). Head blight of wheat caused by *Fusarium graminearum* Group 1 in New South Wales in 1983. *Australas. Plant Pathol.* 16, 72–78. doi:10.1071/APP9870072.

Chakraborty, S. A., Liu, C. J. A., Mitter, V. A., Scott, J. B. A., Akinsanmi, O. A. B., Ali, S. C., et al. (2006). Pathogen population structure and epidemiology are keys to wheat crown rot and Fusarium head blight management. *Austral. Plant Pathol.* 35, 643–655.

Challis, G. L., and Hopwood, D. A. (2003). Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc. Natl. Acad. Sci.* 100, 14555–14561. doi:10.1073/pnas.1934677100.

Chater, K. F. (1972). A morphological and genetic mapping study of white colony mutants of *Streptomyces coelicolor*.

Chater, K. F., Biró, S., Lee, K. J., Palmer, T., and Schrempf, H. (2010). The complex extracellular biology of *Streptomyces*. *FEMS Microbiol. Rev.* 34, 171–198. doi:10.1111/j.1574-6976.2009.00206.x.

Clark, A. J., Sarti-Dvorjak, D., Brown-Guedira, G., Dong, Y., Baik, B. K., and Van Sanford, D. A. (2016). Identifying rare FHB-resistant segregants in intransigent backcross and F2 winter wheat populations. *Front. Microbiol.* 7, 277. doi:10.3389/fmicb.2016.00277.

Colombo, E. M., Pizzatti, C., Kunova, A., Gardana, C., Saracchi, M., Cortesi, P., et al. (2019). Evaluation of in-vitro methods to select effective streptomycetes against toxigenic fusaria. *PeerJ* 7, e6905. doi:10.7717/peerj.6905.

Comby, M., Gacoin, M., Robineau, M., Rabenoelina, F., Ptas, S., Dupont, J., et al. (2017). Screening of wheat endophytes as biological control agents against Fusarium head blight using two different *in vitro* tests. *Microbiol. Res.* 202, 11–20. doi:10.1016/j.micres.2017.04.014.

Covarelli, L., Beccari, G., Steed, A., and Nicholson, P. (2012). Colonization of soft wheat following infection of the stem base by *Fusarium culmorum* and translocation of

deoxynivalenol to the head. *Plant Pathol.* 61, 1121–1129. doi:10.1111/j.1365-3059.2012.02600.x.

Curtis, T., and Halford, N. G. (2014). Food security: the challenge of increasing wheat yield and the importance of not compromising food safety. *Ann. Appl. Biol.* 164, 354–372. doi:10.1111/aab.12108.

Dietz, A., and Mathews, J. (1971). Classification of *Streptomyces* spore surfaces into five groups. *Appl. Environ. Microbiol.* 21, 527–533.

Dill-Macky, R., and Jones, R. K. (2000). The effect of previous crop residues and tillage on Fusarium head blight of wheat. *Plant Dis.* 84, 71–76. doi:10.1094/pdis.2000.84.1.71.

Doumbou, C. L., Salove, M. K. H., Crawford, D. L., and Beaulieu, C. (2001). Actinomycetes, promising tools to control plant diseases and to promote plant growth. *Phytoprotection* 82, 85–102. doi:10.7202/706219ar.

Dubos, T., Pasquali, M., Pogoda, F., Hoffmann, L., and Beyer, M. (2011). Evidence for natural resistance towards trifloxystrobin in *Fusarium graminearum*. *Eur. J. Plant Pathol.* 130, 239–248. doi:10.1007/s10658-011-9749-7.

Dunlap, C. A., Schisler, D. A., Price, N. P., and Vaughn, S. F. (2011). Cyclic lipopeptide profile of three *Bacillus subtilis* strains; antagonists of Fusarium head blight. *J. Microbiol.* 49, 603–609. doi:10.1007/s12275-011-1044-y.

Edwards, S. G. (2004). Influence of agricultural practices on *Fusarium* infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. *Toxicol. Lett.* 153, 29–35. doi:10.1016/J.TOXLET.2004.04.022.

Elliot, M. A., Buttner, M. J., and Nodwell, J. R. (2008). "Multicellular development in *Streptomyces*," in *Myxobacteria: Multicellularity and Differentiation*, ed. D. E. Whitworth (ASM Press, Washington, D.C.), 419–437.

Ellner, F. M. (2005). Results of long-term field studies into the effect of strobilurin containing fungicides on the production of mycotoxins in several winter wheat varieties. *Mycotoxin Res.* 21, 112–115. doi:10.1007/BF02954432.

European Commission (2006). Commission regulation (EC) 1881/2006. *Off. J. Eur. Union* L 364, 5–24.

European Commission (2009a). Commission directive (EC) 128/2009. *Off. J. Eur. Union* L 309/71.

European Commission (2009b). Commission regulation (EC) 1107/2009. *Off. J. Eur. Union* L 309/1.

Figueroa, M., Hammond-Kosack, K. E., and Solomon, P. S. (2018). A review of wheat diseases-a field perspective. *Mol. Plant Pathol.* 19, 1523–1536. doi:10.1111/mpp.12618.

Foroud, N. A., and Eudes, F. (2009). Trichothecenes in cereal grains. *Int. J. Mol. Sci.* 10, 147–73. doi:10.3390/ijms10010147.

Fuchs, E., Binder, E., Heidler, D., and Krska, R. (2000). Characterisation of metabolites after the microbial degradation of A- and B-trichothecenes by BBSH 797. *Mycotoxin Res.* 16, 66–69. doi:10.1007/BF02942984.

Goswami, R. S., and Kistler, H. C. (2004). Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* 5, 515–525. doi:10.1111/J.1364-3703.2004.00252.X.

Guo, Y. P., Zheng, W., Rong, X. Y., and Huang, Y. (2008). A multilocus phylogeny of the *Streptomyces griseus* 16S rRNA gene clade: use of multilocus sequence analysis for streptomycete systematics. *Int. J. Syst. Evol. Microbiol.* 58, 149–159. doi:10.1099/ijs.0.65224-0.

He, J., Boland, G. J., and Zhou, T. (2009). Concurrent selection for microbial suppression of *Fusarium graminearum*, Fusarium head blight and deoxynivalenol in wheat. *J. Appl. Microbiol.* 106, 1805–1817. doi:10.1111/j.1365-2672.2009.04147.x.

Heier, T., Jain, S. K., Kogel, K. H., and Pons-Kühnemann, J. (2005). Influence of N-fertilization and fungicide strategies on Fusarium head blight severity and mycotoxin content in winter wheat. *J. Phytopathol.* 153, 551–557. doi:10.1111/j.1439-0434.2005.01021.x.

Ilgen, P., Hadeler, B., Maier, F. J., and Schäfer, W. (2009). Developing kernel and rachis node induce the trichothecene pathway of *Fusarium graminearum* during wheat head infection. *Mol. Plant-Microbe Interact.* 22, 899–908. doi:10.1094/MPMI-22-8-0899.

Islam, R., Zhou, T., Young, J. C., Goodwin, P. H., and Pauls, K. P. (2012). Aerobic and anaerobic de-epoxydation of mycotoxin deoxynivalenol by bacteria originating from agricultural soil. *World J. Microbiol. Biotechnol.* 28, 7–13. doi:10.1007/s11274-011-0785-4.

Jansen, C., von Wettstein, D., Schafer, W., Kogel, K.-H., Felk, A., and Maier, F. J. (2005). Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proc. Natl. Acad. Sci.* 102, 16892–16897. doi:10.1073/pnas.0508467102.

Jochum, C. C., Osborne, L. E., and Yuen, G. Y. (2006). Fusarium head blight biological control with *Lysobacter enzymogenes* strain C3. *Biol. Control* 39, 336–344. doi:10.1016/j.biocontrol.2006.05.004.

Jung, B., Park, S. Y., Lee, Y. W., and Lee, J. (2013). Biological efficacy of *Streptomyces* sp. strain BN1 against the cereal head blight pathogen *Fusarium graminearum*. *Plant Pathol. J.* 29, 52–58. doi:10.5423/PPJ.OA.07.2012.0113.

Khan, M. R., and Doohan, F. M. (2009). Bacterium-mediated control of Fusarium head blight disease of wheat and barley and associated mycotoxin contamination of grain. *Biol. Control* 48, 42–47. doi:10.1016/j.biocontrol.2008.08.015.

Khan, N. I., Schisler, D. A., Boehm, M. J., Lipps, P. E., and Slininger, P. J. (2004). Field testing of antagonists of Fusarium head blight incited by *Gibberella zeae*. *Biol. Control* 29, 245–255. doi:10.1016/S1049-9644(03)00157-9.

Klix, M. B., Verreet, J. A., and Beyer, M. (2007). Comparison of the declining triazole sensitivity of *Gibberella zeae* and increased sensitivity achieved by advances in triazole fungicide development. *Crop Prot.* 26.4, 683–690. doi:10.1016/j.cropro.2006.06.006.

Korn-Wendish, and F. and Kutzner H.J. (1992). The Family *Streptomycetaceae*. *Prokaryotes A Handb. Biol. Bact. Ecophysiol. Identification,Applications*, 921–955.

Labeda, D. P., Dunlap, C. A., Rong, X., Huang, Y., Doroghazi, J. R., Ju, K.-S., et al. (2016). Phylogenetic relationships in the family *Streptomycetaceae* using multi-locus sequence analysis. *Antonie Van Leeuwenhoek*. doi:10.1007/s10482-016-0824-0.

Labeda, D. P., Goodfellow, M., Brown, R., Ward, A. C., Lanoot, B., Vanncanneyt, M., et al. (2012). Phylogenetic study of the species within the family *Streptomycetaceae*. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* 101, 73–104. doi:10.1007/s10482-011-9656-0.

Lamichhane, J. R., Bischoff-Schaefer, M., Bluemel, S., Dachbrodt-Saaydeh, S., Dreux, L., Jansen, J. P., et al. (2017). Identifying obstacles and ranking common biological control research priorities for Europe to manage most economically important pests in arable, vegetable and perennial crops. *Pest Manag. Sci.* 73, 14–21. doi:10.1002/ps.4423.

Langham, C. D., Williams, S. T., Sneath, P. H. A., and Mortimer, A. M. (1989). New probability matrices for identification of *Streptomyces*. *Microbiology* 135, 121–133. doi:10.1099/00221287-135-1-121.

Lee, T., Han, Y. K., Kim, K. H., Yun, S. H., and Lee, Y. W. (2002). Tri13 and tri7 determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. *Appl. Environ. Microbiol.* 68, 2148–2154. doi:10.1128/AEM.68.5.2148-2154.2002.

Lemmens, M., Haim, K., Lew, H., and Ruckenbauer, P. (2004). The effect of nitrogen fertilization on Fusarium head blight development and deoxynivalenol contamination in wheat. *J. Phytopathol.* 152, 1–8. doi:10.1046/j.1439-0434.2003.00791.x.

Liu, X., Bolla, K., Ashforth, E. J., Zhuo, Y., Gao, H., Huang, P., et al. (2012). Systematics-guided bioprospecting for bioactive microbial natural products. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* 101, 55–66. doi:10.1007/s10482-011-9671-1.

Locci R. (1989). "*Streptomyces* and related genera," in *BERGEY'S Manual of Systematic Bacteriology*, eds. S. T. Williams, M. E. Sharpe, and J. G. Holt (Williams & Wilkins, Baltimore USA), 2451–2508.

Luongo, L., Galli, M., Corazza, L., Meekes, E., De Haas, L., Van Der Plas, C. L., et al. (2005). Potential of fungal antagonists for biocontrol of *Fusarium* spp. in wheat and maize through competition in crop debris. *Biocontrol Sci. Technol.* 15, 229–242. doi:10.1080/09583150400016852.

Lutz, M. P., Feichtinger, G., Défago, G., and Duffy, B. (2003). Mycotoxigenic *Fusarium* and deoxynivalenol production repress chitinase gene expression in the biocontrol agent *Trichoderma atroviride* P1. *Appl. Environ. Microbiol.* 69, 3077–3084. doi:10.1128/AEM.69.6.3077-3084.2003.

Maresca, M. (2013). From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. *Toxins (Basel).* 5, 784–820. doi:10.3390/toxins5040784.

Maresca, M., Mahfoud, R., Garmy, N., and Fantini, J. (2002). The mycotoxin deoxynivalenol affects nutrient absorption in human intestinal epithelial cells. *J Nutr* 132, 2723–2731.

Matarese, F., Sarrocco, S., Gruber, S., Seidl-Seiboth, V., and Vannacci, G. (2012). Biocontrol of Fusarium head blight: interactions between *Trichoderma* and mycotoxigenic *Fusarium*. *Microbiology* 158, 98–106. doi:10.1099/mic.0.052639-0.

McCartney, C. A., Brûlé-Babel, A. L., Fedak, G., Martin, R. A., McCallum, B. D., Gilbert, J., et al. (2016). Fusarium head blight resistance QTL in the spring wheat cross Kenyon/86ISMN 2137. *Front. Microbiol.* 7, 1542. doi:10.3389/fmicb.2016.01542.

McCormick, S. P. (2013). Microbial detoxification of mycotoxins. *J. Chem. Ecol.* 39, 907–918. doi:10.1007/s10886-013-0321-0.

McCormick, S. P., Stanley, A. M., Stover, N. A., Alexander, N. J., and Pathogens, B. F. (2011). Trichothecenes: from simple to complex mycotoxins. 802–814. doi:10.3390/toxins3070802.

McMullen, M., Bergstrom, G., De Wolf, E., Dill-macky, R., Hershman, D., Shaner, G., et al. (2012a). Fusarium head blight disease cycle, symptoms, and impact on grain yield and quality frequency and magnitude of epidemics since 1997. *Plant Dis.* 96.

McMullen, M., Bergstrom, G., Dill-Macky, R., and Shaner, G. (2012b). A unified effort to fight an enemy of wheat and barley: Fusarium head blight. *Plant Dis.* 96, 1712–1728. doi:10.1094/PDIS-03-12-0291-FE.

Méndez, C., Brana, A. F., Manzanal, M. B., and Hardisson, C. (1985). Role of substrate mycelium in colony development in *Streptomyces*. *Can. J. Microbiol.* 31, 446–450. doi:10.1139/m85-083.

Merrick, M. J. (1976). A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor*. *J. Gen. Microbiol.* 96, 299–315. doi:10.1099/00221287-96-2-299.

Mesterházy, A. (1995). Types and components of resistance to Fusarium head blight of wheat. *Plant Breed.* 114, 377-386. doi:10.1111/j.1439-0523.1995.tb00816.x.

Miller, J. D., Greenhalgh, R., Wang, Y., and Lu, M. (1991). Trichothecene chemotypes of three *Fusarium* species. *Mycologia* 83, 121–130. doi:10.1080/00275514.1991.12025988.

Mudge, A. M., Dill-Macky, R., Dong, Y., Gardiner, D. M., White, R. G., and Manners, J. M. (2006). A role for the mycotoxin deoxynivalenol in stem colonisation during crown rot disease of wheat caused by *Fusarium graminearum* and *Fusarium pseudograminearum*. *Physiol. Mol. Plant Pathol.* 69, 73–85. doi:10.1016/j.pmpp.2007.01.003.

Niwa, S., Kubo, K., Lewis, J., Kikuchi, R., Alagu, M., Ban, T. (2014). Variations for Fusarium head blight resistance associated with genomic diversity in different sources of the resistant wheat cultivar 'Sumai 3.' *Breed. Sci.* 64, 90–96. doi:10.1270/jsbbs.64.90.

Nourozian, J., Etebarian, H. R., and Khodakaramian, G. (2006). Biological control of *Fusarium graminearum* on wheat by antagonistic bacteria. *Songklanakarin J. Sci. Technol.* 28, 29–38.

Pal, K. K., Tilak, K. V. B. R., Saxcna, A. K., Dey, R., and Singh, C. S. (2001). Suppression of maize root diseases caused by *Macrophomina phaseolina*, *Fusarium moniliforme* and *Fusarium graminearum* by plant growth promoting rhizobacteria. *Microbiol. Res.* 156, 209–223. doi:10.1078/0944-5013-00103.

Palazzini, J. M., Ramirez, M. L., Torres, A. M., and Chulze, S. N. (2007). Potential biocontrol agents for Fusarium head blight and deoxynivalenol production in wheat. *Crop Prot.* 26, 1702–1710. doi:10.1016/j.cropro.2007.03.004.

Palazzini, J. M., Yerkovich, N., Alberione, E., Chiotta, M., and Chulze, S. N. (2017). An integrated dual strategy to control *Fusarium graminearum sensu stricto* by the biocontrol agent *Streptomyces* sp. RC 87B under field conditions. *Plant Gene* 9, 13–18. doi:10.1016/j.plgene.2016.11.005.

Paul, P. A., Lipps, P. E., Hershman, D. E., McMullen, M. P., Draper, M. A., and Madden, L. V. (2008). Efficacy of triazole-based fungicides for Fusarium head blight and deoxynivalenol control in wheat: a multivariate meta-analysis. *Phytopathology* 98, 999–1011. doi:10.1094/PHYTO-98-9-0999.

Pawlik, K., Kotowska, M., Chater, K. F., Kuczek, K., and Takano, E. (2007). A cryptic type I polyketide synthase (cpk) gene cluster in *Streptomyces coelicolor* A3(2). *Arch. Microbiol.* 187, 87–99. doi:10.1007/s00203-006-0176-7.

Pestka, J. J., and Smolinski, A. T. (2005). Deoxynivalenol: toxicology and potential effects on humans. *J. Toxicol. Environ. Health* 8, 39–69. doi:10.1080/10937400590889458.

Pridham, T. G., Hesseltine, C. W., and Benedict, R. G. (1958). A guide for the classification of streptomycetes according to selected groups. Placement of strains in morphological sections. *Appl. Microbiol.* 6, 52–79.

Proctor, R. H., McCormick, S. P., Kim, H. S., Cardoza, R. E., Stanley, A. M., Lindo, L., et al. (2018). Evolution of structural diversity of trichothecenes, a family of toxins produced by plant pathogenic and entomopathogenic fungi. *PLoS Pathog.* 14, e1006946. doi:10.1371/journal.ppat.1006946.

Ruckenbauer, P., Buerstmayr, H., and Lemmens, M. (2001). Present strategies in resistance breeding against scab (*Fusarium* spp.).

Salgado, J. D., Madden, L. V, and Paul, P. A. (2015). Quantifying the effects of Fusarium head blight on grain yield and test weight in soft red winter wheat. *Phytopathology* 105, 295–306.

Sarrocco, S., Valenti, F., Manfredini, S., Esteban, P., Bernardi, R., Puntoni, G., et al. (2019). Is exploitation competition involved in a multitrophic strategy for the biocontrol of Fusarium head blight? *Phytopathology* 109, 560–570. doi:10.1094/PHYTO-04-18-0123-R.

Sato, I., Ito, M., Ishizaka, M., Ikunaga, Y., Sato, Y., Yoshida, S., et al. (2012). Thirteen novel deoxynivalenol-degrading bacteria are classified within two genera with distinct degradation mechanisms. *FEMS Microbiol. Lett.* 327, 110–117. doi:10.1111/j.1574-6968.2011.02461.x.

Schaafsma, A. W., Tamburic-Ilincic, L., and Hooker, D. C. (2005). Effect of previous crop, tillage, field size, adjacent crop, and sampling direction on airborne propagules of *Gibberella zeae*/*Fusarium graminearum*, fusarium head blight severity, and deoxynivalenol accumulation in winter wheat. *Can. J. Plant Pathol* 27, 217–224.

Schatz, A., and Waksman, S. A. (1944). Effect of streptomycin and other antibiotic substances upon *Mycobacterium tuberculosis* and related organisms. *Proc. Soc. Exp. Biol. Med.* 57, 244–248. doi:10.3181/00379727-57-14769.

Schisler, D. A., Khan, N. I., Boehm, M. J., and Slininger, P. J. (2002). Greenhouse and field evaluation of biological control of Fusarium Head Blight on durum wheat. *Plant Dis.* 86, 1350–1356.

Schöneberg, A., Musa, T., Voegele, R. T., and Vogelgsang, S. (2015). The potential of antagonistic fungi for control of *Fusarium graminearum* and *Fusarium crookwellense* varies depending on the experimental approach. *J. Appl. Microbiol.* 118, 1165–1179. doi:10.1111/jam.12775.

Seipke, R. F., Kaltenpoth, M., and Hutchings, M. I. (2012). *Streptomyces* as symbionts: an emerging and widespread theme? *FEMS Microbiol. Rev.* 36, 862–876. doi:10.1111/j.1574-6976.2011.00313.x.

Shi, C., Yan, P., Li, J., Wu, H., Li, Q., and Guan, S. (2014). Biocontrol of *Fusarium graminearum* growth and deoxynivalenol production in wheat kernels with bacterial antagonists. *Int. J. Environ. Res. Public Health* 11, 1094–1105. doi:10.3390/ijerph110101094.

Shirling, E. B., and Gottlieb, D. (1968a). Cooperative description of type cultures of *Streptomyces* II. Species descriptions from first study. *Int. J. Syst. Bacteriol.* 18, 69–189. doi:10.1099/00207713-18-2-69.

Shirling, E. B., and Gottlieb, D. (1968b). Cooperative description of type cultures of *Streptomyces* III. Additional species descriptions from first and second studies. *Int. J. Syst. Bacteriol.* 18, 279–392. doi:10.1099/00207713-18-4-279.

Shirling, E. B., and Gottlieb, D. (1969). Cooperative description of type cultures of *Streptomyces* IV. Species descriptions from the second, third and fourth studies. *Int. J. Syst. Bacteriol.* 19, 391–512. doi:10.1099/00207713-19-4-391.

Shirling, E. B., and Gottlieb, D. (1972). Cooperative description of type strains of *Streptomyces*: V. Additional descriptions. *Int. J. Syst. Bacteriol.* 22, 265–394. doi:10.1099/00207713-22-4-265.

Siegel, D., and Babuscio, T. (2011). Mycotoxin management in the European cereal trading sector. *Food Control* 22, 1145–1153. doi:10.1016/j.foodcont.2011.02.022.

Smiley, R. W., Gourlie, J. A., Easley, S. A., Patterson, L.-M., and Whittaker, R. G. (2005). Crop damage estimates for crown rot of wheat and barley in the Pacific Northwest. *Plant Dis.* 89, 595–604. doi:10.1094/PD-89-0595.

Spolti, P., Del Ponte, E. M., Dong, Y., Cummings, J. A., and Bergstrom, G. C. (2013). Triazole sensitivity in a contemporary population of *Fusarium graminearum* from New York wheat and competitiveness of a tebuconazole-resistant isolate. *Plant Dis.* 98, 607–613. doi:10.1094/pdis-10-13-1051-re.

Studholme, D. J. (2016). Genome update. Let the consumer beware: *Streptomyces* genome sequence quality. *Microb. Biotechnol.* 9, 3–7. doi:10.1111/1751-7915.12344.

Trail, F. (2009). Update on the *Fusarium graminearum* genome for blighted waves of grain: *Fusarium graminearum* in the postgenomics era. *Plant Physiol.* 149, 103–110. doi:10.1104/pp.108.129684.

Viaene, T., Langendries, S., Beirinckx, S., Maes, M., and Goormachtig, S. (2016). *Streptomyces* as a plant's best friend? *FEMS Microbiol. Ecol.* 92. doi:10.1093/femsec/fiw119.

Wachowska, U., Packa, D., and Wiwart, M. (2017). Microbial inhibition of *Fusarium* pathogens and biological modification of trichothecenes in cereal grains. *Toxins (Basel).* 9, 408. doi:10.3390/toxins9120408.

Waksman, S. A., and Henrici, A. T. (1943). The nomenclature and classification of the actinomycetes. *J. Bacteriol.* 46, 337–41.

Waksman, S. A., and Woodruff, H. B. (1940). Bacteriostatic and bactericidal substances produced by a soil *Actinomyces*. *Proc. Soc. Exp. Biol. Med.* 45, 609–614. doi:10.3181/00379727-45-11768.

Waksman, S. A., and Woodruff, H. B. (1942). Selective antibiotic action of various substances of microbial origin. *J. Bacteriol.* 44, 373–84.

Wang, Q., Vera Buxa, S., Furch, A., Friedt, W., and Gottwald, S. (2015). Insights into *Triticum aestivum* seedling root rot caused by *Fusarium graminearum*. *Mol. Plant-Microbe Interact.* 28, 1288–1303. doi:10.1094/MPMI-07-15-0144-R.

Ward, T. J., Bielawski, J. P., Kistler, H. C., Sullivan, E., and O'Donnell, K. (2002). Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. *Proc. Natl. Acad. Sci. U. S. A.* 99, 9278–83. doi:10.1073/pnas.142307199.

Watve, M., Tickoo, R., Jog, M., and Bhole, B. (2001). How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.* 176, 386–390. doi:10.1007/s002030100345.

Wildermuth, H., and Hopwood, D. A. (1970). Septation during sporulation in *Streptomyces coelicolor*. *J. gen. Microbiol* 60, 51–59.

Williams, S. T., Goodfellow, M., and Alderson, G. (1989). "Genus *Streptomyces* Waksman and Henrici 1943," in *Bergey's Manual of Determinative Bacteriology*, eds. S. T. Williams, E. Sharpe, M. Willkins, and J. Holt (Baltimore: Williams & Wilkins), 2452–2492.

Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A., and Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. *Microbiology* 129, 1743–1813. doi:10.1099/00221287-129-6-1743.

Windels, C. E. (2000). Economic and social impacts of fusarium head blight: changing farms and rural communities in the northern great plains. *Phytopathology* 90, 17–21. doi:10.1094/PHYTO.2000.90.1.17.

Xue, A. G., Voldeng, H. D., Savard, M. E., Fedak, G., Tian, X., and Hsiang, T. (2009). Biological control of fusarium head blight of wheat with *Clonostachys rosea* strain ACM941. *Can. J. Plant Pathol.* 31, 169–179. doi:10.1080/07060660909507590.

Yekkour, A., Sabaou, N., Zitouni, A., Errakhi, R., Mathieu, F., and Lebrihi, A. (2012). Characterization and antagonistic properties of *Streptomyces* strains isolated from Saharan soils, and evaluation of their ability to control seedling blight of barley caused by *Fusarium culmorum*. *Lett. Appl. Microbiol.* 55, 427–435. doi:10.1111/j.1472-765x.2012.03312.x.

Yin, Y., Liu, X., Li, B., and Ma, Z. (2009). Characterization of sterol demethylation inhibitor-resistant isolates of *Fusarium asiaticum* and *F. graminearum* collected from wheat in China. *Phytopathology* 99, 487-497. doi:10.1094/phyto-99-5-0487.

Yu, H., Zhou, T., Gong, J., Young, C., Su, X., Li, X. Z., et al. (2010). Isolation of deoxynivalenol-transforming bacteria from the chicken intestines using the approach of PCR-DGGE guided microbial selection. *BMC Microbiol.* 10. doi:10.1186/1471-2180-10-182.

Yuan, Q.-S., Yang, P., Wu, A.-B., Zuo, D.-Y., He, W.-J., Guo, M.-W., et al. (2018). Variation in the microbiome, trichothecenes, and aflatoxins in stored wheat grains in Wuhan, China. *Toxins (Basel).* 10, 171. doi:10.3390/toxins10050171.

Zhao, Y., Selvaraj, J. N., Xing, F., Zhou, L., Wang, Y., Song, H., et al. (2014). Antagonistic action of *Bacillus subtilis* strain SG6 on *Fusarium graminearum*. *PLoS One* 9, e92486. doi:10.1371/journal.pone.0092486.

Aims and objectives

Wheat is one of the most important cereal worldwide, with a global production of 759 million tons reached in 2017/2018. Fungal diseases can affect its production by 15-20%. Among the most important ones, diseases caused by *Fusarium* spp. play a major role. *Fusarium graminearum* together with *F. culmorum* and *F. pseudograminearum* are the main causal agents of Fusarium head blight (FHB), root rot (FRR) and foot rot (FFR). They affect the yield quantity and quality but also contaminate grain production with mycotoxins. In an effort to reach an integrated management of fungal diseases, also due to the latest European regulation, which reduced chemicals in crop protection, the use of biocontrol agents is a feasible option. Recently, many microorganisms have been tested against toxigenic *Fusarium* spp., but the complex interactions occurring within the cereal-*Fusarium* spp. pathosystem are seldomly explored.

Streptomyces spp. are Gram-positive bacteria ubiquitous in soil as free living microorganisms but also as symbionts with inner tissues of plants. Their ability to colonize successfully different substrates is likely due to the production of a wide range of bioactive compounds able to protect them from other competitors as well as indirectly helping plants to counteract pathogens. These features make streptomycetes promising biocontrol candidates in complex pathosystem.

This PhD project aims therefore to select and characterize potential biocontrol *Streptomyces* strains against *Fusarium* pathogens of wheat and its related mycotoxins (focusing on deoxynivalenol that is the major contaminant of cereals). The research has been divided in the following steps:

- Critical analysis of the literature and patents dealing with the use of streptomycetes against toxigenic *Fusarium* spp. (Chapter 1);
- Development of effective screening methods *in vitro* for novel biocontrol agents (Chapter 2). First of all, experimental procedures able to take into account, at the laboratory scale, the variations that

occur when the streptomycetes, the fungus and the plant interact have been set up. In addition, a high-throughput bioassay able to test molecules limiting deoxynivalenol production has been proposed;

- Assessment of plant growth promotion and biocontrol activity *in vitro* and *in planta* of a pool of streptomycetes (Chapter 3). The most promising strains, selected during the first part of the thesis, were tested for their activity against disease severity and deoxynivalenol production in wheat grains;
- Isolation of antifungal compounds produced by three promising biocontrol strains able to inhibit mycelial proliferation and/or deoxynivalenol production (Chapter 4). This work was carried out in collaboration with the research group of Professor Barrie Wilkinson at the Department of Molecular Microbiology (John Innes Centre, Norwich, UK) and the isolated metabolites will likely be patented.

Chapter 1: Critical assessment of *Streptomyces* spp. able to control toxigenic fusaria in cereals: a literature and patent review

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Abstract

Mycotoxins produced by *Fusarium* species on cereals represent a major concern for food safety worldwide. *Fusarium* toxins that are currently under regulation for their content in food include trichothecenes, fumonisins, and zearalenone. Biological control of *Fusarium* spp. has been widely explored with the aim of limiting disease occurrence, but few efforts have focused so far on limiting toxin accumulation in grains. The bacterial genus *Streptomyces* is responsible for the production of numerous drug molecules and represents a huge resource for the discovery of new molecules. *Streptomyces* spp. are also efficient plant colonizers and able to employ different mechanisms of control against toxigenic fungi on cereals. This review describes the outcomes of research using *Streptomyces* strains and/or their derived molecules to limit toxin production and/ or contamination of *Fusarium* species in cereals. Both the scientific and patent literature were analyzed, starting from the year 2000, and we highlight promising results as well as the current pitfalls and limitations of this approach.

Keywords: mycotoxins, deoxynivalenol, fumonisin, biocontrol, antagonism, bioactive compounds, wheat

1. Introduction

Mycotoxins are extracellular metabolites produced by filamentous fungi that contaminate cereals, grains, fruits and vegetables. The most important

Fusarium toxins are trichothecenes, zearalenone (ZEN) and fumonisins (FBs), that are dangerous for human and animal health, and their presence in food is regulated worldwide [1]. Mycotoxin co-occurrence in food is a real and relatively underestimated issue [2], as is the modification of toxins by plant metabolism (creating masked mycotoxins) [3]. Both factors mean that the levels of toxins measured in food, and therefore being ingested, are significantly underestimated. Due to this, it is likely that normative limits will be lowered by the regulatory agencies in the future.

Cereals, the staple foods of diets all over the world, are perfect hosts for pathogenic and toxigenic fungi and represent one of the main sources of mycotoxin contamination for humans and animals [4]. Among toxigenic species, *Fusarium* spp. (Division Ascomycota) are major producers of mycotoxins in cereals [5].

Trichothecenes A-B are mainly associated with Fusarium head blight (FHB) and crown rot (FCR) in wheat and barley. The major group of *Fusarium* spp. responsible for these diseases include *Fusarium graminearum* species complex (FGSC; [6]) which exhibit a diverse distribution of species across the different continents [7]. The most important species are *F. graminearum, F. culmorum* and *F. pseudograminearum* [8,9]. Grain quality decrease and yield are of concern [10]. The trichothecenes type B are the most prevalent and comprise deoxynivalenol (DON) and nivalenol (NIV) and their acetylated forms 3-ADON, 15-ADON and 4-ANIV [11]. They are immunosuppressant, neurotoxic and cause intestinal irritation, leading to feed refusal in livestock [12,13]. In maize, *F. graminearum* and other related species have been found to be associated to Fusarium ear rot (FER), contaminating grains with ZEN. ZEN displays estrogenic activity, causing reproductive problems in animals, in addition to cytotoxic and immunosuppressive effects [14,15].

Ear rot in maize is also caused by *F. moniliforme* (now referred as *F. verticillioides* [16]) and *F. proliferatum*, which produce fumonisins [17]. Fumonisins have been classified as Group 2B carcinogens, i.e. as possibly carcinogenic to humans [18], and fumonisin B1 (FB_1) is the most abundant

analogue found in contaminated samples [19]. Moreover, *Fusarium* spp. infecting cereals can also produce other minor mycotoxins with cytotoxic effects such as enniatins, beauvericin and moniliformin. Knowledge gaps regarding the occurrence, toxicity and toxicokinetic data for these compounds in cereal crops is a major and immediate problem [20].

Fusarium spp. infections of cereal is therefore a major concern for both the growers and the food chains associated with the processing of grains. Several control strategies against this complex group of pathogens have been developed and include host resistance, the application of fungicides, and implementation of specific agricultural practices [21]. However, effective management of *Fusarium* pathogens and the related toxins cannot be achieved using a single control strategy because each has its own limitations [22]. Therefore, at least in Europe, the integrated disease management is urgently needed, favored by European Regulation 1107/2009/EC and European Directive 128/2009/EC [23,24]. Moreover, biocontrol approaches are becoming increasingly important due to the limitation on the use of certain fungicides. Amongst the biocontrol agents (BCA) used to control toxigenic *Fusarium* spp. in cereals, bacteria have shown several successful outcomes. For instance, strains of *Bacillus* spp. [25–28], *Brevibacillus* sp. [29], *Pseudomonas* spp. [27,30], and *Lysobacter enzymogenes* [31] have been applied to limit pathogen development, reducing disease severity and mycotoxin production. Microbial communities or single strains have been also tested to detoxify contaminated substrates as reviewed by McCormick et al. in 2013 [32].

Bacteria of the genus *Streptomyces* display promising plant growth promoting features and biocontrol efficacy against plant pathogens. They belong to the phylum of Gram-positive Actinobacteria, which is one of the largest taxonomic units within the bacterial domain, and include microorganisms relevant to human and veterinary medicine, biotechnology as well as to ecology [33]. Streptomycetes are the most abundant actinobacteria in soil [34]. They display a unique life cycle, and after germination grow through a combination of tip

extension and the branching of hyphae. They first form a vegetative mycelium firmly attached to the growth substrate and, subsequently, due to nutrient depletion and under environmental stress signals, develop an aerial mycelium. Each aerial hypha then differentiates into a long chain of pre-spore compartments which subsequently mature into individual spores [35]. The ability to produce a variety of secondary metabolites, including anti-infective agents, has an important ecological role including the inhibition of competitors during the transition from mycelial to aerial growth [36]. These various characteristics enable them to colonize different substrates and establish symbiotic interactions with plant tissues and other eukaryotes [37]. The ability to produce numerous secondary metabolites means they are the most exploited bacterial genus in natural product research. Notably, more than half of all antibiotics in current clinical use are derived from actinobacterial secondary metabolites [38]. Furthermore, *Streptomyces* spp. have been evaluated as plant growth promoting bacteria (PGPB), as they can inhibit pathogen development, enhance nutrient uptake by mineral solubilization, and increase plant growth by nitrogen fixation and phytohormone synthesis [39]. Streptomycetes have therefore been investigated for their possible use in agriculture including cereal crops [40].

The diversity of secondary metabolites production plus their reported endophytic features make the genus *Streptomyces* a perfect candidate to control toxigenic *Fusarium* spp. development and related toxin production [41,42]. Endophytic microorganisms, able to reduce disease severity on spikelets, have been reported as useful antagonists against Fusarium head blight [43]. Nevertheless, the incredible diversity and potentiality of these microorganisms against mycotoxigenic fungi and their possible influence on toxin accumulation has been rarely explored and deserves further investigation [44]. This review describes reports in which streptomycetes, or molecules derived from them, were exploited against *Fusarium* spp., and will pay special attention to the possible influence on toxin production. The scientific and patent literature were analyzed from the period 2000-2018.

2. Critical assessment of literature

Despite the huge amount of literature regarding the biological control of *Fusarium* mycotoxigenic isolates in cereals, only two products have found a consistent market niche [45]. These are based on *Pseudomonas chlororaphis* and *Pythium oligandrum* and are marketed in Europe as Cerall® (Belchim Crop Protection) and Polyversum® (Biopreparáty/De Sangosse) respectively [46]. Furthermore, no *Streptomyces* product is officially registered to be used for this purpose [47]. The main obstacles for biocontrol agents are due to the lack of consistency when microbial inoculants are applied under complex environmental conditions, and to the complexity of finding appropriate formulation and timing for application [48]. Biological, ecological, toxicological and regulatory cost factors also influence the effectiveness and marketability of biological control products [49].

In order to verify the status of research using *Streptomyces* strains, and their derived molecules, to limit toxigenic *Fusarium* spp. infections and/or toxin contamination we screened the published literature. To critically assess the status of each research paper a set of definitions describing the type of study and their accuracy were established:

1. *Streptomyces* species definition. Species identification is essential as approximately 10 *Streptomyces* species have been described as plant pathogens, causing economically important diseases on underground plant structures such as tuber/root crops. The best studied and characterized of these is *Streptomyces scabies* which causes potato scab [50,51]. Moreover, from a food safety perspective, *Streptomyces* isolates producing antimycin A, which is potentially dangerous to human health, have been found on wheat and barley grains [52]. Therefore, it is essential that species and strain characterisation is performed accurately.
2. *In vitro* testing for antifungal activity. This is generally the first step for identifying antifungal microorganisms or molecules produced by them. Such studies help define the mechanism(s) of action of the *Streptomyces*

species and lead to the identification of potential interactions with the target organism. Assessment of bioactivity should consider the diversity of targets (verifying if pathogen diversity influences the consistency of the BCA or derived product). Indeed, specific interactions occur among bacterial and fungal strains [53] and this may impact the biocontrol capability of a strain [54,55].

3. The effect of culture media in the bioassays *in vitro*. Media composition modulates secondary metabolite production in actinomycetes [55–57] and optimising laboratory selection procedures should broaden the number of interesting BCAs that can be identified.
4. The use of fermentation extracts to perform bioassays. During screening procedures, it would be ideal to identify the metabolite(s) responsible for the observed antifungal effect. The screening of crude extracts is generally followed by further steps of purification, chemical analysis and retesting of purified compound(s) [58].
5. Evaluation of the antifungal mode of action. Risks concerning the use of these antibiotic producing bacteria associated to events of horizontal gene transfer and the development of antibiotic resistance are still under debate within the scientific community [44]. However, given the current legislative requests [59], understanding the mode of action is essential in order to proceed with the registration of a BCA, in order to avoid risks of spreading in the environment dangerous metabolites for human and animal health [60].
6. Assessment of the ability to colonize treated plant organs. Many BCAs are rhizosphere colonizing microorganisms and can be applied as seed coatings [61]. However, some *Streptomyces* spp. can exhibit endophytic behaviour, colonizing different parts of the plant (e.g. roots, stem, leaves) [41]. Some BCAs exhibit activity both in the rhizosphere and after infection of the plant and function inside the root at the same time. Therefore, the colonization niche of the strain should be investigated in order to warrant a consistent protection [62]. These studies are

fundamental to provide an assessment of the durability of the protection warranted by the BCA.

7. Testing the influence of complex environmental conditions. As for pathogens during disease development, antagonist strains are influenced by environmental factors which strongly impact the ability of the BCAs to exert their biocontrol activity [40]. Assessing the impact of environmental parameters on BCA using both greenhouse and field trials is essential to select strains with consistent biocontrol activity.
8. Assessment of antifungal and plant growth promoting effect *in planta*. This step is essential, given that the BCA will ultimately be employed in the field. Very often there is poor correlation between *in vitro* and *in planta* trials [55,63,64]. Moreover, the wide range of metabolites produced may have direct influences on plant development altering growth and plant fitness both positively and negatively [39]. Indeed, negative effects cannot be underestimated: some *Streptomyces* can be pathogens (see before) or produce phytotoxic and herbicidal substances [65].
9. Assessment of the method used for application. Selecting an appropriate delivery system for the BCA as well as an optimized formulation can determine its efficacy in the field [66].
10. The effects of the BCA on the pathogen inoculum *in planta*. Due to the complex epidemiology of *Fusarium* diseases in cereals, quantification of the pathogen *in planta* is important to verify if the treatment can, for example, effectively reduce the source of overwintering inoculum, limiting the infection pressure at the subsequent infection season [67].
11. Quantification of the mycotoxin. It is essential to verify if the BCA limits toxin production specifically given there is a lack of full correlation between the presence of the fungus and the amount of toxin that is found in the grains [68,69]. Moreover, some secondary metabolites can limit toxin production without impairing growth of the pathogen [70]. Biological interactions can also lead to unexpected cross talk between the BCA and

pathogen that can lead to an overproduction of toxins and secondary metabolites [71–75].

3. Literature analysis

To guide future implementation of biocontrol research using *Streptomyces* spp. it is essential to identify the strengths and weaknesses of past and present research in this domain. Therefore, we reviewed the published literature focussing on the methods used for the selection of promising biocontrol streptomycetes and on the results achieved.

We searched the Scopus and Google Scholar databases for articles including the words "*Fusarium*" and "*Streptomyces*" that were published during the timeframe 2000-2018. The resulting articles were read and individually screened leading to the identification of 64 articles that deal with the ability of *Streptomyces* or their secondary metabolites to limit the growth or toxin production of toxigenic *Fusarium* spp. in cereals (Table 1)**.**

Streptomyces spp. or their derived molecules have been tested mostly against *Fusarium* spp. producing trichothecenes, including DON. The species investigated are all usually found to infect cereals and include *F. graminearum, F. culmorum, F. poae, F. crockwellense, F. sporotrichioides* and *F. equiseti.* The most studied interactions address the wheat-*F. graminearum* pathosystem, which is the most important cause of DON (and derivatives) accumulation in grains [76]. Less frequently, streptomycetes have been tested against fumonisin producers in maize, all belonging to the *F. fujikuroi* species complex [77].

3.1 *Streptomyces* identification

Regarding the identification of *Streptomyces* species, most studies focused on the integration of morphological and molecular characteristics. Given the complexity of streptomycete biology [33], the use of 16S rRNA alone as molecular marker is not sufficient to achieve species discrimination. Multi-locus sequence typing [78] integrated with biochemical and morphological identification would be a preferred option, but none of the studies used this

approach. On this basis, all the species identifications reported in the selected papers should be treated with caution. Looking forward, the increasing number of *Streptomyces* strain genomes now available may help in correct species identification [79].

3.2 Screening for antifungal activity: *in vitro* tests

Amongst the selected articles, *in vitro* testing is the most commonly used first line screening method. Indeed, dual culture assays on solid media are exploited in all the studies as a preliminary screen, evaluating the inhibition halo between the growth of the streptomycete and the fungal target or measuring the radial growth of the *Fusarium* colony in comparison with an untreated control to obtain a percentage of growth inhibition. Rather than use these standard *in vitro* inhibition assays, some research groups [80–82] characterize the type of interactions occurring in dual culture by using the Index of dominance (Id) [83]. The Id consists of visually observe antagonist and pathogen growth in dual-culture, testing different media or water activity (a_w) of the culture medium, and to classify the type of interactions occurring based on predefined scores: mutual intermingling (1/1), mutual inhibition on contact (2/2), mutual inhibition at a distance (3/3), dominance of one species on contact (4/0) and dominance at a distance (5/0). This method evaluates if the inhibition is due to the production of antifungal metabolites diffusible in the media or if the mycelium is parasitized by the antagonists. Moreover, it can be noted the negative effect of the target pathogen on the potential antagonists. Therefore, the selection of biocontrol agents is carried out by evaluating the biocontrol interactions (e.g. mycoparasitism, competition or antibiosis) established at different growth conditions.

For most reports, growth of the *Streptomyces* strain inoculum to some predefined point usually takes place on agar media before addition of the pathogen in order to allow a complete establishment of these growing bacteria [80,84].

The use of a diverse range of growth media and fungal strains was evaluated in our analysis given the importance that these criteria have in the estimation

of the biocontrol activity *in vitro* [55]. Interestingly, the influence of growth media was seldomly evaluated in these types of experiments [84,85] as well as the assessment of antifungal activity on different *Fusarium* strains belonging to a single species [80,86–88].

Given the lack of a standardized protocol when performing dual culture assays (e.g. *Fusarium* strains on which the biocontrol activity should be tested, position and distance between streptomycetes and *Fusarium* strain inoculum, timing of observation after pathogen inoculum, culture medium) it is difficult to compare the results between studies. However, here we report some examples of the wide range of activities recorded against mycelial proliferation. For instance, growth inhibition percentages against *F. graminearum* and *F. verticillioides* ranged from the weakest (< of 20%) [84,89] up to 60-90% of inhibition [87,90]. Yekkour et al. [91] obtained different levels of inhibition in dual culture for isolated streptomycetes: indeed, only 6 out of 133 isolates displayed an anti-*Fusarium* activity and in particular only *F. culmorum* was significantly inhibited (inhibition halo > 20 mm). Less sensitive fungal species were *F. moniliforme*, *F. sporotrichoides*, *F. graminearum* and *F. proliferatum* [91].

3.3 Evaluation of antifungal mechanism of action

The importance of the identification of any antifungal molecules involved in the bioactivity led some researchers to achieve a complete characterization of the compounds involved. The fermentation process and the optimization of all the parameters (e.g. medium, agitation rate, pH, temperature) were strain and laboratory dependent [92–95]. For instance, it has been reported that some of the *Streptomyces* strains which are active against *F. moniliforme* on solid media lack antibiotic production in submerged liquid culture, highlighting the importance of an appropriate optimization of laboratory procedures and media in the stimulation of secondary metabolites [96]. The first attempt of compound purification is commonly carried out by crude extract fractionation [97,98]. Often the bioactivity of the selected strain is not related to a single mechanism:

different metabolites, enzymes or volatile organic compounds likely contribute to the overall antifungal activity. Many studies exploited the fermentation broth as a source of bioactive compounds [99–101]. Therefore, several compounds have been purified and tested against toxigenic *Fusarium* spp. For example, strain PAL114 produce saquayamycins A and C which inhibited the growth of *F. culmorum* at the minimum inhibitory concentrations of 75 ug/mL [102]. Three allelochemicals [5,7-dihydroxyflavone, 5-hydroxy-7-methoxy- flavone and di (2-ethylhexyl) phthalate] able to inhibit mycelial growth of *F. graminearum* were isolated and purified from the fermentation broth of *Streptomyces* sp. 6803 [103]. *In vitro* cultures of *Streptomyces* sp. 201, produce 2-methylheptyl isonicotinate able to inhibit the growth of *F. moniliforme* more efficiently than a natural analogue (isoniazid) [98]. On the other hand, modest activity has been observed by the metabolites extracted from *Streptomyces* LZ35 against *F. verticillioides* [104]. For several studies chitinase activity, rather than antibiotic production, was shown to play a role in the antifungal mechanism [105–108]. In addition, new antifungal proteins have been characterized, such as the one isolated from *Streptomyces* sp. C/33-6 culture supernatants which displayed a fungicidal activity, determining complete inhibition of conidia germination of *F. graminearum* [109].

Secondary metabolites exhibiting anti *Fusarium* activity can also include volatile organic compounds (VOCs). For example, *Streptomyces alboflavus* TD-1 was able to reduce the mycelial growth of *F. moniliforme* when volatile metabolites were applied as fumigants [110]. Inhibition of growth, sporulation and conidial germination has been recorded when culturing this strain on wheat seeds. In addition, the VOCs activity increased the fungal membrane permeability as observed by significant leakage of mycelial materials. Chemical analysis of these VOCs identified a high quantity of 2-methylisoborneol and 2-methyl disulphide, which were further tested for their antifungal activity [110,111]. VOCs production was also linked to the antagonist activity of *Streptomyces philanthi* RM-1-138 cultured on wheat seeds, which inhibited mycelium growth of *F. fujikuroi* by 50% [112]. Chemical

analysis showed that a complex mixture of volatile metabolites was involved [112].

It is evident from our analysis that the biocontrol activity of *Streptomyces* strains involves a large range of bioactive molecules. The exploitation of *Streptomyces* spp. has been, and will in future also be, hindered by the variability of the production of these metabolites. Therefore, to exploit the huge diversity of streptomycetes for successful disease management different factors, such as the age of the fungal colony, culture conditions, temperature, and other environmental parameters will have to be carefully studied, even at the very early stages of investigation. Transferring the outputs of these laboratory studies to the field remains one of the major challenges in exploiting *Streptomyces* spp. as BCAs for tackling toxigenic *Fusarium* spp.

3.4 Assessment of streptomycete effects *in planta*

Literature reports lack of durable and consistent effects when streptomycetes or commercially available formulations have been applied in greenhouse experiments and field trials [40]. Likely ability to cope in a complex environment that comprise the plant, the presence of the pathogens as well as several abiotic factors varies depending on the fitness of the strain and its formulation in the field. For this reasons, verifying the level of colonization achieved by the strain when used as BCA is essential to confirm its ecological fitness. Only a few papers have addressed this question in detail. Notable, most of these were published recently which indicates an increasing level of attention regarding *Fusarium*/plant/*Streptomyces* interactions [113,114].

Moreover, *in planta* experiments are essential during the process of BCA selection to confirm their ability to significantly decrease *Fusarium* spp. infections. Indeed, BCAs can influence crop growth and disease severity, reduce *Fusarium* inoculum levels on stubble after harvest as well as ideally the presence of mycotoxins [115]. However, only a limited number of studies (*N* = 16) performed complete *in planta* studies. The application of streptomycetes was tested on seeds [84,116–118], on the main emerged

spike [80,84,86,87] as well as wheat stubble [86]. Indeed, these bacteria can contribute to the reduction of FHB on wheat at different times in the *Fusarium* spp. life cycle. In a research conducted by Palazzini et al. [80] in 2007, isolates from wheat anthers, were applied to wheat heads grown in greenhouse and, after 16 days, their influence on FHB severity was estimated. Despite the slight reduction of diseases symptoms in comparison to the control, streptomycete BRC 87B decreased under undetectable level the DON content in spikes. For this reason, in a subsequent study it was tested in field, showing the ability to decrease FHB severity and DON amounts, as well as the *F. graminearum* inoculum on wheat stubble [86].

Testing the efficacy in the field requires also specific assessments of the way the strains are inoculated. For example, the use of a Korean strain isolated from rice kernels led to a significant reduction of the disease severity after its inoculation using a spore spraying method that was not achieved using point inoculation method on wheat heads [84]. This is actually the only study where the influence of the BCA application method was taken into account, and shows that, depending on the application of the BCA, different results can be obtained [47].

Differences in the level crop protection have been reported also against other *Fusarium* spp. For instance, two *Streptomyces* strain designated as DAUFPE 11470 and DAUFPE 14632 were isolated from maize rhizosphere in Brazil and tested against maize seed pathogenic fungi. Treatments on seeds with biomass deriving from streptomycete fermentation or with cell free filtrate reduced significantly *Fusarium subglutinans* incidence on stored maize seeds [119]. The same strains were also tested as spore suspension to assess their effects on seedling blight caused by *F. moniliforme* in greenhouse [120]. Bacterial treatments significantly reduced disease incidence compared with the controls, with protection level variable according to the tested pathogen inoculum concentrations. Indeed, the disease incidence has been significantly reduced at low and high antagonists and pathogen concentrations respectively. Moreover, their ability to reduce chlamydospores germination

was assessed: the percentage of germinated propagules was evaluated after antagonist treatments in sterilized soil added with glucose, to recreate the natural environment and enhance spore germination. The addition of glucose increased propagules germination in all the treatments, but the presence of the antagonists decreased this parameter up to 65%. This study stressed therefore the important influence of both antagonist and pathogen concentration and the presence of nutrients in the final biocontrol efficacy obtained *in planta* [120].

Streptomyces strains as reported above, can be helpful to reduce the disease symptoms, acting also as plant growth promoting bacteria. Despite the wide range of metabolites produced by them, their ability to influence plant development has been seldomly studied by the current literature addressing the biological control properties of the strain. Few positive examples include the report of negative influence on seed germination and seedling development [91] as well as improvement of plant growth parameters [87].

3.5 Evaluation of streptomycete activity against mycotoxin production

As noted above, it is essential to accurately determine the concentration of mycotoxins present in grains destined for human or animal consumption. Similarly, verification of the toxin content under experimental conditions is vital for the future of potential streptomycete biocontrol agents. Indeed, it should be possible that reduction of disease severity does not positively correlate with a reduction of the mycotoxin content in grain samples. So far only one research group has evaluated the reduction of DON mycotoxins by *Streptomyces* strains isolated from wheat anthers, in comparison to the level of infection, *in vitro*, in greenhouse and in the field [80,86]. Indeed, they showed that their streptomycete strains (BRC 87B and BRC 273) were able to significantly reduce DON levels on wheat grains, without influencing disease severity caused by *Fusarium* infections [80]. This suggests the existence of specific mechanism of inhibition uncoupling fungal fitness and toxin production. Follow-up research by the same group evaluated in the field

the use of BRC 87B which showed strong inhibition of DON production in wheat spikes [86].

Preliminary *in vitro* studies have also been conducted to verify the ability of streptomycetes to limit fumonisin accumulation. Strains isolated from soil samples amended with different organic manures by Nguyen et al. have been tested against fumonisins FB_1 and FB_2 production by *F. verticillioides* [121]. They significantly decreased (by up to 98.2%) the level of FB_1 and FB_2 in agar plate cultures [121]. Inhibition of FB_1 accumulation on milled maize agar was also demonstrated in another *in vitro* study using *Streptomyces* sp. AS1 [122], a strain isolated from peanuts in Egypt. Further, El-Naggar et al. [123] showed the ability of *Streptomyces* isolates to reduce accumulation of a wide range of mycotoxins including total aflatoxins, fumonisin, zearalenone, T-2 toxin, alternariol, and alternariol monomethyl ether. However, the identity of the *Fusarium* spp. producers was based only on morphological characteristics and should be considered with caution.

Table 1 Published studies regarding the efficacy of Streptomyces *spp. (and derived molecules) against* Fusarium *toxigenic species* in vitro, in planta *and under different environmental conditions. The methods used for the identification of the* Streptomyces *strain are also reported. Data were obtained combining the results of Scopus and Google scholar searches with the following search words "*Fusarium Streptomyces*" limiting the period of publication from 2000 to 2018.*

*Legend: M (Morphological identification), B (Biochemical identification), BCA/s (Biocontrol agent/s), GC (Growth chamber), G (Greenhouse), F (Field), *possibly misleading identification*

Fusarium spp. studied	Streptomycete identification	*In vitro* tests for antifungal activity	Influence of pathogen diversity	Influence of culture media on BCAs	*In vitro* tests using BCA extracts	Evaluation of antifungal mode of action	BCAs survival on plants	Environment of trials *in planta*	Evaluation of BCA application	BCAs effects on plants	BCAs effects on disease	BCAs effects on fusaria inoculum	Toxin measurement	References
F. avenaceum		x			x	x								[95]
F. avenaceum										x	x			[124]
F. avenaceum*, *F. oxysporum*, *F. solani					x	x								[125]
F. coeruleum*; *Gibberella saubinetii	16S rRNA	x			x	x								[101]
F. crookwellense*, *F. oxysporum		x				x								[107]
F. culmorum		x			x	x								[102]
F. culmorum	M/B/ 16S rRNA	x												[126]
F. culmorum		x			x	x								[99]
F. culmorum	M	x				x		GC		x	x			[116]
F. culmorum						x	x	GC		x	x			[114]
F. culmorum*, *F. moniliforme*, *F. sporotrichoides*, *F. graminearum*, *F. proliferatum	16S rRNA	x						GC		x	x			[91]

Fusarium spp. studied	Streptomycete identification	*In vitro* tests for antifungal activity	Influence of pathogen diversity	Influence of culture media on BCAs	*In vitro* tests using BCA extracts	Evaluation of antifungal mode of action	BCAs survival on plants	Environment of trials *in planta*	Evaluation of BCA application	BCAs effects on plants	BCAs effects on disease	BCAs effects on fusaria inoculum	Toxin measurement	References
F. culmorum*, *F. equiseti*, *F. proliferatum*, *F. graminearum*, *F. sporotrichioides*, *F. moniliforme*, *F. oxysporum	M/ 16S rRNA	x			x	x								[127]
F. culmorum*, *F. graminearum*, *F. proliferatum*, *F. oxysporum	M/B/ 16S rRNA	x				x								[128]
F. culmorum*, *F. oxysporum	16S rRNA	x			x	x								[129]
F. culmorum*, *F. oxysporum		x		x	x	x		GC		x				[130]
F. culmorum*, *F. graminearum*, *F. oxysporum								G			x			[118]
F. fujikuroi		x			x	x								[112]
F. graminearum	M/B/ 16S rRNA	x			x	x		G		x	x			[131]

Fusarium spp. studied	Streptomycete identification	*In vitro* tests for antifungal activity	Influence of pathogen diversity	Influence of culture media on BCAs	*In vitro* tests using BCA extracts	Evaluation of antifungal mode of action	BCAs survival on plants	Environment of trials *in planta*	Evaluation of BCA application	BCAs effects on plants	BCAs effects on disease	BCAs effects on fusaria inoculum	Toxin measurement	References
F. graminearum		x				x								[106]
F. graminearum		x			x	x				x				[103]
F. graminearum	M/B/ 16S rRNA	x			x	x	**x**			**x**				[113]
F. graminearum	M/16S rRNA	x		x				G	x	x	x			[84]
F. graminearum	M/B/ 16S rRNA	x			x	x								[132]
F. graminearum	M/B/ 16S rRNA	x	x					G			x		x	[80]
F. graminearum			x					F			x	x	x	[86]
F. graminearum			x					F		x	x	x	x	[133]
F. graminearum		x		x	x	x								[85]
F. graminearum								G; F				x		[134]
F. graminearum		x	x			x		G		x	x			[87]
F. graminearum, F. culmorum	16S rRNA	x												[135]

Fusarium spp. studied	Streptomycete identification	*In vitro* tests for antifungal activity	Influence of pathogen diversity	Influence of culture media on BCAs	*In vitro* tests using BCA extracts	Evaluation of antifungal mode of action	BCAs survival on plants	Environment of trials *in planta*	Evaluation of BCA application	BCAs effects on plants	BCAs effects on disease	BCAs effects on fusaria inoculum	Toxin measurement	References
F. graminearum*, *F. culmorum*, *F. oxysporum	M/B	x				x							x	[136]
F. graminearum*, *F. oxysporum		x			x	x				x				[137]
F. graminearum*, *F. oxysporum		x			x	x								[138]
F. graminearum*, *F. oxysporum*, *F. solani		x			x	x								[105]
F. graminearum*, *F. proliferatum*, *F. sporotrichioides*, *F. oxysporum		x			x	x								[109]
F. graminearum*, *F. verticilloides*, *F. culmorum	M/ 16S rRNA	x						G		x	x			[117]
F. graminearum*, *F. moniliforme*, *F. oxysporum*, *F. solani	M/B/ 16S rRNA	x			x	x								[100]

Fusarium spp. studied	Streptomycete identification	*In vitro* tests for antifungal activity	Influence of pathogen diversity	Influence of culture media on BCAs	*In vitro* tests using BCA extracts	Evaluation of antifungal mode of action	BCAs survival on plants	Environment of trials *in planta*	Evaluation of BCA application	BCAs effects on plants	BCAs effects on disease	BCAs effects on fusaria inoculum	Toxin measurement	References
F. graminearum*, *F. moniliforme	M/B/ 16S rRNA	x		x	x	x								[139]
F. moniliforme	M/B	x				x								[140]
F. moniliforme		x						G			x			[120]
F. moniliforme										x	x			[119]
F. moniliforme	16S rRNA	x												[89]
F. moniliforme	M/16S rRNA				x	x								[141]
F. moniliforme	M/B	x			x	x								[96]
F. moniliforme	M/16S rRNA	x			x	x								[93]
F. moniliforme	B	x				x								[94]
F. moniliforme	M/B/ 16S rRNA	x			x	x								[110]
F. moniliforme		x			x	x								[111]

Fusarium spp. studied	Streptomycete identification	*In vitro* tests for antifungal activity	Influence of pathogen diversity	Influence of culture media on BCAs	*In vitro* tests using BCA extracts	Evaluation of antifungal mode of action	BCAs survival on plants	Environment of trials *in planta*	Evaluation of BCA application	BCAs effects on plants	BCAs effects on disease	BCAs effects on fusaria inoculum	Toxin measurement	References
F. moniliforme*, *F. oxysporum	M/B/ 16S rRNA	x			x	x				x	x			[142]
F. moniliforme*, *F. oxysporum*, *F. semitectum	M/B	x			x	x								[98]
F. moniliforme*, *F. oxysporum*, *F. semitectum*, *F. solani	M/B/ 16S rRNA	x			x	x				x				[97]
F. oxysporum**, *F. solani*		x											x	[123]
F. poae	M/B	x			x	x								[143]
F. poae	M/ 16S rRNA	x												[144]
F. poae*, *F. avenaceum*, *F. culmorum		x												[145]
F. proliferatum	M/B/ 16S rRNA	x				x								[146]
F. subglutinans*, *F. sambucinum		x												[147]

Fusarium spp. studied	Streptomycete identification	*In vitro* tests for antifungal activity	Influence of pathogen diversity	Influence of culture media on BCAs	*In vitro* tests using BCA extracts	Evaluation of antifungal mode of action	BCAs survival on plants	Environment of trials *in planta*	Evaluation of BCA application	BCAs effects on plants	BCAs effects on disease	BCAs effects on fusaria inoculum	Toxin measurement	References
F. verticillioides	16S rRNA	x			x	x								[92]
F. verticillioides		x			x	x								[104]
F. verticillioides	16S rRNA	x			x	x							x	[121]
F. verticillioides		x		x									x	[122]
F. verticillioides	16S rRNA	x			x	x								[148]
F. verticillioides, F. oxysporum	M/B/ 16S rRNA	x			x	x								[90]
F. graminearum, F. culmorum, F. oxysporum, F. sporotrichiella, F. moniliforme		x				x		F	x	x	x			[149]

4. Patent search

To have a complete overview of the work using *Streptomyces* against toxigenic fusaria, a research of the major patent databases was carried out. Using both Espace-net and Orbit intelligence a total of 233 results were obtained using the keywords "*Fusarium Streptomyces*". By manual screening of the titles and abstracts a total of 25 patents were retained and added to Table 2. Given the use of different languages (most not English) only abstracts could be accessed so it was not possible to apply the same critical criteria used in our literature search. Most of the patent claimed general activity of strains and derived molecules against a large set of microorganisms including toxigenic fusaria. Only a single patent in its claim directly addressed the ability to limit *F. graminearum* growth on cereals [150]. Two documents patented the antifungal metabolites isolated from streptomycete strains and tested against toxigenic *Fusarium* spp. [151,152]. The other patents are related to specific formulation methods, using live streptomycetes, proposed as biocontrol products against plant pathogens, among them *Fusarium* spp. of cereal crops. Interestingly most of the patents are concentrated in the last 5 years (Table 2), therefore further developments could be expected also towards novel industrial applications in the near future.

Table 2 Patent lists of Streptomyces *spp. (and derived molecules) against* Fusarium *toxigenic species. Data were obtained combining the results of Espacenet and Orbit Intelligence with the following search words "*Fusarium Streptomyces*" limiting the period of publication from 2000 to 2018.*

Publication number	Publication date	Target *Fusarium* spp.	Source	Reference
RU2003100579 A	27/07/2004	*F. moniliforme, F. sambucinum, F. avenaceum*	Espacenet	[153]
KR100914225 B1	26/08/2009	*F. graminearum*	Espacenet	[154]
CN101698827B; CN101698827A	28/04/2010	*F. moniliforme*	Espacenet	[155]
CN101822272A	8/09/2010	*F. avenaceum, F. semitectum*	Orbit intelligence	[156]
KR101098280	23/12/2011	*F. proliferatum*	Orbit intelligence	[157]
CN102433281A; CN102433281 B	02/05/2012	*F. graminearum*	Espacenet	[158]
KR101211681	12/12/2012	*F. fujifuroi*	Orbit intelligence	[159]
CN102835423B; CN102835423A	26/12/2012	*F. nivale, F. graminearum*	Espacenet	[160]
CN103114064B; CN103114064A	22/05/2013	*F. moniliforme, F. graminearum*	Espacenet	[161]
CN103820351A; CN103820351B	28/05/2014	*F. moniliforme, F. graminearum*	Espacenet	[151]
CN104130965A	05/11/2014	*F. moniliforme*	Espacenet	[162]

Publication number	Publication date	Target *Fusarium* spp.	Source	Reference
CN104140982A	12/11/2014	*F. moniliforme*	Espacenet	[163]
CN105060951A	18/11/2015	*F. moniliforme*	Espacenet	[164]
EP3048890A1	3/08/2016	*F. culmorum*	Orbit intelligence	[165]
CN105886428A	24/08/2016	*F. verticillioides*	Espacenet	[166]
CN106676040	17/05/2017	*F. graminearum*	Orbit intelligence	[167]
CN107058131	18/08/2017	*F. graminearum*	Orbit intelligence	[150]
CN107164259A	15/09/2017	*F. culmorum*	Espacenet	[168]
CN107287130A	24/10/2017	*F. verticillioides*	Espacenet	[169]
WO201553482A1	16/04/2018	*F. proliferatum*	Orbit intelligence	[170]
CN108048380A	18/05/2018	*F. graminearum*	Espacenet	[171]
CN108102961A	1/06/2018	*F. graminearum*	Espacenet	[172]
CN108165506	15/06/2018	*F. graminearum*	Orbit intelligence	[173]
CN108208016	29/06/2018	*F. graminearum*	Orbit intelligence	[152]
CN108587981	28/09/2018	*F. graminearum*	Orbit intelligence	[174]

5. Conclusion and perspectives

Our review of the literature and patent clearly identifies a growing interest in the use of *Streptomyces* spp. as biological control agents against toxigenic *Fusarium* spp., both to inhibit growth and to limit toxin accumulation (contamination). However, it is clear that for the majority of the available studies, the findings are preliminary. In most cases a clear understanding of the role of the BCA, the identification of the molecules or mechanisms of inhibition, as well as the fungal targets are lacking [175]. Moreover, most of the data are limited to laboratory *in vitro* experiments and lack validation *in planta* or in the field.

The future of research on streptomycetes as biocontrol agents for *Fusarium* will need to integrate diverse expertise and may profit from new methods able to better mimic in the laboratory interactions occurring in the field [55]. Novel formulation and application techniques will be needed to enable individual beneficial microbes and microbial consortia to exert their activity in a consistent manner for different crops and soils [176]. For instance, one biocontrol approach to further investigate could be combining multiple strains to build consortia able to exert complementary activities [177]. Indeed, understanding the ecological role, including specific interactions with other microorganisms and the host, is essential for developing effective and long-lasting approaches of biocontrol. Reaching a better understanding of microbes-*Fusarium* interactions could help to provide effective biocontrol strains among natural endophytes present in the wheat microbiome [178] and within graminaceous plant rhizosphere [179]. The effect of specific interactions as well as the ability to shift metabolic profiles within the same *Streptomyces* species, niche and also among individuals [180] suggest that studies on the efficacy of strains should encompass a broad range of conditions mimicking the agricultural milieu [55]. Appropriate fitness tests able to predict the behaviour in the field are needed at the selection level. Novel BCAs or their metabolites could also be identified and produced integrating appropriate novel genome editing [181] as well as adaptive evolution

techniques [182]. A better understanding of secondary metabolites regulation during the interaction with fungi will help to increase their discovery for agricultural purposes [53].

Our analysis of the literature leads to the observation that each single paper only addresses a few aspects of the proposed criteria that would have to be evaluated in identifying effective *Streptomyces* based BCAs. This review may serve as a proposal for future research efforts which will likely profit from an integrated analysis of the different parameters that we have identified.

The increasing interest with industry proved by the raising number of patents addressing and referring to the use of *Streptomyces* spp. to limit *Fusarium* spp. in grains is a further indication of the potential role that this powerful group of microorganisms can play in the future of agricultural research. In conclusion, by performing a complete analysis of the literature regarding the use of *Streptomyces* spp. for the biological control of mycotoxigenic fusaria, we identified a set of parameters that we consider essential for enabling their implementation for biological and toxin contamination control. Our review suggests that streptomycetes have the potential to play a crucial role both as BCA, and as producers of novel inhibitory molecules, for the combined control of *Fusarium* infection and to limit the accumulation of mycotoxins in crops [183,184].

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References

1. Wu, F.; Groopman, J.D.; Pestka, J.J. Public health impacts of foodborne mycotoxins. *Annu. Rev. Food Sci. Technol.* 2014, *5*, 351–372.
2. Smith, M.C.; Madec, S.; Coton, E.; Hymery, N. Natural co-occurrence of mycotoxins in foods and feeds and their *in vitro* combined toxicological effects. *Toxins (Basel).* 2016, *8*, 94.
3. Stoev, S.D. Foodborne mycotoxicoses, risk assessment and underestimated hazard of masked mycotoxins and joint mycotoxin effects or interaction. *Environ. Toxicol. Pharmacol.* 2015, *39*, 794–809.

4. Pinotti, L.; Ottoboni, M.; Giromini, C.; Dell'Orto, V.; Cheli, F. Mycotoxin contamination in the EU feed supply chain: a focus on cereal by products. *Toxins (Basel).* 2016, *8*, 45.
5. Lee, H.J.; Ryu, D. Worldwide occurrence of mycotoxins in cereals and cereal-derived food products: public health perspectives of their co-occurrence. *J. Agric. Food Chem.* 2017, *65*, 7034–7051.
6. O'Donnell, K.; Ward, T.J.; Geiser, D.M.; Corby Kistler, H.; Aoki, T. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genet. Biol.* 2004, *41*, 600–623.
7. Aoki, T.; Ward, T.J.; Kistler, H.C.; O'Donnell, K. Systematics, phylogeny and trichothecene mycotoxin potential of Fusarium head blight cereal pathogens. *Mycotoxins* 2012, *62*, 91–102.
8. Pasquali, M.; Beyer, M.; Logrieco, A.; Audenaert, K.; Balmas, V.; Basler, R.; Boutigny, A.L.; Chrpová, J.; Czembor, E.; Gagkaeva, T.; et al. A European database of *Fusarium graminearum* and *F. culmorum* trichothecene genotypes. *Front. Microbiol.* 2016, *7*, 406.
9. Summerell, B.A.; Laurence, M.H.; Liew, E.C.Y.; Leslie, J.F. Biogeography and phylogeography of *Fusarium*: a review. *Fungal Divers.* 2010, *44*, 3–13.
10. Salgado, J.D.; Madden, L.V; Paul, P.A. Quantifying the effects of Fusarium head blight on grain yield and test weight in soft red winter wheat. *Phytopathology* 2015, *105*, 295–306.
11. Bakker, M.G.; Brown, D.W.; Kelly, A.C.; Kim, H.S.; Kurtzman, C.P.; Mccormick, S.P.; O'Donnell, K.L.; Proctor, R.H.; Vaughan, M.M.; Ward, T.J. Fusarium mycotoxins: a trans-disciplinary overview. *Can. J. Plant Pathol.* 2018, *40*, 161–171.
12. Eriksen, G.S.; Pettersson, H. Toxicological evaluation of trichothecenes in animal feed. *Anim. Feed Sci. Technol.* 2004, *114*, 205–239.
13. Maresca, M. From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. *Toxins (Basel)* 2013, *5*, 784–820.
14. Kostro, K.R.; Gajecka, M.; Lisiecka, U.R.; Majer-Dziedzic, B.A.; Obremski, K.; Zielonka, L.; Gajecki, M. Subpopulation of lymphocytes CD4+ and CD8+ in peripheral blood of sheep with zearalenone mycotoxicosis. *Bull. Vet. Inst. Pulawy* 2011, *55*, 241–246.
15. El-Makawy, A.; Hassanane, M.S.; Alla, E.-S.A.A. Genotoxic evaluation for the estrogenic mycotoxin zearalenone. *Reprod. Nutr. Dev.* 2001, *41*, 79–89.
16. Seifert, K.A.; Aoki, T.; Baayen, R.P.; Brayford, D.; Burgess, L.W.; Chulze, S.; Gams, W.; Geiser, D.; De Gruyter, J.; Leslie, J.F.; Logrieco, A., Marasas, W.F.O., Nirenberg, H.I., O'Donnell, K., Rheeder, J., Samuels, G.J., Summerell, B.A., Thrane, U., Waalwijk, C. The name *Fusarium moniliforme* should no longer be used. *Mycol. Res.* 2003, *107*, 643–644.
17. Rheeder, J.P.; Marasas, W.F.O.; Vismer, H.F. Production of fumonisin analogs by *Fusarium* species. *Appl. Environ. Microbiol.* 2002, *68*, 2101–2105.
18. International Agency for Research on Cancer. *Toxins derived from* Fusarium moniliforme*: fumonisins B1 and B2 and Fusarin C*; 1993; pp. 445–466;.
19. Marasas, W.F.O. Fumonisins: history, world-wide occurrence and impact. In *Fumonisins in food. Advances in Experimental medicine and Biology*; Jackson, L.S., DeVries, J.W., Bullerman, L.B., Eds.; Springer: Boston. Massachussets, USA, 1996; pp. 1–17 ISBN 978-1-4899-1381-4.
20. Fraeyman, S.; Croubels, S.; Devreese, M.; Antonissen, G. Emerging *Fusarium* and *Alternaria* mycotoxins: occurrence, toxicity and toxicokinetics. *Toxins (Basel)* 2017, *9*, 1–26.
21. Dweba, C.C.; Figlan, S.; Shimelis, H.A.; Motaung, T.E.; Sydenham, S.; Mwadzingeni, L.; Tsilo, T.J. Fusarium head blight of wheat: pathogenesis and control strategies. *Crop Prot.* 2017, *91*, 114–122.
22. Edwards, S.G. Influence of agricultural practices on *Fusarium* infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. *Toxicol. Lett.* 2004, *153*, 29–35.
23. European Commission Commission regulation (EC) 1107/2009. *Off. J. Eur. Union* 2009,

L 309/1.
24. European Commission Commission directive (EC) 128/2009. *Off. J. Eur. Union* 2009, *L 309/71*.
25. Pan, D.; Mionetto, A.; Tiscornia, S.; Bettucci, L. Endophytic bacteria from wheat grain as biocontrol agents of *Fusarium graminearum* and deoxynivalenol production in wheat. *Mycotoxin Res.* 2015, *31*, 137–143.
26. Palazzini, J.M.; Dunlap, C.A.; Bowman, M.J.; Chulze, S.N. *Bacillus velezensis* RC 218 as a biocontrol agent to reduce Fusarium head blight and deoxynivalenol accumulation: genome sequencing and secondary metabolite cluster profiles. *Microbiol. Res.* 2016, *192*, 30–36.
27. Schisler, D.A.; Khan, N.I.; Boehm, M.J.; Lipps, P.E.; Slininger, P.J.; Zhang, S. Selection and evaluation of the potential of choline-metabolizing microbial strains to reduce Fusarium head blight. *Biol. Control* 2006, *39*, 497–506.
28. Zhao, Y.; Selvaraj, J.N.; Xing, F.; Zhou, L.; Wang, Y.; Song, H.; Tan, X.; Sun, L.; Sangare, L.; Minnie, Y.; et al. Antagonistic action of *Bacillus subtilis* strain SG6 on *Fusarium graminearum*. *PLoS One* 2014, *9*, e92486.
29. Palazzini, J.M.; Ramirez, M.L.; Alberione, E.J.; Torres, A.M.; Chulze, S.N. Osmotic stress adaptation, compatible solutes accumulation and biocontrol efficacy of two potential biocontrol agents on Fusarium head blight in wheat. *Biol. Control* 2009, *51*, 370–376.
30. Khan, M.R.; Doohan, F.M. Bacterium-mediated control of Fusarium head blight disease of wheat and barley and associated mycotoxin contamination of grain. *Biol. Control* 2009, *48*, 42–47.
31. Jochum, C.C.; Osborne, L.E.; Yuen, G.Y. Fusarium head blight biological control with *Lysobacter enzymogenes* strain C3. *Biol. Control* 2006, *39*, 336–344.
32. McCormick, S.P. Microbial detoxification of mycotoxins. *J. Chem. Ecol.* 2013, *39*, 907–918.
33. Barka, E.A.; Vatsa, P.; Sanchez, L.; Gaveau-Vaillant, N.; Jacquard, C.; Klenk, H.-P.; Clément, C.; Ouhdouch, Y.; van Wezel, G.P. Taxonomy, physiology, and natural products of Actinobacteria. *Microbiol. Mol. Biol. Rev.* 2016, *80*, 1–43.
34. Williams, S.T.; Vickers, J.C. Detection of actinomycetes in the natural environment: problems and perspectives. In *Biology of actinomycetes*; Okami, Y., Beppu, T., Ogawara, H., Eds.; Japan Scientific Societies Press: Tokyo, Japan, 1988; pp. 165–270.
35. Bush, M.J.; Tschowri, N.; Schlimpert, S.; Flärdh, K.; Buttner, M.J. C-di-GMP signalling and the regulation of developmental transitions in streptomycetes. *Nat. Rev. Microbiol.* 2015, *13*, 749–760.
36. Challis, G.L.; Hopwood, D.A. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc. Natl. Acad. Sci.* 2003, *100*, 14555–14561.
37. Seipke, R.F.; Kaltenpoth, M.; Hutchings, M.I. *Streptomyces* as symbionts: an emerging and widespread theme? *FEMS Microbiol. Rev.* 2012, *36*, 862–876.
38. Bérdy, J. Bioactive microbial metabolites: a personal view. *J. Antibiot. (Tokyo)* 2005, *58*, 1–26.
39. Viaene, T.; Langendries, S.; Beirinckx, S.; Maes, M.; Goormachtig, S. *Streptomyces* as a plant's best friend? *FEMS Microbiol. Ecol.* 2016, *92*, fiw119.
40. Newitt, J.T.; Prudence, S.M.M.; Hutchings, M.I.; Worsley, S.F.; Newitt, J.T.; Prudence, S.M.M.; Hutchings, M.I.; Worsley, S.F. Biocontrol of cereal crop diseases using streptomycetes. *Pathogens* 2019, *8*, 78.
41. Qin, S.; Xing, K.; Jiang, J.-H.; Xu, L.-H.; Li, W.-J. Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. *Appl. Microbiol. Biotechnol.* 2011, *89*, 457–473.
42. Zamoum, M.; Goudjal, Y.; Sabaou, N.; Barakate, M.; Mathieu, F.; Zitouni, A. Biocontrol capacities and plant growth-promoting traits of endophytic actinobacteria isolated from native plants of Algerian sahara. *J. Plant Dis. Prot.* 2015, *122*, 215–233.
43. Comby, M.; Gacoin, M.; Robineau, M.; Rabenoelina, F.; Ptas, S. Screening of wheat endophytes as biological control agents against Fusarium head blight using two different

in vitro tests. *Microbiol. Res.* 2017, *202*, 11–20.
44. Rey, T.; Dumas, B. Plenty is no plague: *Streptomyces* symbiosis with crops. *Trends Plant Sci.* 2017, *22*, 30–37.
45. Nguyen, P.A.; Strub, C.; Fontana, A.; Schorr-Galindo, S. Crop molds and mycotoxins: alternative management using biocontrol. *Biol. Control* 2017, *104*, 10–27.
46. Todd, J.; Antonet, K.; Svircev, M.; Goettel, M.S.; Woo, S.G. *The use and regulation of microbial pesticides in representative jurisdictions worldwide*; Todd, J., Antonet, K., Svircev, M., Goettel, M.S., Woo, S.G., Eds.; IOBC Global, 2010; pp 99.
47. Vurukonda, S.S.K.P.; Giovanardi, D.; Stefani, E. Plant growth promoting and biocontrol activity of *Streptomyces* spp. as endophytes. *Int. J. Mol. Sci.* 2018, *19*, 952.
48. Alabouvette, C.; Olivain, C.; Steinberg, C. Biological control of plant diseases: the European situation. *Eur. J. Plant Pathol.* 2006, *114*, 329–341.
49. Kagot, V.; Okoth, S.; De Boevre, M.; De Saeger, S. Biocontrol of *Aspergillus* and *Fusarium* mycotoxins in Africa: benefits and limitations. *Toxins (Basel)* 2019, *11*, 109.
50. Loria, R.; Kers, J.; Joshi, M. Evolution of plant pathogenicity in *Streptomyces*. *Annu. Rev. Phytopathol.* 2006, *44*, 469–487.
51. Zhang, Y.; Bignell, D.R.D.; Zuo, R.; Fan, Q.; Huguet-Tapia, J.C.; Ding, Y.; Loria, R. Promiscuous pathogenicity islands and phylogeny of pathogenic *Streptomyces* spp. *Mol. Plant-Microbe Interact.* 2016, *29*, 640–650.
52. Rasimus-Sahari, S.; Mikkola, R.; Andersson, M.A.; Jestoi, M.; Salkinoja-Salonen, M. *Streptomyces* strains producing mitochondriotoxic antimycin A found in cereal grains. *Int. J. Food Microbiol.* 2016, *218*, 78–85.
53. van der Meij, A.; Worsley, S.F.; Hutchings, M.I.; van Wezel, G.P. Chemical ecology of antibiotic production by actinomycetes. *FEMS Microbiol. Rev.* 2017, *41*, 392–416.
54. Schisler, D.A.; Slininger, P.J.; Hanson, L.E.; Loria, R. Potato cultivar, pathogen isolate and antagonist cultivation medium influence the efficacy and ranking of bacterial antagonists of *Fusarium* dry rot. *Biocontrol Sci. Technol.* 2000, *10*, 267–279.
55. Colombo, E.M.; Pizzatti, C.; Kunova, A.; Gardana, C.; Saracchi, M.; Cortesi, P.; Pasquali, M. Evaluation of *in-vitro* methods to select effective streptomycetes against toxigenic fusaria. *PeerJ* 2019, *7*, e6905.
56. Sánchez, S.; Chávez, A.; Forero, A.; García-Huante, Y.; Romero, A.; Sánchez, M.; Rocha, D.; Sánchez, B.; Valos, M.; Guzmán-Trampe, S.; Rodríguez-Sanoja, R., Langley, E., Ruiz, B. Carbon source regulation of antibiotic production. *J. Antibiot. (Tokyo)* 2010, *63*, 442–459.
57. Abdelmohsen, U.R.; Grkovic, T.; Balasubramanian, S.; Kamel, M.S.; Quinn, R.J.; Hentschel, U. Elicitation of secondary metabolism in actinomycetes. *Biotechnol. Adv.* 2015, *33*, 798–811.
58. Bucar, F.; Wube, A.; Schmid, M. Natural product isolation – how to get from biological material to pure compounds. *Nat. Prod. Rep.* 2013, *30*, 525–545.
59. European Commission Commission regulation (EU) 546/2011. *Off. J. Eur. Union* 2011.
60. Cook, R.J.; Bruckart, W.L.; Coulson, J.R.; Goettel, M.S.; Humber, R.A.; Lumsden, R.D.; Maddox, J. V; Mcmanus, M.L.; Moore, L.; Meyer, S.F.; et al. Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. *Biol. Control* 1996, *7*, 333–351.
61. O'Callaghan, M. Microbial inoculation of seed for improved crop performance: issues and opportunities. *Appl. Microbiol. Biotechnol.* 2016, *100*, 5729–5746.
62. Pliego, C.; Ramos, C.; de Vicente, A.; Cazorla, F.M. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. *Plant Soil* 2011, *340*, 505–520.
63. Colombo, E.M.; Pizzatti, C.; Kunova, A.; Saracchi, M.; Cortesi, P.; Pasquali, M. Selection of an endophytic *Streptomyces* sp. strain DEF09 from wheat roots as a biocontrol agent against *Fusarium graminearum*. *Front. Microbiol.* 2019, *10*, 2356.
64. Whitaker, B.K.; Bakker, M.G. Bacterial endophyte antagonism toward a fungal pathogen in vitro does not predict protection in live plant tissue. *FEMS Microbiol. Ecol.* 2018, *95*, fiy237.
65. Omura, S.; Iwai, Y.; Takahashi, Y.; Sadakane, N.; Nakagawa, A.; Oiwa, H.; Hasegawa,

Y. Herbimycin, a new antibiotic produced by a strain of *Streptomyces*. *J. Antibiot. (Tokyo)* 1979, *32*, 255–261.
66. Jambhulkar, P.P.; Sharma, P.; Yadav, R. Delivery systems for introduction of microbial inoculants in the field. In *Microbial Inoculants in Sustainable Agricultural Productivity: Vol. 2: Functional Applications*; Singh, D., Singh, H., Prabha, R., Eds.Springer. New Delhi. India, 2016; pp. 199–218 ISBN 9788132226444.
67. Legrand, F.; Picot, A.; Cobo-Díaz, J.F.; Chen, W.; Le Floch, G. Challenges facing the biological control strategies for the management of Fusarium head blight of cereals caused by *F. graminearum*. *Biol. Control* 2017, *113*, 26–38.
68. He, J.; Boland, G.J.; Zhou, T. Concurrent selection for microbial suppression of *Fusarium graminearum*, Fusarium head blight and deoxynivalenol in wheat. *J. Appl. Microbiol.* 2009, *106*, 1805–1817.
69. Dalié, D.; Pinson-Gadais, L.; Atanasova-Penichon, V.; Marchegay, G.; Barreau, C.; Deschamps, A.; Richard-Forget, F. Impact of *Pediococcus pentosaceus* strain L006 and its metabolites on fumonisin biosynthesis by *Fusarium verticillioides*. *Food Control* 2012, *23*, 405–411.
70. Sakuda, S. Mycotoxin production inhibitors from natural products. *Mycotoxins* 2010, *60*, 79–86.
71. Schroeckh, V.; Scherlach, K.; Nützmann, H.W.; Shelest, E.; Schmidt-Heck, W.; Schuemann, J.; Martin, K.; Hertweck, C.; Brakhage, A.A. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci. U. S. A.* 2009, *106*, 14558–14563.
72. Zuck, K.M.; Shipley, S.; Newman, D.J. Induced production of N-formyl alkaloids from *Aspergillus fumigatus* by co-culture with *Streptomyces peucetius*. *J. Nat. Prod.* 2011, *74*, 1653–1657.
73. Ola, A.R.B.; Thomy, D.; Lai, D.; Brötz-Oesterhelt, H.; Proksch, P. Inducing secondary metabolite production by the endophytic fungus *Fusarium tricinctum* through coculture with *Bacillus subtilis*. *J. Nat. Prod.* 2013, *76*, 2094–2099.
74. Rateb, M.E.; Hallyburton, I.; Houssen, W.E.; Bull, A.T.; Goodfellow, M.; Santhanam, R.; Jaspars, M.; Ebel, R. Induction of diverse secondary metabolites in *Aspergillus fumigatus* by microbial co-culture. *RSC Adv.* 2013, *3*, 14444-14450.
75. Marmann, A.; Aly, A.H.; Lin, W.; Wang, B.; Proksch, P. Co-cultivation - a powerful emerging tool for enhancing the chemical diversity of microorganisms. *Mar. Drugs* 2014, *12*, 1043–1065.
76. Pasquali, M.; Migheli, Q. Genetic approaches to chemotype determination in type B-trichothecene producing Fusaria. *Int. J. Food Microbiol.* 2014, *189*, 164–182.
77. O'Donnell, K.; Nirenberg, H.I.; Aoki, T.; Cigelnik, E. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. *Mycoscience* 2000, *41*, 61–78.
78. Guo, Y.P.; Zheng, W.; Rong, X.Y.; Huang, Y. A multilocus phylogeny of the *Streptomyces griseus* 16S rRNA gene clade: use of multilocus sequence analysis for streptomycete systematics. *Int. J. Syst. Evol. Microbiol.* 2008, *58*, 149–159.
79. Yoon, S.H.; Ha, S.M.; Kwon, S.; Lim, J.; Kim, Y.; Seo, H.; Chun, J. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* 2017, *67*, 1613–1617.
80. Palazzini, J.M.; Ramirez, M.L.; Torres, A.M.; Chulze, S.N. Potential biocontrol agents for Fusarium head blight and deoxynivalenol production in wheat. *Crop Prot.* 2007, *26*, 1702–1710.
81. Sultan, Y.; Magan, N. Impact of a *Streptomyces* (AS1) strain and its metabolites on control of *Aspergillus flavus* and aflatoxin B1 contamination *in vitro* and in stored peanuts. *Biocontrol Sci. Technol.* 2011, *21*, 1437–1455.
82. Verheecke, C.; Liboz, T.; Darriet, M.; Sabaou, N.; Mathieu, F. *In vitro* interaction of actinomycetes isolates with *Aspergillus flavus*: impact on aflatoxins B1 and B2 production. *Lett. Appl. Microbiol.* 2014, *58*, 597–603.
83. Magan, N.; Lacey, J. Effect of water activity, temperature and substrate on interactions between field and storage fungi. *Trans. Br. Mycol. Soc.* 1984, *82*, 83–93.

84. Jung, B.; Park, S.Y.; Lee, Y.W.; Lee, J. Biological efficacy of *Streptomyces* sp. strain BN1 against the cereal head blight pathogen *Fusarium graminearum*. *Plant Pathol. J.* 2013, *29*, 52–58.
85. Pei-Sheng, Y.; Cui-Juan, S.; Chun-Chun, H.; Guang-Feng, K. Inhibition of vomitoxin-producing *Fusarium graminearum* by marine actinomycetes and the extracellular metabolites. In Proceedings of the International Conference on Human Health and Biomedical Engineering; 2011; pp. 454–456.
86. Palazzini, J.M.; Yerkovich, N.; Alberione, E.; Chiotta, M.; Chulze, S.N. An integrated dual strategy to control *Fusarium graminearum sensu stricto* by the biocontrol agent *Streptomyces* sp. RC 87B under field conditions. *Plant Gene* 2017, *9*, 13–18.
87. Nourozian, J.; Etebarian, H.R.; Khodakaramian, G. Biological control of *Fusarium graminearum* on wheat by antagonistic bacteria. *Songklanakarin J. Sci. Technol.* 2006, *28*, 29–38.
88. Palazzini, J.; Roncallo, P.; Cantoro, R.; Chiotta, M.; Yerkovich, N.; Palacios, S.; Echenique, V.; Torres, A.; Ramírez, M.; Karlovsky, P.; Chulze, S.. Biocontrol of *Fusarium graminearum sensu stricto*, reduction of deoxynivalenol accumulation and phytohormone induction by two selected antagonists. *Toxins (Basel)* 2018, *10*, 88.
89. Ranjbariyan, A.R.; Shams-Ghahfarokhi, M.; Kalantari, S.; Razzaghi-Abyaneh, M. Molecular identification of antagonistic bacteria from Tehran soils and evaluation of their inhibitory activities toward pathogenic fungi. *Iran. J. Microbiol.* 2011, *3*, 140–146.
90. Solans, M.; Scervino, J.M.; Messuti, M.I.; Vobis, G.; Wall, L.G. Potential biocontrol actinobacteria: rhizospheric isolates from the Argentine pampas lowlands legumes. *J. Basic Microbiol.* 2016, *56*, 1289–1298.
91. Yekkour, A.; Sabaou, N.; Zitouni, A.; Errakhi, R.; Mathieu, F.; Lebrihi, A. Characterization and antagonistic properties of *Streptomyces* strains isolated from Saharan soils, and evaluation of their ability to control seedling blight of barley caused by *Fusarium culmorum*. *Lett. Appl. Microbiol.* 2012, *55*, 427–435.
92. Al-Askar, A.A.; Khair, A.; Rashad, W.M. In vitro antifungal activity of *Streptomyces spororaveus* RDS28 against some phytopathogenic fungi. *African J. Agric. Res.* 2011, *6*, 2835–2842.
93. Singh, L.S.; Mazumder, S.; Bora, T.C. Optimisation of process parameters for growth and bioactive metabolite produced by a salt-tolerant and alkaliphilic actinomycete, *Streptomyces tanashiensis* strain A2D. *J. Mycol. Med.* 2009, *19*, 225–233.
94. Tripathi, C.K.M.; Praaven, V.; Singh, V.; Bihari, V. Production of antibacterical and antifungal metabolites by *Streptomyces violaceusniger* and media optimization studies for the maximum metabolite production. *Med. Chem. Res.* 2004, *13*, 790–799.
95. Mitrovi, I.Ž.; Grahovac, J.A.; Dodi, J.M.; Grahovac, M.S. Effect of agitation rate on the production of antifungal. *Acta Period. Technol.* 2017, *48*, 231–244.
96. Singh, L.S.; Barauah, I.; Bora, T.C. Actinomycetes of Loktak habitats: isolation and screening for antimicrobial activities. *Biotechnology* 2006, *5*, 217–221.
97. Alam, M.; Dharni, S.; Abdul-Khaliq; Srivastava, S.K.; Samad, A.; Gupta, M.K. A promising strain of *Streptomyces* sp. with agricultural traits for growth promotion and disease management. *Indian J. Exp. Biol.* 2012, *50*, 559–568.
98. Bordoloi, G.N.; Kumari, B.; Guha, A.; Bordoloi, M.; Yadav, R.N.S.; Roy, M.K.; Bora, T.C. Isolation and structure elucidation of a new antifungal and antibacterial antibiotic produced by *Streptomyces* sp. 201. *Biosci. Biotechnol. Biochem.* 2001, *65*, 1856–1858.
99. Koch, E.; Löffler, I. Partial characterization of the antimicrobial activity of *Streptomyces antimycoticus* FZB53. *J. Phytopathol.* 2009, *157*, 235–242.
100. Pan, H.Q.; Yu, S.Y.; Song, C.F.; Wang, N.; Hua, H.M.; Hu, J.C.; Wang, S.J. Identification and characterization of the antifungal substances of a novel *Streptomyces cavourensis* NA4S. *J. Microbiol. Biotechnol.* 2015, *25*, 353–357.
101. Wang, T.; Jiang, Y.; Ma, K.X.; Li, Y.Q.; Huang, R.; Xie, X.S.; Wu, S.H. Two new butenolides produced by an actinomycete *Streptomyces* sp. *Chem. Biodivers.* 2014, *11*, 929–933.
102. Aouiche, A.; Bijani, C.; Zitouni, A.; Mathieu, F.; Sabaou, N. Antimicrobial activity of saquayamycins produced by *Streptomyces* spp. PAL114 isolated from a Saharan soil.

J. Mycol. Med. 2014, *24*, e17–e23.
103. Chen, M.; Xie, L. J. ; Zhou, J. R.; Song, Y. Y.; Wang, R. L.; Chen, S. ; Su, Y. J.; Zheng, R.S. Collection, purification and structure elucidation of allelochemicals in *Streptomyces* sp. 6803. *Allelopath. J.* 2010, *25*, 93–106.
104. Deng, J.; Lu, C.; Li, Y.; Li, S. Cuevaenes C – E: three new triene carboxylic derivatives from *Streptomyces* sp. LZ35 Δ gdmAI. *Beilstein J. Org. Chem.* 2014, *10*, 858–862.
105. Gomes, R.C.; Semedo, L.T.A.S.; Soares, R.M.A.; Linhares, L.F.; Ulhoa, C.J.; Alviano, C.S.; Coelho, R.R.R. Purification of a thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. *J. Appl. Microbiol.* 2001, *90*, 653–661.
106. Baharlouei, A.; Sharifi-Sirchi, G.R.; Shahidi Bonjar, G.H. Identification of an antifungal chitinase from a potential biocontrol agent, *Streptomyces plicatus* strain 101, and its new antagonistic spectrum of activity. *Philipp. Agric. Sci.* 2010, *93*, 439–445.
107. Shi, P.; Yao, G.; Yang, P.; Li, N.; Luo, H.; Bai, Y.; Wang, Y.; Yao, B. Cloning, characterization, and antifungal activity of an endo-1,3-beta-D: -glucanase from *Streptomyces* sp. S27. *Appl. Microbiol. Biotechnol.* 2010, *85*, 1483–90.
108. Swiontek Brzezinska, M.; Jankiewicz, U.; Burkowska, A. Purification and characterization of *Streptomyces albidoflavus* antifungal components. *Appl. Biochem. Microbiol.* 2013, *49*, 451–457.
109. Fulgueira, C.L.; Amigot, S.L.; Magni, C. Growth inhibition of toxigenic fungi by a proteinaceous compound from *Streptomyces* sp. C/33-6. *Curr. Microbiol.* 2004, *48*, 135–139.
110. Wang, C.; Wang, Z.; Qiao, X.; Li, Z.; Li, F.; Chen, M.; Wang, Y.; Huang, Y.; Cui, H. Antifungal activity of volatile organic compounds from *Streptomyces alboflavus* TD-1. *FEMS Microbiol. Lett.* 2013, *341*, 45–51.
111. Wang, Z.; Wang, C.; Li, F.; Li, Z.; Chen, M.; Wang, Y.; Qiao, X.; Zhang, H. Fumigant activity of volatiles from *Streptomyces alboflavus* TD-1 against *Fusarium moniliforme* Sheldon. *J. Microbiol.* 2013, *51*, 477–483.
112. Boukaew, S.; Plubrukam, A.; Prasertsan, P. Effect of volatile substances from *Streptomyces philanthi* RM-1-138 on growth of *Rhizoctonia solani* on rice leaf. *BioControl* 2013, *58*, 471–482.
113. Han, D.; Wang, L.; Luo, Y. Isolation, identification, and the growth promoting effects of two antagonistic actinomycete strains from the rhizosphere of *Mikania micrantha* Kunth. *Microbiol. Res.* 2018, *208*, 1–11.
114. Toumatia, O.; Compant, S.; Yekkour, A.; Goudjal, Y.; Sabaou, N.; Mathieu, F.; Sessitsch, A.; Zitouni, A. Biocontrol and plant growth promoting properties of *Streptomyces mutabilis* strain IA1 isolated from a Saharan soil on wheat seedlings and visualization of its niches of colonization. *South African J. Bot.* 2016, *105*, 234–239.
115. Wachowska, U.; Kucharska, K.; Jędryczka, M.; Łobik, N. Microorganisms as biological control agents against *Fusarium* pathogens in winter wheat. *Polish J. Environ. Stud.* 2013, *22*, 591–597.
116. Mouloud, G.; Samir, M.; Hani, B.; Daoud, H. Biocontrol of wheat Fusarium Head Blight (FHB) by *Streptomyces* spp. isolated from the rhizosphere of *Astragalus gombo* Coss. & Dur. and *Ononis angustissima* Lam. *Am-Euras. J. Agric. Environ. Sci.* 2016, *15*, 2499–2511.
117. Orakçi, G.E.; Yamaç, M.; Amoroso, M.J.; Cuozzo, S.A. Selection of antagonistic actinomycete isolates as biocontrol agents against root-rot fungi. *Fresenius Environ. Bull.* 2010, *19*, 417–424.
118. Koch, E.; Weil, B.; Wächter, R.; Wohlleben, S.; Spiess, H.; Krauthausen, H.J. Evaluation of selected microbial strains and commercial alternative products as seed treatments for the control of *Tilletia tritici*, *Fusarium culmorum*, *Drechslera graminea* and *D. teres*. *J. Plant Dis. Prot.* 2006, *113*, 150–158.
119. Bressan, W. Biological control of maize seed pathogenic fungi by use of actinomycetes. *BioControl* 2003, *48*, 233–240.
120. Bressan, W.; Figueiredo, J.E.F. Efficacy and dose-response relationship in biocontrol of Fusarium disease in maize by *Streptomyces* spp. *Eur. J. Plant Pathol.* 2008, *120*, 311–

316.
121. Nguyen, P.-A.; Strub, C.; Durand, N.; Alter, P.; Fontana, A.; Schorr-Galindo, S. Biocontrol of *Fusarium verticillioides* using organic amendments and their actinomycete isolates. *Biol. Control* 2017, *118*, 55–66.
122. Samsudin, N.I.P.; Magan, N. Efficacy of potential biocontrol agents for control of *Fusarium verticillioides* and fumonisin B_1 under different environmental conditions. *World Mycotoxin J.* 2016, *9*, 205–213.
123. El-Naggar, M.A.; Alkahtani, M.D.F.; Thabit, T.M.; Sarhan, E.A.; Morsy, K.M. *In vitro* study on influence of some *Streptomyces* strains isolated from date palm rhizosphere soil on some toxigenic fungi. *Foodborne Pathog. Dis.* 2012, *9*, 646–654.
124. Shirokikh, I.G.; Merzaeva, O. V. Biological activity of *Streptomyces hydroscopicus* against phytopathogenic fungus *Fusarium avenaceum* in rhizosphere. *Mikol. I Fitopatol.* 2008, *42*, 586–591.
125. Mizuhara, N.; Kuroda, M.; Ogita, A.; Tanaka, T.; Usuki, Y.; Fujita, K.I. Antifungal thiopeptide cyclothiazomycin B1 exhibits growth inhibition accompanying morphological changes via binding to fungal cell wall chitin. *Bioorganic Med. Chem.* 2011, *19*, 5300–5310.
126. Aouar, L.; Lerat, S.; Ouffroukh, A.; Boulahrouf, A.; Beaulieu, C. Taxonomic identification of rhizospheric actinobacteria isolated from Algerian semi-arid soil exhibiting antagonistic activities against plant fungal pathogens. *Can. J. Plant Pathol.* 2012, *34*, 165–176.
127. Khebizi, N.; Boudjella, H.; Bijani, C.; Bouras, N.; Klenk, H.P.; Pont, F.; Mathieu, F.; Sabaou, N. Oligomycins A and E, major bioactive secondary metabolites produced by *Streptomyces* sp. strain HG29 isolated from a Saharan soil. *J. Mycol. Med.* 2018, *28*, 150–160.
128. Passari, A.K.; Mishra, V.K.; Gupta, V.K.; Saikia, R.; Singh, B.P. Distribution and identification of endophytic *Streptomyces* species from *Schima wallichii* as potential biocontrol agents against fungal plant pathogens. *Polish J. Microbiol.* 2016, *65*, 319–329.
129. Swiontek Brzezinska, M.; Jankiewicz, U.; Burkowska, A. Purification and characterization of *Streptomyces albidoflavus* antifungal components. *Appl. Biochem. Microbiol.* 2013, *49*, 451–457.
130. Golińska, P.; Dahm, H. Antagonistic properties of *Streptomyces* isolated from forest soils against fungal pathogens of pine seedlings. *Dendrobiology* 2013, *69*, 87–97.
131. Al-Askar, A.A. Endophytic *Streptomyces olivaceiscleroticus* Endo-1: biocontrol agent and growth promoter of wheat. *J. Pure Appl. Microbiol.* 2014, *8*, 307–317.
132. Khieu, T.N.; Liu, M.J.; Nimaichand, S.; Quach, N.T.; Chu-Ky, S.; Phi, Q.T.; Vu, T.T.; Nguyen, T.D.; Xiong, Z.; Prabhu, D.M.; et al. Characterization and evaluation of antimicrobial and cytotoxic effects of *Streptomyces* sp. HUST012 isolated from medicinal plant *Dracaena cochinchinensis* Lour. *Front. Microbiol.* 2015, *6*, 1–9.
133. Palazzini, J.; Roncallo, P.; Cantoro, R.; Chiotta, M.; Yerkovich, N.; Palacios, S.; Echenique, V.; Torres, A.; Ramirez, M.; Karlovsky, P.; et al. Biocontrol of *Fusarium graminearum sensu stricto*, reduction of deoxynivalenol accumulation and phytohormone induction by two selected antagonists. *Toxins (Basel)* 2018, *10*.
134. Perez, C.; Dill-Macky, R.; Kinkel, L.L. Management of soil microbial communities to enhance populations of *Fusarium graminearum*-antagonists in soil. *Plant Soil* 2008, *302*, 53–69.
135. Comby, M.; Gacoin, M.; Robineau, M.; Rabenoelina, F.; Ptas, S.; Dupont, J.; Profizi, C.; Baillieul, F. Screening of wheat endophytes as biological control agents against Fusarium head blight using two different *in vitro* tests. *Microbiol. Res.* 2017, *202*, 11–20.
136. Ursan, M.; Boiu-Sicuia, O.A.; Voaides, C.; Stan, V.; Bubueanu, C.; Cornea, C.P. The potential of new *Streptomyces* isolates as biocontrol agents against *Fusarium* spp. In Proceedings of the “Agriculture for Life, Life for Agriculture”; 2018; pp. 594–600.
137. Chen, M.; Zhou, J.R.; Li, C.Y.; Song, Y.Y.; Xie, L.J.; Chen, S.; Zeng, R.S. Isolation, identification and bioactivity of allelochemicals of *Streptomyces* sp. strain 6803. *Allelopath. J.* 2009, *23*, 411–424.

138. Ye, L.; Zhu, H.; Tian, M.; Huang, X. Structure elucidation and activity of compound H6794-A, a fungal cell wall inhibitor. *Chinese J. Antibiot.* 2010, *32*, 77–80.
139. Wei, Z.; Xu, C.; Wang, J.; Lu, F.; Bie, X.; Lu, Z. Identification and characterization of *Streptomyces flavogriseus* NJ-4 as a novel producer of actinomycin D and holomycin. *PeerJ* 2017, *5*, e3601.
140. Apichaisataienchote, B.; Altenbuchner, J.; Buchenauer, H. Isolation and identification of *Streptomyces fradiae* SU-1 from Thailand and protoplast transformation with the chitinase B gene from *Nocardiopsis prasina* OPC-131. *Curr. Microbiol.* 2005, *51*, 116–121.
141. Silva-Lacerda, G.R.; Santana, R.C.F.; Vicalvi-Costa, M.C.V.; Solidônio, E.G.; Sena, K.X.F.R.; Lima, G.M.S.; Araújo, J.M. Antimicrobial potential of actinobacteria isolated from the rhizosphere of the Caatinga biome plant *Caesalpinia pyramidalis* Tul. *Genet. Mol. Res.* 2016, *15*, 1–12.
142. Kaur, T.; Manhas, R.K. Antifungal, insecticidal, and plant growth promoting potential of *Streptomyces hydrogenans* DH16. *J. Basic Microbiol.* 2014, *54*, 1175–1185.
143. Charousová, I.; Medo, J.; Halenárová, E.; Maková, J.; Javoreková, S. Effect of fertilization on biological activity of community of soil streptomycetes. *J. Cent. Eur. Agric.* 2016, *17*, 1134–1149.
144. Kovácsová, S.; Javoreková, S.; Medo, J.; Charousová, I.; Elbl, J.; Plošek, L. Characteristic of *Streptomyces* species with antimicrobial activity against selected phytopathogenic bacteria and fungi. *J. Microbiol. Biotechnol. Food Sci.* 2015, *05*, 55–59.
145. Paškevičius, A.; Švediene, J.; Levinskaite, L.; Repečkiene, J.; Raudoniene, V.; Melvydas, V. The effect of bacteria and essential oils on mycotoxin producers isolated from feed of plant origin. *Vet. ir Zootech.* 2014, *65*, 52–60.
146. Guoying, Z.; Guangtao, S.; Lei, Y. Fungistatic activity and identification of antagonistic actinomycetes to camellia diseases from soil. *Int. Conf. Challenges Environ. Sci. Comput. Eng. CESCE 2010* 2010, *1*, 475–478.
147. Sadeghy, B.; Hatami, N. Screening biological activities of soil-borne *Streptomyces* sp. against several phytopathogenic fungi. *Arch. Phytopathol. Plant Prot.* 2014, *47*, 954–958.
148. Wardecki, T.; Brötz, E.; De Ford, C.; Von Loewenich, F.D.; Rebets, Y.; Tokovenko, B.; Luzhetskyy, A.; Merfort, I. Endophytic *Streptomyces* in the traditional medicinal plant *Arnica montana* L.: secondary metabolites and biological activity. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* 2015, *108*, 391–402.
149. Novikova, I.I.; Litvinenko, A.I.; Boikova, I. V.; Yaroshenko, V.A.; Kalko, G. V. Biological activity of new microbiological preparations alirins B and S designed for plant protection against diseases. I. Biological activity of alirins against diseases of vegetable crops and potato. *Mikol. I Fitopatol.* 2003, *37*, 92–98.
150. Xu, J.; Shi, J.; Kou, C.; Wang, G.; Xu, L.; Pan, L. Strain of *Streptomyces netropsis* and application thereof. 2017. C.N. Patent No 107,058,131. Beijing: State Intellectual Property Office of the P.R.C.
151. Pan, H.; Yu, S.; Hu, J.; Wang, S. *Streptomyces cavourensis* and application thereof 2014. C.N. Patent No 103,820,351 A; 103,820,351 B. Beijing: State Intellectual Property Office of the P.R.C.
152. Xing, M.; Zhang, R.; Liu, T. Application of volatile substance produced by *Streptomyces fimicarius* in control of plant diseases 2018. C.N. Patent No 108,208,016. Beijing: State Intellectual Property Office of the P.R.C.
153. Gromovykh, T.I.; Litovka, J.A.; Sadykova, V.S. Actinomyces strain *Streptomyces lateritious* 19/97-m used for stimulating growth and protecting coniferous seedling against disease pathogen caused by fungi of genus *Fusarium* and *Alternaria* 2004 RU Patent No 200,310,057,9 A. Federal Service for Intellectual Property (ROSPATENT).
154. Lee, W.K.; Lee, H.K.; Park, K.B. *Streptomyces* sp. Sm008s strain and method of controlling plant pathogens using the same 2009. K.R. Patent No 100,914,225 B1. Korean Intellectual Property Office
155. Wu, W.; Guo, Y.; Meng, F.; Qi, L.; Xia, N.; Jiao, Z. Erythrochromogenes and use thereof

in biological control of diseases 2010. C.N. Patent No 101,698,827 A; 101,698,827 B. Beijing: State Intellectual Property Office of the P.R.C.
156. Caixia, D.; Gao, S.; Jing, R.; Peng, L.; Wei, L.; Aiping, L.; Xing, L.; Yaxue, L.; Yaqin, S.; Na, T.; et al. *Streptomyces griseoflavus* and application thereof in biological prevention and control of plant diseases 2010. C.N. Patent No 101,822,272 A. Beijing: State Intellectual Property Office of the P.R.C.
157. Suh, J.W.; Yoon, T.M.; Yang, S.H.; Kim, J.Y.; Lee, S.K.; Cheng, J.H. *Streptomyces cinnamoneus* Mjm8987 producing antifungal substances Ys-822a and its use 2010. K.R. Patent No 101,098,280. Korean Intellectual Property Office.
158. Yang, M.; Shu, C.; Zhou, E.; Gao, Y.; Zhang, D.; Fan, J. *Streptomyces katrae* NB20, as well as culture method and application thereof. 2012. C.N. Patent No 102,433,281 A; 102,433,281 B. Beijing: State Intellectual Property Office of the P.R.C.
159. Shin, J.G.; Shin, M.U.; Im, Y.M.; Park, S.W.; Son, H.N.; Shim, N.G.; Cho, J.H. Biological agent for plant diseases using *Streptomyces nigrogriseolus* Cmc0647 2012. K.R. Patent No 101,211,681. Korean Intellectual Property Office.
160. Ma, G.; Pu, Z.; Wang, S.; Wu, S.; Fu, H.; Ge, P. *Streptomyces mediolani* ZW-1 bacterial strain, and bacteriostatic application of fermentation broth thereof. 2012. C.N. Patent No 102,835,423 A; 102,835,423 B. Beijing: State Intellectual Property Office of the P.R.C.
161. Lu, L.; Du, D.; Pu, Z.; Hu, X.; Chen, G.; Huang, Z.; Zhang, X.; Zhang, L. Marine actinomycete with antibacterial activity to multiple plant pathogens 2013. C.N. Patent No 103,114,064 A; 103,114,064 B. Beijing: State Intellectual Property Office of the P.R.C.
162. Liu, X.; Ma, L.; Liu, X. *Streptomyces* with inhibiting effect on poplar gray leaf spot pathogen 2014. C.N. Patent No 104,130,965 A. Beijing: State Intellectual Property Office of the P.R.C.
163. Liu, X.; Liu, X.; Ma, L.; Zhang, J.; Shi, F. Method for preparing *Streptomyces* fermentation liquor for preventing and treating alamo grey speck disease germs 2014. C.N. Patent No 104,140,982 A. Beijing: State Intellectual Property Office of the P.R.C.
164. Huang, H.; Tao, T. Active enzyme biological leaf fertilizer and preparation method thereof 2015. C.N. Patent No 105,886,428 A. Beijing: State Intellectual Property Office of the P.R.C.
165. Errakhi, R.; Attia, F.; Cabanes, C. Isolated bacterium of the genus *Streptomyces* 2016. E.P. Patent No 3,048,890 A1. Munich, Germany: European Patent Office.
166. Shaojie, L.; Zhenying, Z.; Xianyun, S. *Streptomyces albidoflavus* and applications thereof in microbial fertilizers 2016. C.N. Patent No 105,886,428 A. Beijing: State Intellectual Property Office of the P.R.C.
167. Wang, C.; Zhao, C.; Cui, J.; Liu, S.; Lu, Z.; Dou, S.; Xie, X.; Wang, Y.; Li, L.; Ma, X.; et al. *Streptomyces griseoplanus*, application thereof and microbial agent 2017. C.N. Patent No 106,676,040. Beijing: State Intellectual Property Office of the P.R.C.
168. Yu, J.; Wang, X.; Guo, C.; Liu, C.; Liu, A.; Li, X.; Al., E. *Streptomyces samsunensis* and application thereof. 2017. C.N. Patent No 107,164,259 A. Beijing: State Intellectual Property Office of the P.R.C.
169. Li, S.; Zhang, Z.; Sun, X. *Streptomyces albidoflavus* strain as well as application thereof in pesticide 2017. C.N. Patent No 107,287,130 A. Beijing: State Intellectual Property Office of the P.R.C.
170. Kim, S.H.; Yun, Y.H. Novel strain of *Streptomyces* sp., ducc501, having antifungal activity and plant disease controlling composition comprising said novel strain 2018. W.O. Patent No 201,553,482 A1. Geneva: World Intellectual Property Organization.
171. Li, X.; Liu, Z.; Gu, L.; Zhang, C.; Zhang, N.; Zhang, Y.; Wu, M.; Al., E. *Streptomyces deccanensis* QY-3 and application thereof. 2018. C.N. Patent No 108,048,380 A. Beijing: State Intellectual Property Office of the P.R.C.
172. Jing, T.; Zang, X.; Xie, J.; Wang, L.; HE, Y.; Ding, Z.; Zhou, D.; Chen, Y.; et al. *Streptomyces samsunensis* and application thereof 2018. C.N. Patent No 108,102,961 A. Beijing: State Intellectual Property Office of the P.R.C.
173. Zhou, D.; Chen, Y.; Xie, J.; Wang, F.; Zhang, M..; Qi, D.; Feng, R.; Wang, W.; Jing, T.; Zang, X. *Streptomyces sanglieri* and application thereof. 2018. C.N. Patent No 108,165,506. Beijing: State Intellectual Property Office of the P.R.C.

174. Shunpeng, H.L.; Yu, Y.; Ceng, Y.; Luo, F.; Yang, C.; Zhang, X.H. Multifunctional algae strains of mold *Streptomyces amritsarensis* and application thereof. 2018. C.N. Patent No 108,587,981. Beijing: State Intellectual Property Office of the P.R.C.
175. Chen, Y.; Wang, J.; Yang, N.; Wen, Z.; Sun, X.; Chai, Y.; Ma, Z. Wheat microbiome bacteria can reduce virulence of a plant pathogenic fungus by altering histone acetylation. *Nat. Commun.* 2018, *9*, 3429.
176. Syed Ab Rahman, S.F.; Singh, E.; Pieterse, C.M.J.; Schenk, P.M. Emerging microbial biocontrol strategies for plant pathogens. *Plant Sci.* 2018, 102–111.
177. Jain, A.; Singh, A.; Singh, B.N.; Singh, S.; Upadhyay, R.S.; Sarma, B.K.; Singh, H.B. Biotic stress management in agricultural crops using microbial consortium. In *Bacteria in Agrobiology: Disease Management*; Maheshwari D. Eds. Springer-Verlag Berlin Heidelberg, 2013; pp. 427–448 ISBN 9783642336393.
178. Rojas, E.C.; Sapkota, R.; Jensen, B.; Jørgensen, H.J.L.; Henriksson, T.; Jørgensen, L.N.; Nicolaisen, M.; Collinge, D.B. Fusarium Head Blight modifies fungal endophytic communities during infection of wheat spikes. *Microb. Ecol.* 2019, 1–12.
179. Essarioui, A.; LeBlanc, N.; Kistler, H.C.; Kinkel, L.L. Plant community richness mediates inhibitory interactions and resource competition between *Streptomyces* and *Fusarium* populations in the rhizosphere. *Microb. Ecol.* 2017, *74*, 157–167.
180. Seipke, R.F. Strain-level diversity of secondary metabolism in *Streptomyces albus*. *PLoS One* 2015, *10*, e0116457.
181. Alberti, F.; Corre, C. *Editing streptomycete genomes in the CRISPR/Cas9 age*; Royal Society of Chemistry (RSC), 2019; *36*, 1237-1248
182. LaCroix, R.A.; Palsson, B.O.; Feist, A.M. A model for designing adaptive laboratory evolution experiments. *Appl. Environ. Microbiol.* 2017, *83*, e03115-16
183. Harir, M.; Bendif, H.; Bellahcene, M.; Fortas and Rebecca Pogni, Z. *Streptomyces* secondary metabolites. In *Basic Biology and Applications of Actinobacteria*; Enany, S., Ed.; IntechOpen, 2018.
184. Manteca, Á.; Yagüe, P. *Streptomyces* as a source of antimicrobials: novel approaches to activate cryptic secondary metabolite pathways. In *Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods*; Kırmusaoğlu, S., Ed.; IntechOpen, 2019.

Chapter 2: Development of novel screening approaches for biocontrol streptomycetes

- Colombo, E.M., Pizzatti, C., Kunova, A., Gardana, C., Saracchi, M., Cortesi, P., Pasquali, M. (2019). Evaluation of *in-vitro* methods to select effective streptomycetes against toxigenic fusaria. *PeerJ* 7, e6905. doi:10.7717/peerj.6905.

- Colombo, E.M., Gardana, C., Kunova, A., Pizzatti, C., Saracchi, M., Cortesi, P., Gardiner, D., Pasquali, M. Selection of *Streptomyces* strains limiting trichothecene B production using a *TRI5::GFP* biosensor assay.

Evaluation of *in vitro* methods to select effective streptomycetes against toxigenic fusaria

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Abstract

Biocontrol microorganisms are emerging as an effective alternative to pesticides. Ideally, biocontrol agents (BCAs) for the control of fungal plant pathogens should be selected by an *in vitro* method that is high-throughput and is predictive of *in planta* efficacy, possibly considering environmental factors, and the natural diversity of the pathogen. The purpose of our study was (1) to assess the effects of *Fusarium* strain diversity (*N* = 5) and culture media (*N* = 6) on the identification of biological control activity of *Streptomyces* strains (*N* = 20) against *Fusarium* pathogens of wheat *in vitro* and (2) to verify the ability of our *in vitro* screening methods to simulate the activity *in planta*. Our results indicate that culture media, *Fusarium* strain diversity, and their interactions affect the results of an *in vitro* selection by dual culture assay. The results obtained on the wheat-based culture media resulted in the highest correlation score (r = 0.5) with the *in planta* root rot (RR) inhibition, suggesting that this *in vitro* method was the best predictor of *in planta* performance of streptomycetes against Fusarium RR of wheat assessed as extension of the necrosis on the root. Contrarily, none of the *in vitro* plate assays using the media tested could appropriately predict the activity of the streptomycetes against Fusarium foot rot symptoms estimated as the necrosis at the crown level. Considering overall data of correlation, the activity *in planta* cannot be effectively predicted by dual culture plate studies, therefore improved *in vitro* methods are needed to better mimic the activity of biocontrol strains in natural conditions. This work

contributes to setting up laboratory standards for preliminary screening assays of *Streptomyces* BCAs against fungal pathogens.

Keywords: Fusarium root rot, antagonism, seed treatment, Fusarium foot rot; Actinobacteria, *Streptomyces*, wheat, biocontrol, agriculture, bayesian analysis

Introduction

The use of biocontrol agents (BCAs) against major plant pathogens is becoming a valuable alternative to chemical control of plant diseases and represents an important resource for the future of agriculture (Bardin et al., 2015). The identification of antagonists effective in agricultural environments is essential and requires trustworthy and rapid *in vitro* selection (Le Cocq et al., 2017) that should be able to simulate as much as possible the conditions that the antagonist will encounter in the field.

Streptomyces, a taxonomically wide genus of the phylum *Actinobacteria*, are gram-positive bacteria ubiquitous in soil as free-living microorganisms and as symbionts of plants, animals, and fungi (reviewed in Seipke, Kaltenpoth & Hutchings, 2012). In most cases, these interactions are positive, as they combine *in planta* endophytic behavior (Sardi et al., 1992) with the ability to produce an array of specialized metabolites with biological activities (reviewed in Barka et al., 2016). In fact, they have been studied for their antibacterial and antifungal effects (Wei et al., 2017), as well as their plant growth promoting capabilities (reviewed in Viaene et al., 2016), and therefore they can be exploited in agriculture.

Fusarium graminearum and *Fusarium culmorum* are the major determinants of severe diseases in cereal crops (Goswami & Kistler, 2004; Scherm et al., 2013), such as Fusarium head blight or, under specific climatic conditions, Fusarium foot rot and Fusarium crown rot (Balmas et al., 2015; Obanor & Chakraborty, 2014). The presence of these pathogens in field affects the yield and the seed quality. In addition, mycotoxins such as trichothecenes type B represent a threat to agricultural production and human health (Maresca,

2013). Three chemotypes can be identified among trichothecene B producing fusaria: (1) producers of nivalenol and acetylated derivatives (NIV chemotype), (2) producers of deoxynivalenol and 3-acetyldeoxynivalenol (3ADON chemotype), and (3) producers of deoxynivalenol and 15-acetyldeoxynivalenol (15ADON chemotype) (Miller et al., 1991). Although the evolutionary history of most of the trichothecene biosynthesis genes is discordant with the species phylogeny, this profile variability can affect the fitness and toxicity of the pathogen population (Ward et al., 2002; Aoki et al., 2012). Diversity of *Fusarium* spp. chemotypes has not been considered in the screening of BCAs to date.

The beneficial effect of Actinobacteria on plant disease suppression is well known (Palaniyandi et al., 2013) and documented by an array of reports on the activity of streptomycetes against toxigenic *Fusarium* spp. (Nourozian, Etebarian & Khodakaramian, 2006; Wang et al., 2013; Jung et al., 2013; Samsudin & Magan, 2016). Even today, after years of research, the lack of appropriate selection methods remains one of the main barriers to the identification of new BCAs (Pliego et al., 2011). Screening for antibiosis is still the most common method adopted in the lab for a high-throughput *in vitro* screening of biocontrol strains, even though it excludes the identification of other biocontrol mechanisms (Palazzini et al., 2007; Jung et al., 2013). Interestingly, the majority of studies concerning biological control of *Fusarium* spp. used potato dextrose agar medium for assessing the efficacy of BCAs (Faheem et al., 2015; Dubey, Suresh & Singh, 2007; Khan et al., 2006), without taking into account the influence of the culture media on the antifungal activity. Although the final scope of these studies is to withstand pathogen attack in field, selecting effective BCAs directly in the agricultural environment is not always feasible due to inconsistent abiotic and biotic factors encountered in field studies which are also time-and space-consuming. Therefore, in an effort to identify quick and effective methods of selecting *Streptomyces* strains against *Fusarium* diseases in wheat, we evaluated the effect of *Fusarium* strains, one *F. culmorum* and four *F. graminearum*

characterized by different chemotypes, and culture media on the activity of streptomycete strains. Moreover, we assessed the correlation of biocontrol activity of S*treptomyces* strains obtained *in vitro* with their *in planta* efficacy. The aims of our work therefore were to identify the most effective medium for *in vitro* selection of streptomycetes with biocontrol activity to be employed on wheat; test the hypothesis that *Fusarium* strain diversity plays a role in identifying effective BCA strains; and verify if dual culture assays are able to predict the *in planta* activity of the BCAs.

Materials and methods

Culture media

The antibiosis assays were carried out to test the antifungal activity of 20 *Streptomyces* strains on six different culture media types. PDA1 was prepared using 39 g/L potato dextrose agar (Difco Laboratories, Detroit, MI, USA). One liter of PDA2 was prepared using the extract derived from 200 g of potatoes boiled in distilled water, 20 g glucose (Oxoid Limited, Basingstoke, Hempshire, UK) and 18 g agar (Amresco, Solon, OH, USA). CZY (Czapek-Yeast Extract) contained 35 g/L czapek dox broth, (Difco Laboratories, Detroit, MI, USA), two g/L yeast extract (Difco Laboratories, Detroit, MI, USA), and 15 g/L agar (Amresco, Solon, OH, USA). MMNAG, a modified Minimal Medium (Kieser et al., 2000), was prepared using 4.55 g mannitol (Difco Laboratories, Detroit, MI, USA), 0.5 g KH_2PO_4 (Carlo Erba, Cornaredo, Milan, Italy), 0.2 g $MgSO_4$ $7H_2O$ (Carlo Erba, Cornaredo, Italy), 10 g agar (Amresco, Solon, OH, USA), to which 11.06 g N-acetylglucosamine (Jarrow Formulas, Los Angeles, CA, USA) per liter were added as it has been reported that it may increase secondary metabolite production in *Streptomyces* spp. (Dashti et al., 2017). One liter of wheat meal agar (WMA) was obtained adding the extract derived from 50 g of wheat seeds blended and boiled in distilled water and 15 g agar (Amresco, Solon, OH, USA). One liter of wheat fusarium agar (WFA) was prepared using the extract derived from 50 g of flour boiled in distilled water and 15 g agar (Amresco, Solon, OH, USA). The flour was obtained from wheat

seeds inoculated with a mixture of agar-mycelium plugs derived from colonies of *F. culmorum* (FcUK) and *F. graminearum* (PH1) incubated at 24 °C in 250 mL Erlenmeyer flasks in the dark at 100% humidity. The contaminated wheat seeds, after 11-day-incubation, were lyophilized (model Heto-EPD3; Thermo Scientific, San Jose, CA, USA) for 24 h and blended to obtain a homogenous flour. To determine the amount of toxins in the WFA medium, the flour (two g) was extracted with 12 mL of a solution water:CH_3CN (20:80, v/v), under sonication for 30 min. Then, the mixture was centrifuged at 1,600 g for 10 min, and supernatant transferred in a 10 mL flask and the volumes were adjusted by a solution water:CH_3CN (20:80, v/v). The residues were extracted again as described above. The solutions were centrifuged at 1,000 g for 2 min, diluted with water:CH_3CN (20:80, v/v) and five mL injected into the UHPLC system.

The analysis was carried out on a UHPLC model Acquity (Waters, Milford, MA, USA) coupled with a high resolution fourier transform orbitrap mass spectrometer (model Exactive; Thermo Scientific, San Jose, CA, USA), equipped with a HESI-II probe for ESI and a collision cell (HCD). The operative conditions were as follows: spray voltage -3.5 kV, sheath gas flow-rate 50 (arbitrary units), auxiliary gas flow-rate 15 (arbitrary units), capillary temperature 220 C, capillary voltage -47.5 V, tube lens -120 V, skimmer -16 V formic acid dimer Na adduct $[2M+Na-2H]^-$, was used as lock mass.

A 1.9 mm Hypersil Gold C_{18} column (100 2.1 mm; Thermo Scientific, Waltham, MA, USA) was used for the separation. The column and samples were maintained at 40 and 20 °C, respectively. The flow-rate was 0.5 mL/min, and the eluents were 0.05% formic acid in water (A) and 0.05% formic acid in CH_3CN (B). The UHPLC separation was accomplished by the following linear elution gradient: 5–50% B in 6 min and then 50–95% B in 4 min. The acquisition was made in the full-scan mode in the range m/z 100–1,000 u, using an isolation window of ±2 ppm. The automatic gain control target, injection time and mass resolution were 1 10^6, 100 ms, and 50 K, respectively. The MS data were processed using Xcalibur software (Thermo Scientific, Waltham, MA, USA). The calibration curves were constructed by dissolving

10 mg of each dried standard in 10 mL methanol. The working solutions were prepared in methanol in the range of 0.02–2 mg/mL for Niv, DON, 4-Ac-Niv, and 3-Ac-DON. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Streptomyces *and fungal strains*

Streptomyces strains

The *Streptomyces* strains were part of a collection of endophytic isolates maintained in the laboratory of Plant Pathology at the Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy (Table 1). Strains were grown on CZY for 2 weeks at 24 °C. Spores were collected adding five mL of a water solution containing 10% sterile glycerol (ICN Biomedicals, Irvine, California, USA) + 0.01% Tween20 (Sigma-Aldrich, St. Louis, MO, USA) to the plate and scraping the surface of the colonies with a sterile loop. The concentration was determined and the spore suspension was stored at -20 °C in small aliquots.

Streptomyces identification

DNA from *Streptomyces* isolates DEF04, DEF13, DEF15, DEF25, DEF38, DEF43, DEF44, and DEF47 was extracted following the method described in Sun et al. (2014). A CTAB extraction protocol was used for DEF07, DEF09, DEF10, DEF14, DEF16, DEF19, DEF20, DEF26, DEF35, DEF39, DEF41, DEF48 (Kelly et al., 1998). Analysis of 16S rRNA gene of streptomycetes was conducted using primers 27F (5′-AGAGTTTGATCCTGGC TCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′). PCR was performed in a total volume of 50 µL, which contained 0.3 µL of GoTaq DNA Polymerase 5 U/mL (Promega, Madison, WI, USA), 10 µL of Green GoTaq Reaction Buffer 5 (Promega, Madison, WI, USA), 1 µL of 10 mM dNTP (Promega, Madison, WI, USA), 1 µL of 10 mM primer forward, 1 µL of 10 mM primer reverse, 1 mL of template DNA and nuclease free water. The reaction conditions were initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C

for 20 s, annealing at 56 °C for 30 s and extension at 72 °C for 90 s. A final extension was performed at 72 °C for 7 min. Reaction products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized under UV light. The PCR products were sequenced in both directions (Eurofins Genomics, Ebersberg, Germany) using 27F and rP2 primers and two internal primers 16s_p692f (5′-ATTCCTGGTGTAGCGGT-3′) and 16s_p782r (5′-ACCAGGGTATCTAATCCTGT-3′). The sequences were analyzed using EzBioCloud database, which contains quality-controlled 16S rRNA gene and genome sequences (Yoon et al., 2017).

Pathogens

Four *F. graminearum* isolates (NRRL 28336 (Pasquali et al., 2016), PH1 (Cuomo et al., 2007), Fg8/1 (Boenisch & Schäfer, 2011), 453 (Pasquali et al., 2013a) and one *F. culmorum*: FcUK (Pasquali et al., 2013b) were used in this study. They are representatives of different geographical origins and toxin chemotypes (Pasquali & Migheli, 2014). NRRL 28336 and FcUK belong to the 3ADON chemotype, 453 belongs to the NIV chemotype, PH1 and Fg8/1 are characterized by a 15ADON chemotype.

The pathogens were grown on slant agar containing PDA1 and further stored at 5 °C. Four days before the inoculation for the dual culture assay the strains were transferred on PDA1 dishes at 24 °C.

Dual culture assay

The influence of culture media on the antifungal activity of the streptomycete strains was evaluated using a dual culture assay in Petri plates (90 mm diameter). The first experiment consisted of a 21x5x6 factorial with 21 treatments (20 *Streptomyces* and 1 no- *Streptomyces* control), five *Fusarium* strains and six culture media types.

The *Streptomyces* strains were inoculated as 10 mL of agar-spore suspension (10^6 CFU/mL) 3 days before the pathogen to allow their growth as proposed by Kunova et al. (2016). A plug of pathogen agar-mycelium (six mm diameter), taken from the edge of an actively growing *Fusarium* colony, was inoculated

upside down in the center of the Petri plate at 25 mm distance from the streptomycete. Three replicate plates were prepared for each strain treatment and plates inoculated only with the pathogen were used as control. Following the inoculation, plates were randomly distributed in an incubator at 24 °C in dark. The radius of *Fusarium* mycelial growth was measured in the direction toward the streptomycete point of inoculation, 3 days after the pathogen application on all six media types. On WMA medium, the incubation was extended in order to assess the antifungal activity up to 7 days.

A repetition of the dual culture experiment was carried out using Fg8/1 strain on WMA medium measuring inhibition rate of the 10 most effective streptomycetes identified in the first experiment.

The antifungal activity was expressed as the percentage of mycelium growth inhibition compared to the control, according to the formula: $(R_1-R_2)/R_1$ 100, where R_1 and R_2 were the radius of the pathogen colony in the control and in the presence of the antagonist, respectively.

In planta *assessment of root rot and foot rot severity*

To verify the efficacy of the streptomycetes *in planta*, a modified method from Covarelli et al. (2013) using young plantlets was used. This protocol has been proven to mimic foot and root rot (RR) symptoms development in aged plants (Pasquali et al., 2013b).

A total of 10 *Streptomyces* strains (DEF07, DEF09, DEF14, DEF16, DEF19, DEF20, DEF39, DEF41, DEF47, DEF48), showing higher than 50% inhibitory activity against the target pathogen in dual culture assay, were selected for the seedling assay. The experiment was carried out in glass dishes used as seed trays (diameter 150 mm) sterilized in oven at 160 °C for 2 h. A sterile filter paper was placed in each dish and soaked with 10 mL of sterile water before sowing the seeds. Seeds of wheat “Bandera,” were surface-disinfested in 0.7% sodium hypochlorite for 5 min and then rinsed three times in sterile deionized water using sterile beakers. In a sterile Petri dish, 40 seeds were inoculated with one mL of streptomycete spore suspension (10^7 CFU/mL).

Seeds were left to dry under the laminar flow hood. Control seeds were treated with sterile water. Four dishes were prepared for each treatment containing 10 seeds placed in three rows. The dishes were placed at 5 °C for 24 h to simulate a period of vernalization, then transferred to 20 °C in the dark. After 72 h from treatment with the streptomycete strains, dishes were randomly placed in the growth chamber (21 °C, 16 h photoperiod). Germinating seeds were watered every 2 days with sterile water. After 4 days of growth, when the roots reached approximately 30 mm, an agar-mycelium plug (six mm diameter) taken from the edge of an actively growing *F. graminearum* Fg8/1 colony, was inoculated upside down on the roots at a 10 mm distance from the seed.

Disease assessments were carried out 4 days after pathogen inoculation, measuring for each plant (*N* = 20 for each treatment) the extension of the necrosis on the root (RR assessment) (Figs. 1A–1C). These data were transformed to percent of necrosis inhibition using the formula (CN-TN)/CN 100, where CN and TN were the measurements of necrosis on the control and streptomycete treated seedlings, respectively. In addition, 6 day post inoculation (DPI) Foot Rot assessment (FR) was carried out by evaluating the symptoms at the crown level with a zero to four scale (0 = symptomless; 1 = slightly necrotic; 2 = moderately necrotic; 3 = severely necrotic; 4 = completely necrotic) (Figs. 1D–1H). All the observations were used to obtain the disease severity using the subsequent formula:

$$\left[\frac{\sum(Disease\ grade \times Number\ of\ plants\ in\ each\ grade)}{(Total\ number\ of\ plants) \times (Highest\ disease\ grade)}\right] \times 100$$

The obtained disease severity for each treatment was used to calculate the percentages of protection using the formula [(DC-DT)/DC] x 100, where DC and DT were the disease severity of the control and the treated seedlings, respectively.

Statistical analyses

Statistical analyses were performed using R software, version 3.5.1 (R Core Team, 2018, accessed July 2018). R codes and datasets were included in Data S1. A dual culture assay evaluating the growth inhibition of four *F. graminearum* and one *F. culmorum* strains by a population of streptomycetes was carried out. To identify the best model able to predict the most active *Streptomyces* strains *in vitro* considering as parameters media, *Fusarium* strain, and media x *Fusarium* strain, a stepwise forward selection from the simplest linear model -including only one factor-to the most complex one -including all possible interactions was carried out (Table S1). ANOVA was eventually used to test the goodness of the models. At the same time, model selection using a Bayesian analysis was performed. Consistency of the two approaches was verified. Bayes Factor and the posterior probability of each model were computed with R package "BayesFactor" (Morey & Rouder, 2018). To assess the repeatability of the dual culture assay, a subpopulation of the ten best performing *Streptomyces* strains was selected. Growth inhibition results of the first and the second experiment (both at 3 DPI and 7 DPI), were compared with a t-test. Given the lack of statistical difference between the two experiments, data from the first screening were used to correlate *in vitro* with *in planta* results. The objective of the correlation was to understand if dual culture assay carried out with different media could approximate the *in planta* streptomycete activity.

Table 1 Streptomyces *strains used in the study.*

Strain code	Source of isolation	Environment of sample collection	Sampling site	Year of sample collection	Closest match as similarity % in EzBioCloud database	Completeness (%)	Genbank accession number
DEF04	*Homo sapiens*	Crypt	S. Fruttuoso (GE, Italy)	1960	99.36%: *Streptomyces marokkonensis*	100%	MK412000
DEF07	*Camellia japonica*	Greenhouse	Arona (NO, Italy)	1988	99.36%: *Streptomyces venetus*	100%	MK412001
DEF09	*Triticum aestivum*	Botanic garden	Milano (Italy)	1990	99.93%: *Streptomyces fulvissimus*	100%	MK412002
DEF10	*Hordeum vulgare* var. *distichum*	Botanic garden	Milano (Italy)	1989	99.86%: *Streptomyces zaomyceticus*	100%	MK412003
DEF13	*Polyporus* sp.	Plane tree	Monza (Italy)	1980	100%: *Streptomyces coelicoflavus*	99.9%	MK412004
DEF14	*Arundo* sp.	Lake shores	Ansedonia (GR, Italy)	1996	99.93%: *Streptomyces fulvissimus*	100%	MK412005
DEF15	*Secale cereale*	Botanic garden	Milano (Italy)	1989	100%: *Streptomyces setonii*	100%	MK412006
DEF16	*Zea mays*	Cultivated field	Cantù (CO, Italy)	1985	99.71%: *Streptomyces albidoflavus*	99.7%	MK412007
DEF19	*Camellia japonica*	Greenhouse	Arona (NO, Italy)	1988	99.37%: *Streptomyces venetus*	100%	MK412008
DEF20	*Carex* sp.	Lake shores	Mergozzo (NO, Italy)	1989	99.37%: *Streptomyces venetus*	100%	MK412009
DEF25	*Homo sapiens*	Crypt	S.Fruttuoso (GE, Italy)	1961	99.36%: *Streptomyces marokkonensis*	100%	MK412010
DEF26	*Triticum aestivum*	Botanic garden	Milano (Italy)	1989	100%: *Streptomyces fulvissimus*	100%	MK412011
DEF35	*Secale cereale*	Botanic garden	Milano (Italy)	1992	99.21%: *Streptomyces neopeptinius*	96.8%	MK412012
DEF38	*Secale cereale*	Botanic garden	Milano (Italy)	1989	100%: *Streptomyces canus*	100%	MK412013
DEF39	*Secale cereale*	Botanic garden	Milano (Italy)	1990	100%: *Streptomyces setonii*	100%	MK412014

DEF41	unknown	Natural environment (savanna)	Canaima (Venezuela)	1993	100%: Streptomyces costaricanus	100%	MK412015
DEF43	*Triticum aestivum*	Botanic garden	Milano (Italy)	1989	100%: *Streptomyces costaricanus*	100%	MK412016
DEF44	*Secale cereale*	Botanic garden	Milano (Italy)	1991	99.36%: *Streptomyces marokkonensis*	100%	MK412017
DEF47	unknown	Natural environment (savanna)	Canaima (Venezuela)	1994	100%: *Streptomyces costaricanus*	100%	MK412018
DEF48	*Zea mays*	Cultivated field	Cantù (CO, Italy)	1986	99.36%: *Streptomyces venetus*	100%	MK412019

Note: *Streptomyces* code, the source of their isolation, year, environment and site of sample collection, the percentages of similarity and completeness of the 16S rRNA compared with the EzBioCloud database together with GenBank accession number.

Figure 1 Example of root rot (RR) and foot rot (FR) symptoms on wheat seedlings. (A–C) Length of necrosis of RR measured from the inoculation point (zero, five, or 10 mm from the seed). (D–H) Scale used in FR severity evaluation at the crown level: 0 = symptomless; 1 = slightly necrotic; 2 = moderately necrotic; 3 = severely necrotic; 4 = completely necrotic.

Results

Effect of culture medium and fungal strain diversity on the selection of active Streptomyces spp.

A total of 20 *Streptomyces* strains were tested in dual culture assays against five *Fusarium* strains on six culture media types at 3 DPI (Fig. 2). The flour used to prepare the WFA medium had a concentration of DON, 3-AcDON, and NIV of 4.45, 25.8, 0.03 mg/g respectively. Therefore, in a single Petri plate, a total amount of ca. 35 mg of type B trichothecenes was present.

The reproducibility of the plate culture media testing was evaluated by a t-test, which confirmed the lack of significant differences between the repeated dual culture assays both at 3 (P = 0.9519) and at 7 DPI (P = 0.0758) on WMA (raw data in Data S1). To investigate which model could better explain variations occurring in our experiment we compared classical and Bayesian (Table S2) assessments of models. Both approaches confirmed the role of strain, medium and their interaction in explaining the ability of the *Streptomyces* strains to suppress mycelial growth at 3 DPI (Table S3; raw data in Data S1). Based on the output of the bayesian analysis that suggested the importance of interactions, a multiple regression model was fitted to estimate the effect of each medium, fungal strain and their interactions compared to a selected standard control assay that we defined to be PDA1-Fg8/1 (Table 2, raw data in Data S1). By looking overall at the interactions, PDA2 had similar effects compared to PDA1 confirming that the two media, as expected, are very similar. Also CZY did not significantly differ from PDA1 medium nor did it affect any interaction with fungal species (Table 2). The other media (MMNAG, WFA, WMA) had a significant role in at least some of the interactions. WMA was the most effective medium in highlighting the activity of the *Streptomyces* strains. On this medium, the estimated inhibition for FcUK, NRRL28336, and PH1 strains was significantly different from Fg8/1 (P = 0.0001; P = 0.0002; P = 0.0232) (Table 2). There was a significant effect (*P-value* < 0.05) of each *Fusarium* strains (on at least one culture media type) on the ability of streptomycetes to suppress mycelial growth in culture (Table 2).

Correlation between dual culture assay and* in planta *activity

Evaluation of potential BCAs using plant-based assays is essential to verify tripartite interactions (pathogen-BCA-plant) that occur in the field. We assessed the disease inhibition on wheat plantlets infected with *F. graminearum* strain Fg8/1 and treated with a subpopulation of streptomycetes (DEF07, DEF09, DEF14, DEF16, DEF19, DEF20, DEF39, DEF41, DEF47, DEF48) on wheat plantlets and compared the results obtained in the dual-plate experiments. Scoring the protection level granted by each streptomycete on each medium, we assessed which medium best predicted the results obtained on wheat plantlets applying the 10 most effective *Streptomyces* strains.

By correlating the protection against wheat RR at 4 days (RR) (Table S4, raw data in Data S1) with the pathogen inhibition in dual culture at 3 days we observed that the majority of the culture media types had low or negative correlation values, therefore not corresponding with the *in planta* results (Fig. 3A). Only WMA was able to reach a biologically meaningful positive correlation, r = 0.5. The disease assessment on seedlings at 6 days was carried out to evaluate the incidence of a simulated foot rot, estimating the level of necrosis at the crown (Table S4, raw data in Data S1). The results *in planta* did not correlate significantly with the pathogen inhibition measured at 3 days in the different media plates (Fig. 3B). We evaluated if the WMA antifungal activity recorded at 7 DPI was able to simulate in planta FR protection (Table S5, raw data in Data S1). No biologically significant correlation between the FR protection and the percentages of inhibition at 7 DPI was observed (r = 0.2).

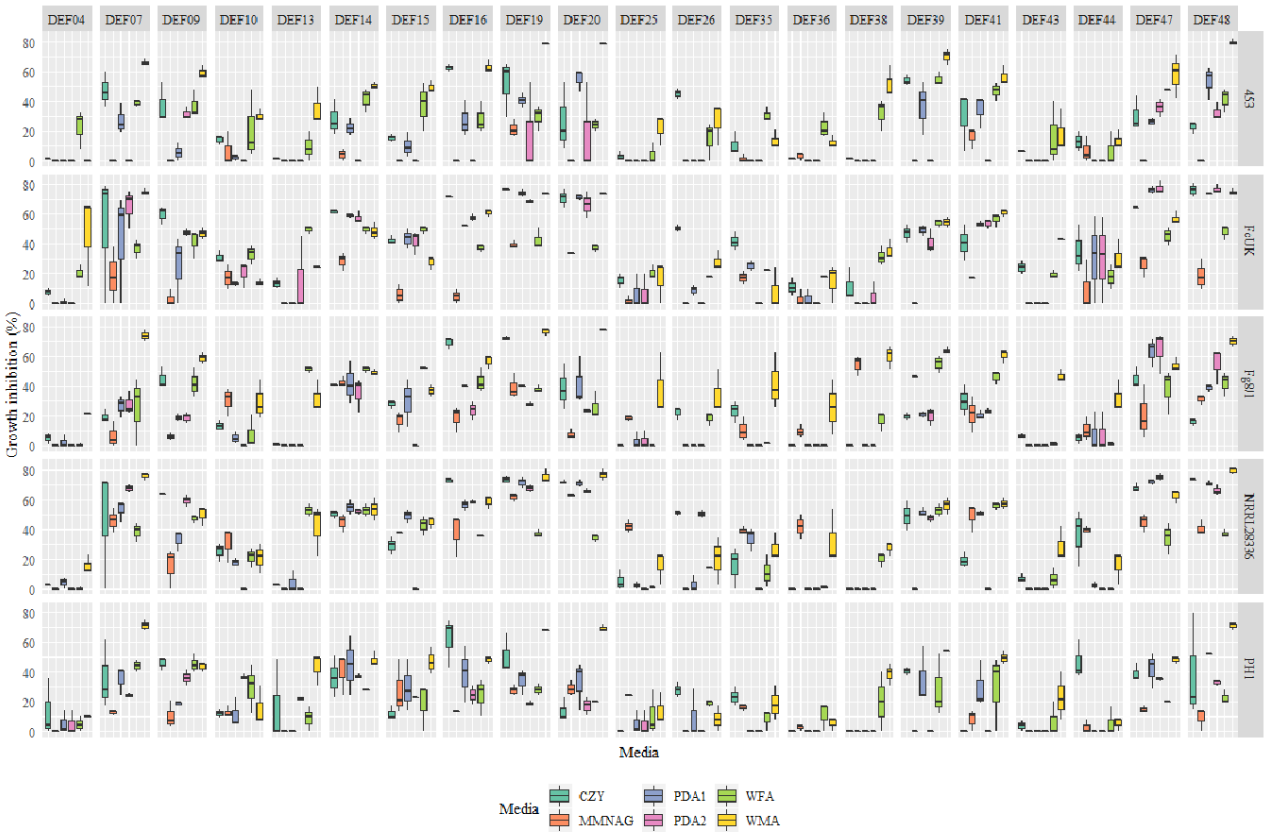


Figure 2 Growth inhibition of five Fusarium *strains (right labels) by 20* Streptomyces *strains (top labels) on six media in dual culture assay, measured at 3 DPI (days post inoculation). The six media used for dual culture were: CZY, Czapeck Yeast Agar; MMNAG, Minimal Medium with N-acetylglucosamine; PDA1, Potato Dextrose Agar 1; PDA2, Potato Dextrose Agar 2; WFA, Wheat Fusarium Agar; WMA, Wheat Meal Agar.*

Table 2 Results of multiple regression model on in vitro inhibition of Fusarium *spp. growth obtained with a population of 20 streptomycetes at 3 DPI (days post inoculation).*

Parameter	**Coefficient**	**Std. Error**	**t-value**	**P-value**
Intercept	20.16	2.73	7.38	2.35e-13*
453	-3.22	3.86	-0.83	0.40
FcUK	15.10	3.86	3.91	9.53e-05*
NRRL28336	14.71	3.86	3.81	0.00*
PH1	1.02	3.86	0.26	0.79
CZY	5.01	3.86	1.30	0.19
MMNAG	-0.94	3.86	-0.24	0.81
PDA2	-3.54	3.86	-0.92	0.36
WFA	7.81	3.86	2.02	0.04*
WMA	30.73	3.86	7.96	3.09e-15*
453-CZY	2.98	5.46	0.55	0.58
FcUK-CZY	4.28	5.46	0.78	0.43
NRRL28336-CZY	-1.87	5.46	-0.34	0.73
PH1-CZY	-0.36	5.46	-0.07	0.95
453-MMNAG	-13.21	5.46	-2.42	0.01*
FcUK-MMNAG	-22.31	5.46	-4.08	4.62e-05*
NRRL28336-MMNAG	-4.53	5.46	-0.83	0.41
PH1-MMNAG	-7.81	5.46	-1.43	0.15
453-PDA2	-6.64	5.46	-1.21	0.22
FcUK-PDA2	4.71	5.46	0.86	0.39
NRRL28336-PDA2	-0.91	5.46	-0.17	0.87
PH1-PDA2	-1.83	5.46	-0.33	0.74
453-WFA	4.44	5.46	0.81	0.42
FcUK-WFA	-6.72	5.46	-1.23	0.22
NRRL28336-WFA	-12.86	5.46	-2.35	0.02*
PH1-WFA	-8.00	5.46	-1.46	0.14

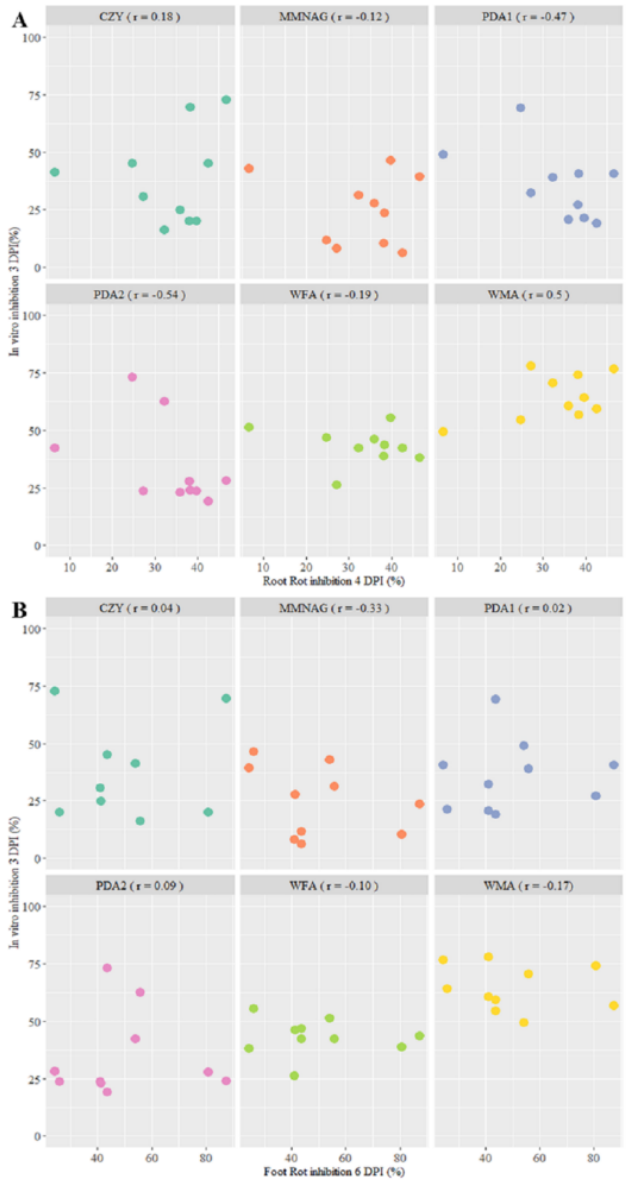
453-WMA	-1.81	5.46	-0.33	0.74
FcUK-WMA	-21.17	5.46	-3.88	0.00*
NRRL28336-WMA	-20.66	5.46	-3.78	0.00*
PH1-WMA	-12.40	5.46	-2.27	0.02*

Notes: Parameters include fungal strains, media, and their interaction (Model 4, Table S1–S2).The medium PDA1 and the *Fusarium* strain Fg8/1 were set as baseline. Other parameters include the fungal strains 453, FcUK, NRRL28336, PH1, and the media.

CZY, Czapeck Yeast Agar; MMNAG, Minimal Medium with N-acetylglucosamine; PDA2, Potato Dextrose Agar 2; WFA, Wheat Fusarium Agar; WMA, Wheat Meal Agar.

* *P < 0.05 is considered significant.

Figure 3 Correlation between in vitro *inhibition of* Fusarium graminearum *Fg8/1 growth by 10 streptomycetes on six culture media and* in vivo *root rot (RR) reduction induced by the same strains (A) and* in vivo *foot rot (FR) reduction. The streptomycete strains used were DEF07, DEF09, DEF14, DEF16, DEF19, DEF20, DEF39, DEF41, DEF47, and DEF48. The six media used for dual culture were: CZY, Czapeck Yeast Agar; MMNAG, Minimal Medium with N-acetylglucosamine; PDA1, Pota-to Dextrose Agar 1; PDA2, Potato Dextrose Agar 2; WFA, Wheat Fusarium Agar; WMA, Wheat Meal Agar. Relationship is expressed as correlation value (r) in the boxes.*



Discussion

Selecting the best dual culture assay medium

By assessing multiple media types (*N* = 6) and *Fusarium* targets representing several chemotypes (*N* = 5) we confirmed the complexity of interactions occurring in dual culture assays used for streptomycetes selection as antagonists in agricultural settings (Cao et al., 2004; Boukaew, Chuenchit & Petcharat, 2011; Yekkour et al., 2012; Kunova et al., 2016). Given the potential significant influence of culture medium on the activity of BCAs (Peighamy-Ashnaei et al., 2008) one of our objectives was to verify whether an optimal medium could be identified for selecting *Streptomyces* strains active against toxigenic fusaria. Therefore, in addition to standard media routinely used for dual culture assays (PDA, CZY), we included also newly developed media able to better simulate possible conditions that would be found in the environment. The scope of using a toxin-containing medium was to select strains able to exert their activity also in conditions where the fungus may have already produced the toxins. Interestingly, we observed no difference in the efficacy of individual streptomycetes based on the toxin content in the medium. Our results suggest that the streptomycetes collection used in our study had similar ability to cope with trichothecene type B. Whether this is due to similar detoxifying capability (Ji, Fan & Zhao, 2016) would require further studies. Although WFA medium was not the most indicative of biocontrol activity, it can be used to test for the sensitivity of BCAs to toxins that may contribute to assess preventively the fitness in real conditions.

Among the other media used, WMA and MMNAG were effective in showing differences between highly active and mildly active *Streptomyces* strains. Taking into consideration all possible combinations of fungal strains and the most effective media WMA, our study shows that antifungal activity of a single streptomycete can vary up to 70%, confirming preliminary observations of Jung et al. (2013), who observed minor effects of different standard media on triggering the ability of streptomycetes to inhibit fungal growth. Our results

indicate that more effective media are needed to assess the activity of streptomycetes and BCAs against fusaria.

Importance of fungal population diversity for BCA identification

The spectrum of activity of an antagonistic strain is often tested against a variety of fungal species (Koch & Löffler, 2009; Al-Askar, Khair & Rashad, 2011; Solans et al., 2016; Kunova et al., 2016). However, the antagonist is rarely tested against a set of diverse representative strains of the pathogen. A positive exception is the recent work of Palazzini et al. (2017), who used a mixture of two *Fusarium* strains to validate the effect in the field. The biological diversity of the target may strongly influence the disease incidence and the response of the pathogen to the BCA treatment (Bardin et al., 2015). In addition, the use of multiple strains can overcome poor outcomes in selection. In our study we included chemotype diversity of *F. graminearum* and *F. culmorum* which are also frequently encountered in different geographical areas as a main cause of *Fusarium* diseases in wheat. Our results indicate that fungal strain diversity contributes to a lower extent than medium diversity in evidencing streptomycetes activity. In addition, the diversity observed between the two species is comparable to the diversity observed within the *F. graminearum* strains. Nonetheless, the different effects observed among fungal strains are likely due to the complex interactions occurring during the hyphae development and the beginning of sporulation (Hopwood, 1988; Chater, 2006). In particular, different metabolites synthesized by the streptomycetes may play a crucial role in these interactions. Their synthesis can be modulated in different ways (Liu et al., 2013; Abdelmohsen et al., 2015; Antoraz et al., 2015) by fungal compounds that are often strain or group specific (Lind et al., 2017). Therefore, it is possible that the fungal molecules may act as inducers or modulators of streptomycete secondary metabolite production (Fguira et al., 2008), leading to different outcomes in the specific interactions occurring in the plate. Further studies on these metabolic interactions are warranted.

The complexity of mimicking* in planta *activity in dual culture assays

The objective of all laboratory approaches to select appropriate BCAs is to better mimic the interactions with the plant that will occur in the field. A large number of successful antagonistic strains selected in laboratories fails when the plant and other environmental factors are taken in consideration (Folman, Postma & Van Veen, 2003). In order to select methodologies able to simulate the interactions between the streptomycetes and the fungus in the natural environment, we decided to verify the correlation of the pathogen inhibition by the biocontrol strain *in planta* (using a simplified laboratory model) with the in plate co-culture on artificial media. The composition of the culture medium can affect secondary metabolite production leading to a modulation of the synthesis of biocontrol involved molecules (Bode et al., 2002). In particular, the effect of different carbon sources has been considered important for this purpose (Sánchez et al., 2010). Our results indicate that the majority of the media are not good predictors of the streptomycete activity *in planta* with correlation values that are close to 0 or negative. In general, the presence of the plant can strongly influence the BCAs metabolism resulting in different expression of antibiotic production and this is important to take into account when we want to compare the *in vivo* and *in vitro* activity (Haas & Keel, 2003). Interestingly, in our study the only meaningful positive correlation was observed using the WMA, a medium containing wheat metabolites resembling the most *in planta* conditions. Indeed, plant materials added to the media can elicit the production of specialized metabolites in streptomycetes (Beauséjour et al., 1999).

We assessed both RR and FR in sterile soilless conditions, without considering other environmental interactors that can influence the activity of the BCAs in field. Despite our simplification of the system, we observed only a weak correlation between dual culture and *in planta* assays (Folman, Postma & Van Veen, 2003). The WMA medium, obtained from wheat seeds, could better predict streptomycetes-mediated protection from RR ($r = 0.5$) but

not FR (r = 0.2). It is evident that *in vitro* screening methods play a significant role in our ability to select new BCAs with good chance to succeed in the field. Despite the use of *in vitro* screening as a common laboratory procedure, even the use of innovative agar media is still not able to simulate the complexity of the plant tissue. Moreover, the signaling during the disease development plays an important role in studying these tripartite interactions. In fact, Beccari, Covarelli & Nicholson (2011) showed that the mode of infection and interaction with the plant of RR and FR follows different pathways. In particular, the severity of FR symptoms does not depend directly from the amount of fungal mycelium growing in the area of infection. Likely, therefore, other biocontrol mechanisms -not only the direct antibiosis- could be elicited. A dual culture assay in plate cannot properly simulate this kind of symptom development.

Conclusions

Our results showed that the assessment of the antagonistic activity of biocontrol strain is strongly influenced by the adopted method. Many promising antagonistic strains fail when they are tested *in planta*, due to a lack of appropriate screening procedures (Pliego et al., 2011). Testing the potential biocontrol isolates in field is essential, but not always possible, as it is expensive, time and space consuming and poses procedural challenges given the inconsistency of the abiotic and biotic parameters. While several factors may contribute to diverse activity between the field and the laboratory (Schisler & Slininger, 1997; Folman, Postma & Van Veen, 2003), our results indicate that the selection of the appropriate method of *in vitro* selection should take into account media and fungal strain diversity, increasing the chance to select truly effective BCAs (Bardin et al., 2015). From the practical point of view, a new single medium selection strategy for *Fusarium* pathogens of wheat would benefit from the use of WMA. Possibly, more than a single *Fusarium* toxigenic strain should be tested.

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

Elena Maria Colombo conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Cristina Pizzatti conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Andrea Kunova conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.

Claudio Gardana conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, approved the final draft.

Marco Saracchi conceived and designed the experiments, performed the experiments, contributed reagents/materials/analysis tools, approved the final draft.

Paolo Cortesi conceived and designed the experiments, contributed reagents/materials/ analysis tools, approved the final draft.

Matias Pasquali conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

DNA sequences are available from the NCBI Nucleotide Database: MK412000– MK412019.

Data Availability

The following information was supplied regarding data availability:

The raw data are available in a Supplementary File and are associated to the R codes that used them. All the measure of growth in plate and the symptoms on plantlets are reported.

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References

Abdelmohsen UR, Grkovic T, Balasubramanian S, Kamel MS, Quinn RJ, Hentschel U. 2015. Elicitation of secondary metabolism in actinomycetes. Biotechnology Advances 33(6):798–811 DOI 10.1016/j.biotechadv.2015.06.003.

Al-Askar AA, Khair A, Rashad WM. 2011. In vitro antifungal activity of *Streptomyces spororaveus* RDS28 against some phytopathogenic fungi. African Journal of Agricultural Research 6:2835–2842.

Antoraz S, Santamaría RI, Díaz M, Sanz D, Rodríguez H. 2015. Toward a new focus in antibiotic and drug discovery from the *Streptomyces* arsenal. Frontiers in Microbiology 6:1–8 DOI 10.3389/fmicb.2015.00461.

Aoki T, Ward TJ, Kistler HC, O'Donnell K. 2012. Systematics, phylogeny and trichothecene mycotoxin potential of fusarium head blight cereal pathogens. Mycotoxins 62(2):91–102 DOI 10.2520/myco.62.91.

Balmas V, Scherm B, Marcello A, Beyer M, Hoffmann L, Migheli Q, Pasquali M. 2015. *Fusarium* species and chemotypes associated with fusarium head blight and fusarium root rot on wheat in Sardinia. Plant Pathology 64(4):972–979 DOI 10.1111/ppa.12337.

Bardin M, Ajouz S, Comby M, Lopez-Ferber M, Graillot B, Siegwart M, Nicot PC. 2015. Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? Frontiers in Plant Science 6:566 DOI 10.3389/fpls.2015.00566.

Barka EA, Vatsa P, Sanchez L, Gaveau-Vaillant N, Jacquard C, Klenk H-P, Clément C, Ouhdouch Y, Van Wezel GP. 2016. Taxonomy, physiology, and natural products of actinobacteria. Microbiology and Molecular Biology Reviews 80(1):1–43 DOI 10.1128/MMBR.00019-15.

Beauséjour J, Goyer C, Vachon J, Beaulieu C. 1999. Production of thaxtomin A by *Streptomyces scabies* strains in plant extract containing media. Canadian Journal of Microbiology 45(9):764–768 DOI 10.1139/w99-072.

Beccari G, Covarelli L, Nicholson P. 2011. Infection processes and soft wheat response to root rot and crown rot caused by *Fusarium culmorum*. Plant Pathology 60(4):671–684 DOI 10.1111/j.1365-3059.2011.02425.x.

Bode HB, Bethe B, Höfs R, Zeeck A. 2002. Big effects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem 3(7):619 DOI 10.1002/1439-7633(20020703)3:7<619::aid-cbic619>3.0.co;2-9.

Boenisch MJ, Schäfer W. 2011. *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. BMC Plant Biology 11(1):110 DOI 10.1186/1471-2229-11-110.

Boukaew S, Chuenchit S, Petcharat V. 2011. Evaluation of *Streptomyces* spp. for biological control of Sclerotium root and stem rot and Ralstonia wilt of chili pepper. BioControl 56(3):365–374 DOI 10.1007/s10526-010-9336-4.

Cao L, Qiu Z, You J, Tan H, Zhou S. 2004. Isolation and characterization of endophytic *Streptomyces* strains from surface-sterilized tomato (*Lycopersicon esculentum*) roots. Letters in Applied Microbiology 39(5):425–430 DOI 10.1111/j.1472-765X.2004.01606.x.

Chater KF. 2006. *Streptomyces* inside-out: a new perspective on the bacteria that provide us with antibiotics. Philosophical Transactions of the Royal Society of London B: Biological Sciences 361(1469):761–768 DOI 10.1098/rstb.2005.1758.

Covarelli L, Gardiner D, Beccari G, Nicholson P. 2013. *Fusarium* virulence assay on wheat and barley seedlings. Bio-Protocol 3(7):e446 DOI 10.21769/BioProtoc.446.

Cuomo CA, Güldener U, Xu JR, Trail F, Turgeon BG, Di Pietro A, Walton JD, Ma L-J, Baker SE, Rep M, Adam G, Antoniw J, Baldwin T, Calvo S, Chang Y-L, DeCaprio D, Gale LR, Gnerre S, Goswami RS, Hammond-Kosack K, Harris LJ, Hilburn K, Kennell JC, Kroken S, Magnuson JK, Mannhaupt G, Mauceli E, Mewes H-W, Mitterbauer R, Muehlbauer G, Munsterkotter M, Nelson D, O'Donnell K, Ouellet T, Qi W, Quesneville H, Roncero MIG, Seong K-Y, Tetko IV, Urban M, Waalwijk C, Ward TJ, Yao J, Birren BW, Kistler HC. 2007. The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. Science 317(5843):1400–1402 DOI 10.1126/science.1143708.

Dashti Y, Grkovic T, Abdelmohsen UR, Hentschel U, Quinn RJ. 2017. Actinomycete metabolome induction/suppression with N-Acetylglucosamine. Journal of Natural Products 80(4):828–836 DOI 10.1021/acs.jnatprod.6b00673.

Dubey SC, Suresh M, Singh B. 2007. Evaluation of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. Biological Control 40(1):118–127 DOI 10.1016/j.biocontrol.2006.06.006.

Faheem M, Raza W, Zhong W, Nan Z, Shen Q, Xu Y. 2015. Evaluation of the biocontrol potential of *Streptomyces goshikiensis* YCXU against *Fusarium oxysporum* f. sp. *niveum*. Biological Control 81:101–110 DOI 10.1016/j.biocontrol.2014.11.012.

Fguira LFB, Smaoui S, Karray-Rebai I, Bejar S, Mellouli L. 2008. The antifungal activity of the terrestrial *Streptomyces* US80 strain is induced by heat-killed fungi. Biotechnology Journal 3(8):1058–1066 DOI 10.1002/biot.200700155.

Folman LB, Postma J, Van Veen JA. 2003. Inability to find consistent bacterial biocontrol agents of *Pythium aphanidermatum* in cucumber using screens based on ecophysiological traits. Microbial Ecology 45(1):72–87 DOI 10.1007/s00248-002-2013-0.

Goswami RS, Kistler HC. 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Molecular Plant Pathology 5(6):515–525 DOI 10.1111/j.1364-3703.2004.00252.x.

Haas D, Keel C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annual Review of Phytopathology 41(1):117–153 DOI 10.1146/annurev.phyto.41.052002.095656.

Hopwood DA. 1988. The Leeuwenhoek lecture, 1987—towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. Philosophical Transactions of the Royal Society of London B: Biological Sciences 235(1279):121–138 DOI 10.1098/rspb.1988.0067.

Ji C, Fan Y, Zhao L. 2016. Review on biological degradation of mycotoxins. Animal Nutrition 2(3):127–133 DOI 10.1016/j.aninu.2016.07.003.

Jung B, Park S-Y, Lee Y-W, Lee J. 2013. Biological efficacy of *Streptomyces* sp. strain BN1 against the cereal head blight pathogen *Fusarium graminearum*. Plant Pathology Journal 29(1):52–58 DOI 10.5423/PPJ.OA.07.2012.0113.

Kelly AG, Bainbridge BW, Heale JB, Pérez-Artés E, Jiménez-Dıaz RM. 1998. In planta-polymerase-chain-reaction detection of the wilt-inducing pathotype of *Fusarium oxysporum* f. sp. *cicerisin* chickpea (*Cicer arietinum* L.). Physiological and Molecular Plant Pathology 52:397–409 DOI 10.1006/pmpp.1998.0161.

Khan MR, Fischer S, Egan D, Doohan FM. 2006. Biological control of *Fusarium* seedling blight disease of wheat and barley. Phytopathology 96(4):386–394 DOI 10.1094/PHYTO-96-0386.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. 2000. Practical *Streptomyces* genetics. Norwich: John Innes Foundation.

Koch E, Löffler I. 2009. Partial characterization of the antimicrobial activity of *Streptomyces antimycoticus* FZB53. Journal of Phytopathology 157(4):235–242 DOI 10.1111/j.1439-0434.2008.01484.x.

Kunova A, Bonaldi M, Saracchi M, Pizzatti C, Chen X, Cortesi P. 2016. Selection of *Streptomyces* against soil borne fungal pathogens by a standardized dual culture assay and evaluation of their effects on seed germination and plant growth. BMC Microbiology 16(1):272 DOI 10.1186/s12866-016-0886-1.

Le Cocq K, Gurr SJ, Hirsch PR, Mauchline TH. 2017. Exploitation of endophytes for sustainable agricultural intensification. Molecular Plant Pathology 18(3):469–473 DOI 10.1111/mpp.12483.

Lind AL, Wisecaver JH, Lameiras C, Wiemann P, Palmer JM, Keller NP, Rodrigues F, Goldman GH, Rokas A. 2017. Drivers of genetic diversity in secondary metabolic gene clusters within a fungal species. PLOS Biology 15(11):e2003583 DOI 10.1371/journal.pbio.2003583.

Liu G, Chater KF, Chandra G, Niu G, Tan H. 2013. Molecular regulation of antibiotic biosynthesis in *Streptomyces*. Microbiology and Molecular Biology Reviews 77(1):112–143 DOI 10.1128/MMBR.00054-12.

Maresca M. 2013. From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. Toxins 5(4):784–820 DOI 10.3390/toxins5040784.

Miller JD, Greenhalgh R, Wang Y, Lu M. 1991. Trichothecene chemotypes of three *Fusarium* species. Mycologia 83(2):121–130 DOI 10.1080/00275514.1991.12025988.

Morey RD, Rouder JN. 2018. BayesFactor: computation of Bayes Factors for common designs. R package version 0.9.12-4.2. Available at https://richarddmorey.github.io/BayesFactor/ (accessed July 2018).

Nourozian J, Etebarian HR, Khodakaramian G. 2006. Biological control of *Fusarium graminearum* on wheat by antagonistic bacteria. Songklanakarin Journal of Science and Technology 28:29–38.

Obanor F, Chakraborty S. 2014. Aetiology and toxigenicity of Fusarium graminearum and *F. pseudograminearum* causing crown rot and head blight in Australia under natural and artificial infection. Plant Pathology 63(6):1218–1229 DOI 10.1111/ppa.12200.

Palaniyandi SA, Yang SH, Zhang L, Suh J-W. 2013. Effects of actinobacteria on plant disease suppression and growth promotion. Applied Microbiology and Biotechnology 97(22):9621–9636 DOI 10.1007/s00253-013-5206-1.

Palazzini JM, Ramirez ML, Torres AM, Chulze SN. 2007. Potential biocontrol agents for *Fusarium* head blight and deoxynivalenol production in wheat. Crop Protection 26(11):1702–17

Palazzini JM, Yerkovich N, Alberione E, Chiotta M, Chulze SN. 2017. Reprint of “an integrated dual strategy to control *Fusarium graminearum sensu stricto* by the biocontrol agent *Streptomyces* sp. RC 87B under field conditions”. Plant Gene 11:2–7 DOI 10.1016/j.plgene.2017.07.002.

Pasquali M, Cocco E, Guignard C, Hoffmann L. 2016. The effect of agmatine on trichothecene type B and zearalenone production in *Fusarium graminearum*, *F. culmorum* and *F. poae*. PeerJ 4:e1672 DOI 10.7717/peerj.1672.

Pasquali M, Migheli Q. 2014. Genetic approaches to chemotype determination in type B-trichothecene producing Fusaria. International Journal of Food Microbiology 189:164–182 DOI 10.1016/j.ijfoodmicro.2014.08.011.

Pasquali M, Serchi T, Renaut J, Hoffmann L, Bohn T. 2013a. 2D difference gel electrophoresis reference map of a *Fusarium graminearum* nivalenol producing strain. Electrophoresis 34(4):505–509 DOI 10.1002/elps.201200256.

Pasquali M, Spanu F, Scherm B, Balmas V, Hoffmann L, Hammond-Kosack KE, Beyer M, Migheli Q. 2013b. FcStuA from *Fusarium culmorum* controls wheat foot and root rot in a toxin dispensable manner. PLOS ONE 8(2):e57429 DOI 10.1371/journal.pone.0057429.

Peighamy-Ashnaei S, Sharifi-Tehrani A, Ahmadzadeh M, Behboudi K. 2008. Interaction of media on production and biocontrol efficacy of *Pseudomonas fluorescens* and *Bacillus subtilis* against grey mould of apple. Communications in Agricultural and Applied Biological Sciences 73:249–255.

Pliego C, Ramos C, De Vicente A, Cazorla FM. 2011. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant and Soil 340(1–2):505–520 DOI 10.1007/s11104-010-0615-8.

R Core Team. 2018. R: a language and environment for statistical computing. Vienna: The R Foundation for Statistical Computing. Available at https://www.R-project.org/ (accessed July 2018).

Samsudin NIP, Magan N. 2016. Efficacy of potential biocontrol agents for control of *Fusarium verticillioides* and fumonisin B1 under different environmental conditions. World Mycotoxin Journal 9(2):205–213 DOI 10.3920/WMJ2015.1886.

Sánchez S, Chávez A, Forero A, García-Huante Y, Romero A, Sánchez M, Rocha D, Sánchez B, Valos M, Guzmán-Trampe S, Rodríguez-Sanoja R, Langley E, Ruiz B. 2010. Carbon source regulation of antibiotic production. Journal of Antibiotics 63(8):442–459 DOI 10.1038/ja.2010.78.

Sardi P, Saracchi M, Quaroni S, Petrolini B, Borgonovi GE, Merli S. 1992. Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. Applied and Environmental Microbiology 58:2691–2693.

Scherm B, Balmas V, Spanu F, Pani G, Delogu G, Pasquali M, Migheli Q. 2013. *Fusarium culmorum*: causal agent of foot and root rot and head blight on wheat. Molecular Plant Pathology 14(4):323–341 DOI 10.1111/mpp.12011.

Schisler DA, Slininger PJ. 1997. Microbial selection strategies that enhance the likelihood of developing commercial biological control products. Journal of Industrial Microbiology and Biotechnology 19(3):172–179 DOI 10.1038/sj.jim.2900422.

Seipke RF, Kaltenpoth M, Hutchings MI. 2012. *Streptomyces* as symbionts: an emerging and widespread theme? FEMS Microbiology Reviews 36(4):862–876 DOI 10.1111/j.1574-6976.2011.00313.x.

Solans M, Scervino JM, Messuti MI, Vobis G, Wall LG. 2016. Potential biocontrol actinobacteria: rhizospheric isolates from the Argentine Pampas lowlands legumes. Journal of Basic Microbiology 56(11):1289–1298 DOI 10.1002/jobm.20160032310 DOI 10.1016/j.cropro.2007.03.004.

Sun Z, Huang Y, Wang Y, Zhao Y, Cui Z. 2014. Potassium hydroxide-ethylene diamine tetraacetic acid method for the rapid preparation of small-scale PCR template DNA from actinobacteria. Molecular Genetics, Microbiology and Virology 29(1):42–46 DOI 10.3103/S089141681401008X.

Viaene T, Langendries S, Beirinckx S, Maes M, Goormachtig S. 2016. *Streptomyces* as a plant's best friend? FEMS Microbiology Ecology 92(8):fiw119 DOI 10.1093/femsec/fiw119.

Wang Z, Wang C, Li F, Li Z, Chen M, Wang Y, Qiao X, Zhang H. 2013. Fumigant activity of volatiles from *Streptomyces alboflavus* TD-1 against *Fusarium moniliforme* Sheldon. Journal of Microbiology 51(4):477–483 DOI 10.1007/s12275-013-2586-y.

Ward TJ, Bielawski JP, Kistler HC, Sullivan E, O'Donnell K. 2002. Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. Proceedings of the National Academy of Sciences of the United States of America 99(14):9278–9283 DOI 10.1073/pnas.142307199.

Wei Z, Xu C, Wang J, Lu F, Bie X, Lu Z. 2017. Identification and characterization of *Streptomyces flavogriseus* NJ-4 as a novel producer of actinomycin D and holomycin. PeerJ 5:e3601DOI 10.7717/peerj.3601.

Yekkour A, Sabaou N, Zitouni A, Errakhi R, Mathieu F, Lebrihi A. 2012. Characterization and antagonistic properties of *Streptomyces* strains isolated from Saharan soils, and evaluation of their ability to control seedling blight of barley caused by *Fusarium culmorum*. Letters in Applied Microbiology 55(6):427–435 DOI 10.1111/j.1472-765x.2012.03312.x.

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J. 2017. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. International Journal of Systematic and Evolutionary Microbiology 67(5):1613–1617 DOI 10.1099/ijsem.0.001755.

Selection of *Streptomyces* strains limiting trichothecene B production using a *TRI5::GFP* biosensor assay

Abstract

Fusarium graminearum, the major cause of Fusarium head blight in cereals, contaminates grains with trichothecenes, among which deoxynivalenol (DON). As it can severely reduce the wheat production, both quantitatively and qualitatively, novel approaches for its management, such as biocontrol, are under scrutiny. When searching for novel biocontrol agents (BCAs), their ability to reduce mycotoxin production should be an essential criterion of selection. Implementing high-throughput analysis of trichothecene by chemical means is expensive and time consuming. Therefore, the availability of a screening method able to assess specifically the limitation of trichothecene synthesis would be important. A *F. graminearum* isolate expressing GFP-tagged trichodiene synthase (*TRI5::GFP*) was used developing a fluorimetric assay allowing to measure *TRI5::GFP* signal when the fungus was exposed to different streptomycete filtrates (N = 5). Fungal spores were treated with cell-free filtrates from *Streptomyces* liquid cultures in order to evaluate their influence on fungal growth and DON production, integrating absorbance and fluorescence measurements. The correlation between the fluorimetric assay based on the *TRI5::GFP* detection and the amount of DON measured by UHPLC-Orbitrap MS was assessed (r = 0.7). Weak antifungal effect was observed for DEF09, with no impact on DON production. Three strains (DEF19, DEF20 and DEF48) inhibited fungal growth, while DEF39 suppressed *TRI5* protein expression without affecting fungal growth. The described method reduces the analytical costs and lowers the use of chemicals and consumables in comparison with traditional chemical analyses. In addition, it increases the speed to screen a large set of natural products.

Introduction

Fusarium graminearum is the main causal agent of Fusarium head blight (FHB), a devastating disease of wheat and barley (O'Donnell et al., 2000). Yield losses are worsened by mycotoxin contamination of the harvested product, which becomes unsuitable for human and animal consumption (Goswami and Kistler, 2004). Indeed, trichothecenes B, such as deoxynivalenol (DON), nivalenol (NIV) and their acetylated derivatives, can be found in grains following *F. graminearum* infection (Foroud and Eudes, 2009). A specific cluster of genes is responsible for their biosynthesis, which involves from seven to ten enzymatic modifications in order to transform farnesyl diphosphate in a toxic sesquiterpene epoxoide (McCormick et al., 2011). Differences among trichothecene chemical structures are due to the diversity in gene functionality within the cluster. However, the first reaction encoded by *TRI5* gene, catalyzing the transformation of farnesyl diphosphate in trichodiene, is shared among all trichothecene producers (Desjardins et al., 1993). Therefore, this gene became a marker used to study the induction of DON/NIV biosynthesis (Gardiner et al., 2009a, 2009b) and the role of these mycotoxins in the various steps during the infection process (Boenisch and Schäfer, 2011). Several researches focused on studying how fungal growth parameters (e.g. pH, temperature, reactive oxygen species, carbon and nitrogen sources) or natural products influence the expression of genes related to trichothecene pathway (Kazan et al., 2012).

The increasing demand for novel biocontrol agents (BCAs) against mycotoxigenic fungi lead to the development of screening tests, which often consist of treating contaminated grains and performing chemical extraction and analysis of the grain samples (Palazzini et al., 2007; Sultan and Magan, 2011; Zhao et al., 2014). These procedures are time-consuming, expensive and imply the use of different organic solvents dangerous for human health. The exploitation of fluorescence associated to *TRI5* protein expression measured over time as a predictor of DON production in *F. graminearum* could allow to obtain a high-throughput screening of novel biocontrol agents (BCAs)

or natural products able to inhibit toxin induction and possibly its biosynthesis (Kazan et al., 2012).

Novel antimycotoxigenic metabolites can be potentially discovered within the large arsenal of bioactive compounds produced by genus *Streptomyces*, promising Gram-positive bacteria exploited for their ability to counteract plant pathogens (Rey and Dumas, 2017). To be able to identify the specific metabolites, the first step consists of assessing the activity of the fermentation broth, which contains secondary metabolites to be further characterized (Al-Askar et al., 2011; Alam et al., 2012; Bressan, 2003; Khieu et al., 2015; Nourozian et al., 2006).

The aim of the present research was to develop a 96-well microplate reader assay to screen of *Streptomyces* culture filtrates able to limit toxin production by the fungus *F. graminearum.* In this biosensor assay, we exploited a *F. graminearum* isolate expressing GFP-tagged trichodiene synthase, encoded by *TRI5 (TRI5::GFP)*, which allowed us to monitor the first step of the toxin biosynthesis pathway by fluorimetric assay. In addition, the influence of filtrates on fungal growth was also assessed. This method was cross validated by a chemical analysis of total DON content.

Material and methods

Streptomyces *strain and culture conditions*

The five *Streptomyces* strains (DEF09, DEF19, DEF20, DEF39 and DEF48) used in this work were part of a collection of *Streptomyces* isolates maintained in the laboratory of Plant Pathology at the Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan (Italy). They were selected on the basis of their interesting biocontrol features observed *in vitro* and *in planta* against *F. graminearum* (Colombo et al., 2019b). *Streptomyces* were grown for 14 days at 24°C on agar plates (90 mm diameter) containing Czapek-Dox yeast medium (CZY: 35 g/L czapek dox broth, Difco Laboratories, USA; 2 g/L yeast extract, Difco Laboratories, USA; 15 g/L agar; Amresco, USA). Spores were collected by adding 5 mL of 10% sterile glycerol

(ICN Biomedicals, USA) + 0.01% tween20 solution (Sigma-Aldrich, USA) and scraping the surface of the colonies with a sterile loop. The concentration was determined using haemacytometer and the spore suspension was stored at -20°C in small aliquots.

Culture filtrates from* Streptomyces *strains

In order to obtain culture filtrates to test in microplate assays, 100 mL flasks containing 50 mL of Czaper-Dox yeast broth (CZYB: 35 g/L czapek dox broth, Difco Laboratories, USA; 2 g/L yeast extract, Difco Laboratories, USA) were inoculated with 250 µL of the *Streptomyces* spore suspension (10^7 spores/mL) and incubated at 24 °C for 14 days at 100 rpm. Liquid cultures were then filtrated before use with 0.2 µm syringe filter and an aliquot was plated on CZY agar plates to check the sterility of the culture filtrates. pH was reported as one of the abiotic factors, which induce toxin biosynthesis in culture media (Gardiner et al., 2009b). Therefore, the pH of each filtrate was assessed in order to normalize the pH also of the control treatment (medium-only) and to compare the results. Small aliquots of sterile filtrates were stored at -20°C in.

Fungal isolate and culture conditions

Fusarium graminearum AB47 strain expressing GFP-tagged trichodiene synthase encoded by *TRI5 (TRI5::GFP)* was used in this study (Blum et al., 2016). Conidia were obtained in liquid CMC medium (15 g/L carboxymethyl-cellulose, Sigma-Aldrich, USA; 1 g/L NH_4NO_3, Carlo Erba Reagents, Italy; 1 g/L KH_2PO_4, Carlo Erba Reagents; 0.5 g/L $MgSO_4$ x $7H_2O$, Carlo Erba Reagents; 1 g/L yeast extract, Difco Laboratories, USA) inoculated with 10 agar-mycelium plugs of *F. graminearum* AB47 grown on V8 medium. The incubation was carried out at 22°C for 6 days at 100 rpm. Conidia were collected using sterile filter paper (Miracloth, Calbiochelm, USA). They were subsequently washed in sterile water and resuspended in 20% sterile glycerol (ICN Biomedicals, USA). The concentration was determined with a haemocytometer and small aliquotes were stored at -20°C.

Microplate assay

Liquid culture experiments were carried out in black 96 well polypropylene plates with clear bottom (Greiner Bio-One, Austria) in 150 µL of a basal medium described by Correll et al. (1987) with minor modification. Per liter, the medium contained: 30 g/L sucrose, Merck, Germany; 1 g/L KH_2PO_4, Carlo Erba Reagents, Italy; 0.5 g/L $MgSO_4$, Carlo Erba Reagents, Italy; 0.5 g/L KCl, Merck, Germany; 10 mg $FeSO_4$ x $7H_2O$ Merck, Germany and 200 µL of trace solution (per 100 mL: 5 g $ZnSO_4$ x $7H_2O$, Carlo Erba Reagents, Italy; 5 g KCl, Merck, Germany; 0.25 g $CuSO_4$ x $5H_2O$, Merck, Germany; 50 mg $MnSO_4$ x H_2O, Carlo Erba Reagents, 50 mg H_3BO_3, Carlo Erba Reagents, Italy; 50 mg $NaMoO_4$ x $2H_2O$, Merck, Germany). As nitrogen source this medium contained 2 g/L $NaNO_3$ (Carlo Erba Reagents, Italy) or 2 g/L L-glutamine (Muscleform, UK) to maintain non-inductive or inductive mycotoxin conditions, respectively, as reported in (Gardiner et al., 2009a). The final pH of the media was adjusted to 5.5 with NaOH before autoclaving.

Conidia of *F. graminearum* AB47 were inoculated into the media at a final concentration of $3x10^3$ conidia/mL. Well treatments were performed in quadruplicates adding 50 µL of filtrates from the selected *Streptomyces* strain cultures. Control treatments were performed using CZY broth with a pH adjusted to that of the culture filtrates (pH 9). Blank wells were prepared adding 150 µL of culture media and 50 µL of filtrate or CZY without the presence of fungal inoculum.

In order to evaluate the ability of the *Streptomyces* filtrates to modulate toxin induction in *F. graminearum* AB47, the *TRI5::GFP* fluorescence was detected. The incubation was carried out for 144 h at 22°C in a microplate reader (Synergy H1, Biotek, USA) and fluorescence was recorded every hour using excitation at 479 nm and emission at 520 nm. In addition, the fungal growth (optical density at 620 nm) was assessed to identify filtrates characterized by an antifungal activity. At the end of the incubation, the pH of each well was measured using a pH meter (PC52+ DHS, XS instruments, Italy). The experiment was repeated three times.

Quantification of total DON by UHPLC-Orbitrap MS

The liquid cultures from wells containing L-glutamine were collected in 1.5 mL tubes after the removal of the grown mycelium and centrifuged for 5 min at 10000 rpm. DON amount was quantified by UHPLC model Acquity (Waters) coupled with an HR Fourier transform Orbitrap mass spectrometer (model Exactive, Thermo Scientific, San Jose, CA), equipped with a HESI-II probe for ESI and a collision cell (HCD). The operative procedures were already described (Colombo et al., 2019b). The samples were prepared making the appropriate dilutions in a solution of water:CH_3CN (20:80, v/v). Mycotoxin standards (NIV, DON, 4ANIV, 15ADON, 3ADON) and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was supplied by a Milli-Q apparatus (Millipore, Milford, MA).

Data analysis

All data from fluorescence and absorbance reads were organized in Microsoft Excel 2016.

The fluorescence and absorbance obtained from blank wells (media+culture filtrate or CZY broth) were subtracted to the correspondent fluorescence and absorbance values. Fluorescence was measured using the arbitrary relative fluorescence units (RFU).

In order to correlate the RFU with DON content of each well, total fluorescence from reads obtained hourly for 144 h was calculated by a crude integration of the fluorescence curve for each individual well. Therefore, the area under the curve was calculated by summing each consecutive time point. The area was correlated with the DON content (ppb) obtained from UHPLC-Orbitrap MS. Areas of DEF09/DEF19 treatments of experiment 1 and areas of DEF19 treatments of experiment 2 were not included in the correlation because they presented not complete curves. Data from three experiments were analyzed together.

All the statistical analyses were performed using R software, version R 3.5.3 (2019), unless stated otherwise. $P < 0.05$ was considered significant. Data

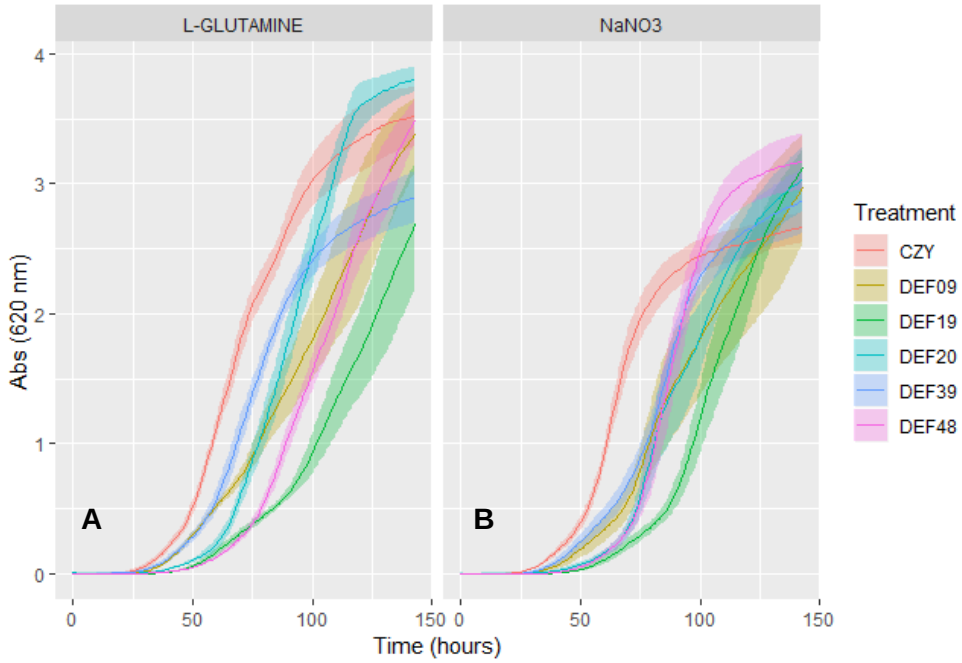
obtained from absorbance and fluorescence reads from the three experiments were combined and submitted to ANOVA. In particular, absorbance data corresponding to the lag time and the middle of exponential phase for CZY treatment (control) and the values of GFP signal at the peak for each treatment were taken into account for statistical elaborations. In order to identify which culture filtrate was able to inhibit the growth and *TRI5::GFP* protein expression of *F. graminearum* in comparison with control treatment a Tukey HSD post-hoc test for multiple comparison was performed. The same analysis was then performed on DON content of each well in order to assess which treatment was able to reduce mycotoxin production.

Results

Effect of culture filtrates on fungal growth

The kinetics of mycelial development absorbance (Figure 1) showed partial growth inhibition in all treated wells in comparison to the control (CZY). In particular, the analysis focused on basal medium containing L-glutamine (Figure 1A), where *TRI5::GFP* protein expression was induced as described below. The kinetics curves showed that the antifungal activity was lost during the period of incubation. The absorbance in all curves reached the level of the control at the end of the period of incubation, except for DEF19 and DEF39 filtrates (Figure 1A). Nevertheless, the treatments had the ability to delay the conidia germination, postponing the exit from the lag phase and strongly reduce the growth during the exponential phase.

Figure 1 Fusarium graminearum *AB47 growth curves obtained by absorbance kinetics measurements (620 nm) for 144 hours in the presence of CZY (Control) or culture filtrates of five* Streptomyces *strains. Conidia were added in basal medium containing L-glutamine (A) or* $NaNO_3$ *(B) as source of nitrogen. Values are the mean of 12 biological replicates, with shade colors representing the 95% confidence interval.*



Culture filtrates of DEF19 and DEF48 were the most effective in reducing the mycelial development. In particular, DEF19, DEF20 and DEF48 filtrates showed the highest ability to reduce the fungal growth at the end of the lag phase of the control in comparison to all the other treatments (Figure 2). At this point of the kinetics, the inhibition reached up to 83%, 78% and 86% for DEF19, DEF20 and DEF48 filtrates, respectively. If absorbance reads are analyzed in the middle of the exponential phase of the control treatment (Figure 3), this trend was maintained for DEF19 and DEF48, with mycelial inhibition reaching up to 83% and 86% respectively. DEF20 at this point was less effective to inhibit the growth (72%). On the contrary, DEF09 and DEF39 culture filtrates showed weak antifungal activity in comparison to the others. Antifungal effect of DEF39 decreased significantly during the exponential growth phase.

Figure 2 Tukey's HSD analysis of absorbance values obtained after 57 hours, corresponding to Fusarium graminearum *AB47 lag phase in wells treated with CZY (Control). Values are the mean of 12 biological replicates.*

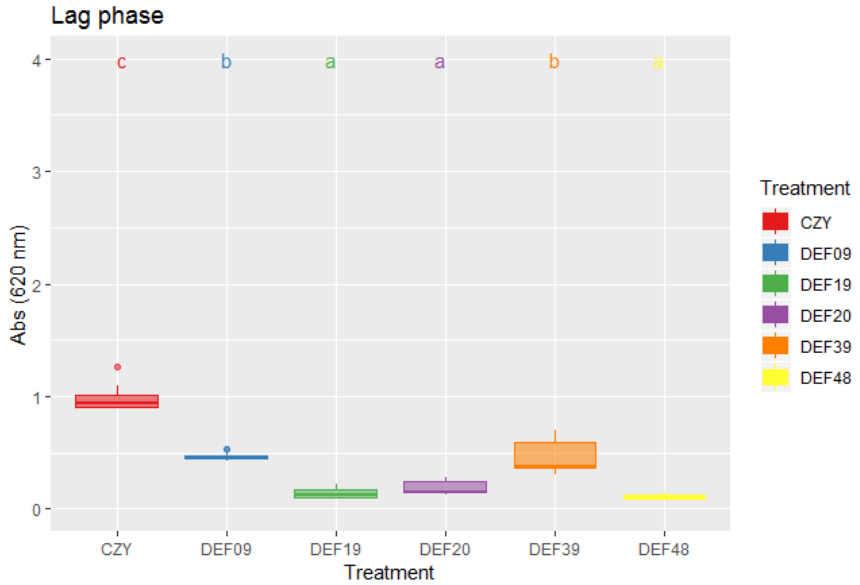
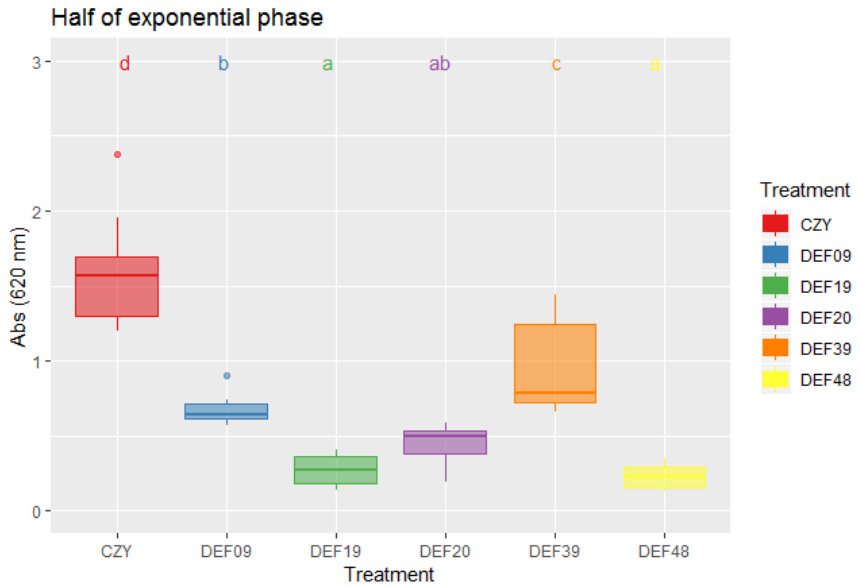


Figure 3 Tukey's HSD analysis of absorbance values obtained after 70 hours, corresponding to Fusarium graminearum *AB47 half of exponential phase of in wells treated with CZY (Control). Values are the mean of 12 biological replicates.*



Effect of culture filtrates on* TRI5::GFP *protein expression

In order to follow the *TRI5::GFP* protein expression of *F. graminearum* treated with culture filtrates from five *Streptomyces* strains, basal medium containing $NaNO_3$ and L-glutamine was tested. $NaNO_3$ used as nitrogen source was not able to trigger the *TRI5::GFP* protein expression (Figure 4). Moreover, none of the culture filtrates induced the *TRI5::GFP* expression in the presence of $NaNO_3$.

Adding L-glutamine in the basal medium allowed us to monitor the expression of *TRI5::GFP,* the first of the gene involved in trichothecene production and determine the ability of filtrates to inhibit the toxin synthesis. The initial phase of fluorescence increase in control wells (CZY) can be observed after 60 hours of incubation and the peak ca. 15 hours later. Effectively, this two important steps of fluorescence reads happened immediately after the end of the lag time and at half of the exponential phase of growth, respectively. In particular, for wells treated with culture filtrates, a delay of the initial phase and peak of fluorescence signal have been observed in comparison to the control, in accordance with the inhibition of mycelial development described above. Interestingly, DEF39 culture filtrate completely inhibited the *TRI5::GFP* fluorescence, resembling fluorescence values of non-inductive conditions, in all the three experiments (Figure 5).

Figure 4 Fusarium graminearum *AB47 fluorescence curves (RFU) obtained by kinetics measurements for 144 hours in the presence of CZY (Control) or culture filtrates of five* Streptomyces *strains. Conidia were added in basal medium containing L-glutamine or $NaNO_3$ as source of nitrogen. Values are the mean of 4 biological replicates for each experiment (EXP1, EXP2, EXP3).*

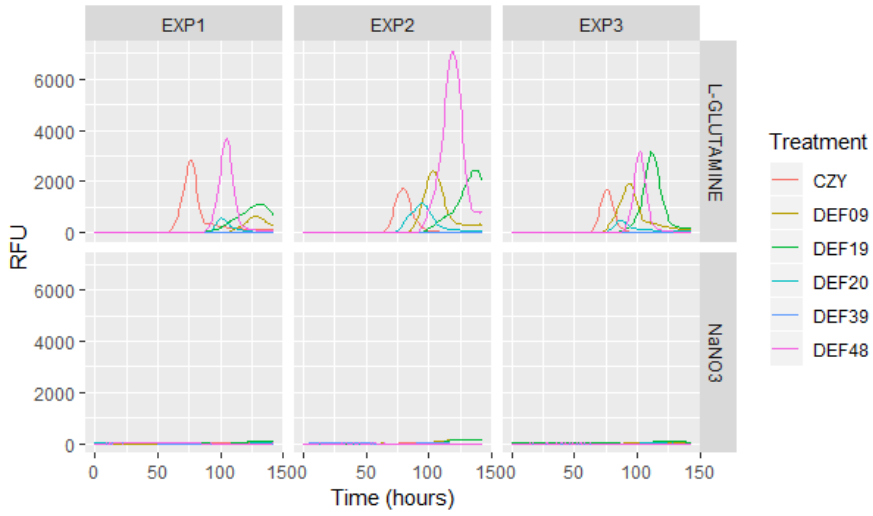
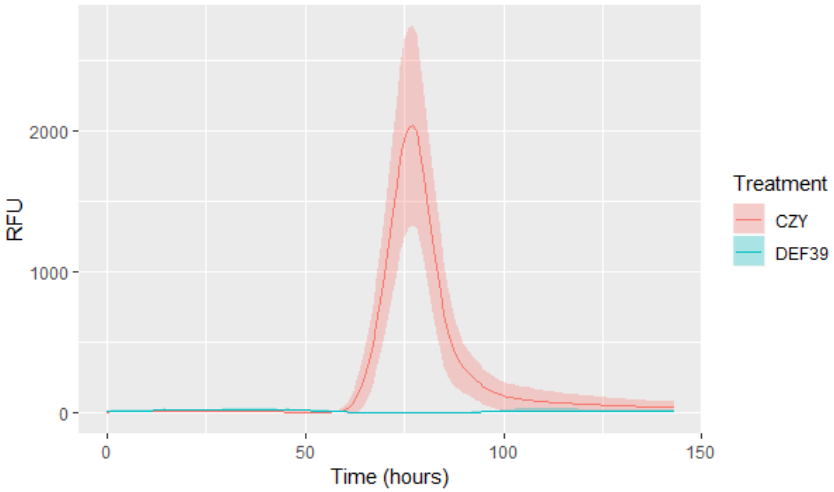
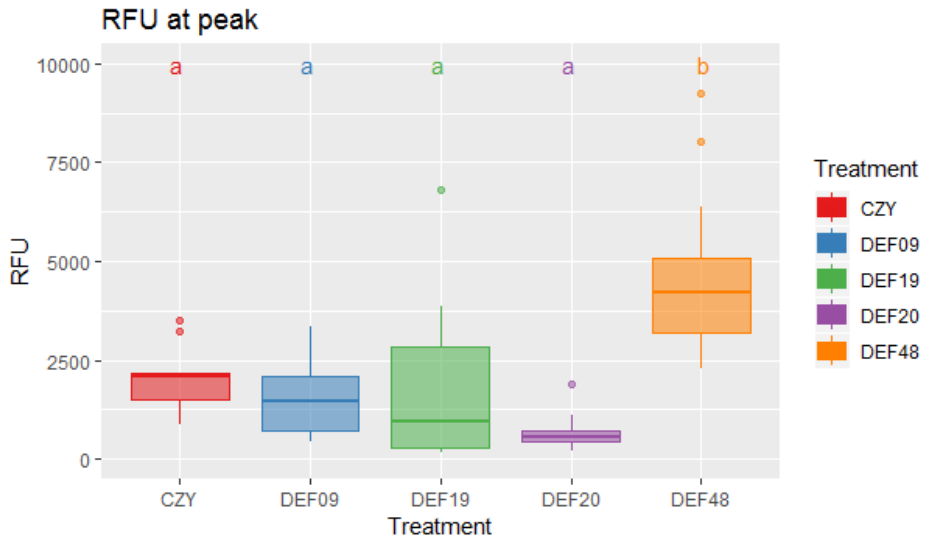


Figure 5 Fusarium graminearum *AB47 fluorescence curve (RFU) obtained by kinetics measurements for 144 hours in the presence of CZY (Control) and DEF39 culture filtrate in basal medium containing L-glutamine. Values are the mean of 12 biological replicates, with shade colors representing the 95% confidence interval.*



None of the other treatments showed activity similar to that of DEF39. Indeed, analyzing the reads at the peak of *TRI5::GFP* protein expression in control (CZY) and culture filtrates of DEF09, DEF19, DEF20 and DEF48 it was clear that none of them was able to significantly decrease the peak RFU value (Figure 6). Instead, DEF48 significantly increased *TRI5::GFP* expression.

Figure 6 Tukey's HSD analysis of values (RFU) recorded at the peak of fluorescence in wells treated with CZY (Control) and streptomycete culture filtrates (DEF09, DEF19, DEF20, DEF48). Values are the mean of 12 biological replicates.



A certain degree of variability has been recorded among the three experiments assessing the inhibitory effects of DEF09, DEF19 and DEF20 on fluorescence production. In particular, the signal inhibition initially recorded for DEF09 and DEF19 was not replicated in the second and third experiment (Figure 4).

The results were compared with DON amount measured in each well at the end of the period of incubation. Only DEF39 was able to reduce total DON amount in the treated wells at the tested conditions (Figure 7). Correlation analysis between the total area under GFP expression (RFU x time) and DON content of each well resulted in r = 0.7 (Figure 8).

Figure 7 Tukey's HSD analysis of DON content (ppb) in wells treated with CZY (Control) or streptomycete culture filtrates after 144 hours of incubation in basal medium added with L-glutamine. Values are the mean of 12 biological replicates.

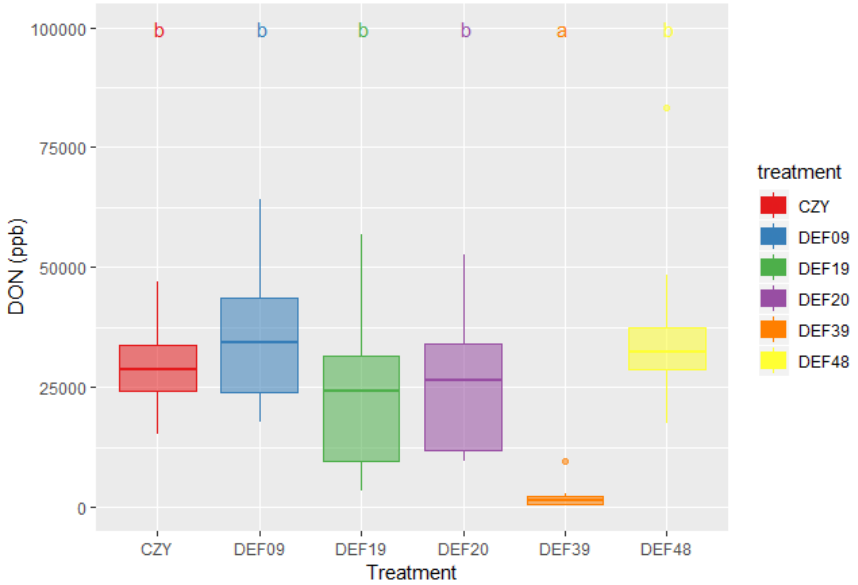
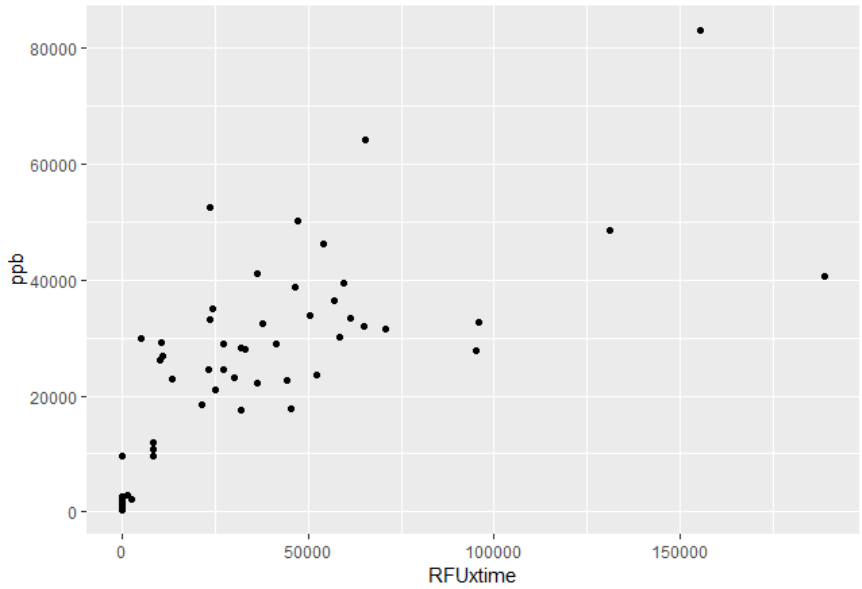


Figure 8 Correlation between data of DON content (ppb) and of the total area under GFP expression (RFUxtime) after 144 hours of incubation (r = 0.7) obtained from wells treated with CZY and streptomycete culture filtrates.



Discussion

The development of this biosensor leads to a substantial improvement over the classical methods of BCA selection: it becomes possible to screen for growth inhibition and toxin inhibition in a 6 days assay analyzing multiple strains at the same time. It reduces the costs associated to the use of other systems relying on chemical extraction and analysis based on HPLC-MS or LC/MS (Bily et al., 2004; Evans et al., 2007). Previous experiments using *TRI5::GFP* strain have been conducted to assess the effect of aminoacids, sugars and pH on toxin induction (Gardiner et al., 2009a, 2009b). The assays correlated well with DON production in the wells (r = 0.82) measured with ELISA. In the present research, the correlation between the fluorescence (RFU x time) and DON (ppb) amount measured via UHPLC Orbitrap MS at the end of the incubation was r = 0.7, confirming that the suggested method can be used to evaluate substances affecting trichothecene pathway. Compared to previous assessments based on the measurement of transcripts via targeted mRNA analysis at different timepoints to assess the effect of exogenous molecules (Ponts et al 2011), our system does not require expensive techniques and laboratory procedures apart from setting the microplate at the initial step of experimental setup.

The microplate bioassay was validated here by testing the activity of culture filtrates from *Streptomyces* spp. against *F. graminearum* growth. We demonstrated that also under microplate growth conditions the activity of DEF19, DEF20 and DEF48 was related to the production of specific antifungal metabolites inhibiting *F. graminearum* development, as observed in dual culture assay and in microsilage conditions (Colombo et al., 2019a, 2019b). The strong antifungal activity of these strains correlated to the low levels of DON present in wheat grains (Colombo et al., 2019a). Further studies will address the characterization of the antifungal metabolites involved, possibly belonging to the large group of polyene macrolides produced by *Streptomyces* spp. against fungal pathogens of eukaryotes (van der Meij et al., 2017).

Surprisingly, in microplate culture conditions, the strains did not inhibit the

TRI5 expression with the exception of DEF39. It might be possible that culture filtrates did not contain the same concentration of antifungal metabolites, or the absence of actively growing BCAs continuously producing these metabolites in turn failed to reduce the DON biosynthesis in liquid culture, that was observed in microsilage conditions (Colombo et al., 2019a). The variation in composition of the culture filtrates or its content stability over the time could explain also the variable outcomes of fluorescence curves among the three experiments reported in Figure 4, which is not observed for control wells. At the end of the fungal growth kinetics, the fungal biomass reached the same amount in the treated wells as in the control. DEF48 filtrate strongly inhibited the conidia germination and initial growth of hyphae but increased significantly the fluorescence at the peak level in comparison to the control. This higher *TRI5* protein expression corresponded to a significant increase of DON (ppb) at the end of incubation. It appears that streptomycete extract can also act as inducers of DON production. Likely the metabolic interactions occurring during the fungal growth in liquid cultures affect the synthesis of DON. Other natural products have been reported to affect fungal growth (50% of growth inhibition) without reducing DON amount, suggesting that an increased mycotoxin production per amount of fungal cells would be an attempt to stimulate host cell death, allowing thus the pathogen to proceed with the infection under stress conditions (Ponts et al., 2011).

Weak antifungal effect and no influence on *TRI5* protein expression was recorded for DEF09 filtrate, despite its ability to counteract FHB symptoms *in planta* (Colombo et al., 2019c). This indicates that the interesting features exerted by this wheat endophyte are probably not related to a direct production of bioactive compounds, but to an indirect defense response elicited *in planta*, as reported for *Streptomyces* sp. EN27 (Conn et al., 2008)

Surprisingly, DEF39 filtrate did not affect mycelial growth but it completely suppressed *TRI5* protein expression during the incubation. These results were confirmed by the low DON content (ppb) in wells treated by DEF39 filtrate in comparison to the other treatments. In another study, a strong reduction of

DON amount by DEF39 in wheat grains was not associated to a strong suppression of the fungal biomass (Colombo et al., 2019a). Further studies are needed to characterize the metabolites responsible for the observed antitoxigenic activity that may differ when the *Streptomyces* strain is exposed to fungal molecules or not. Indeed, it will be of interest to identify molecules that target directly toxigenic ability of the strain as already shown for other natural products able to inhibit aflatoxin production in *Aspergillus parasiticus* and trichothecene in *F. graminearum* without affecting fungal development (Sakuda, 2010).

In conclusion, in effort to find novel biocontrol agents against toxigenic *Fusarium* spp., the selection of microorganisms able to limit DON production is essential. Common screening procedures focus on reducing the mycelium growth, but the antifungal effect not always correspond to the ability to inhibit DON production (He et al., 2009). Therefore, finding a high-throughput method for testing and selecting BCAs with multiple modes of action is crucial for the development of an effective commercial product (Legrand et al., 2017). The described method might be in the future integrated in a robust and fast screening procedure of microbial collections against trichothecene producers, as it combines the evaluation of mycelium growth and mycotoxin production, reducing the cost of the analysis and increasing its speed.

References

Al-Askar, A. A., Khair, A., and Rashad, W. M. (2011). *In vitro* antifungal activity of *Streptomyces spororaveus* RDS28 against some phytopathogenic fungi. *African J. Agric. Res.* 6, 2835–2842. doi:10.5897/AJAR11.320.

Alam, M., Dharni, S., Abdul-Khaliq, Srivastava, S. K., Samad, A., and Gupta, M. K. (2012). A promising strain of *Streptomyces* sp. with agricultural traits for growth promotion and disease management. *Indian J. Exp. Biol.* 50, 559–568.

Bily, A. C., Reid, L. M., Savard, M. E., Reddy, R., Blackwell, B. A., Campbell, C. M., et al. (2004). Analysis of *Fusarium graminearum* mycotoxins in different biological matrices by LC/MS. *Mycopathologia* 157, 117–126. doi:10.1023/B:MYCO.0000012218.27359.ec.

Blum, A., Benfield, A. H., Stiller, J., Kazan, K., Batley, J., and Gardiner, D. M. (2016). High-throughput FACS-based mutant screen identifies a gain-of-function allele of the *Fusarium graminearum* adenylyl cyclase causing deoxynivalenol over-production. *Fungal Genet. Biol.* 90, 1–11. doi:10.1016/j.fgb.2016.02.005.

Boenisch, M. J., and Schäfer, W. (2011). *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. *BMC Plant Biol.* 11, 110. doi:10.1186/1471-2229-11-110.

Bressan, W. (2003). Biological control of maize seed pathogenic fungi by use of actinomycetes.

BioControl 48, 233–240. doi:10.1023/A:1022673226324.
Colombo, E. M., Gardana, C., Kunova, A., Pizzatti, C., Simonetti, P., Cortesi, P., et al. (2019a). *Fusarium*, *Streptomyces* and wheat grains: studying the interaction and the effect of inoculation timing on deoxynivalenol accumulation *in vitro*. unpublished
Colombo, E. M., Pizzatti, C., Kunova, A., Gardana, C., Saracchi, M., Cortesi, P., et al. (2019b). Evaluation of in-vitro methods to select effective streptomycetes against toxigenic fusaria. *PeerJ* 7, e6905. doi:10.7717/peerj.6905.
Colombo, E. M., Pizzatti, C., Kunova, A., Saracchi, M., Cortesi, P., and Pasquali, M. (2019c). Selection of an endophytic *Streptomyces* sp. strain DEF09 from wheat roots as a biocontrol agent against *Fusarium graminearum*. *Front. Microbiol.* 10, 2356. doi:10.3389/fmicb.2019.02356.
Conn, V. M., Walker, A. R., and Franco, C. M. M. (2008). Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* doi:10.1094/mpmi-21-2-0208.
Desjardins, A. E., Hohn, T. M., and McCormick, S. P. (1993). Trichothecene biosynthesis in *Fusarium* species: chemistry, genetics, and significance. *Microbiol. Rev.* 57, 595–604. doi:10113/24882.
Evans, C. K., Xie, W., Dill-Macky, R., and Mirocha, C. J. (2007). Biosynthesis of deoxynivalenol in spikelets of barley inoculated with macroconidia of *Fusarium graminearum*. *Plant Dis.* 84, 654–660. doi:10.1094/pdis.2000.84.6.654.
Foroud, N. A., and Eudes, F. (2009). Trichothecenes in cereal grains. *Int. J. Mol. Sci.* 10, 147–73. doi:10.3390/ijms10010147.
Gardiner, D. M., Kazan, K., and Manners, J. M. (2009a). Nutrient profiling reveals potent inducers of trichothecene biosynthesis in *Fusarium graminearum*. *Fungal Genet. Biol.* 46, 604–613. doi:10.1016/j.fgb.2009.04.004.
Gardiner, D. M., Osborne, S., Kazan, K., and Manners, J. M. (2009b). Low pH regulates the production of deoxynivalenol by *Fusarium graminearum*. *Microbiology* 155, 3149–3156. doi:10.1099/mic.0.029546-0.
Goswami, R. S., and Kistler, H. C. (2004). Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* 5, 515–525. doi:10.1111/J.1364-3703.2004.00252.X.
He, J., Boland, G. J., and Zhou, T. (2009). Concurrent selection for microbial suppression of *Fusarium graminearum*, Fusarium head blight and deoxynivalenol in wheat. *J. Appl. Microbiol.* 106, 1805–1817. doi:10.1111/j.1365-2672.2009.04147.x.
Kazan, K., Gardiner, D. M., and Manners, J. M. (2012). On the trail of a cereal killer: recent advances in *Fusarium graminearum* pathogenomics and host resistance. *Mol. Plant Pathol.* 13, 399–413. doi:10.1111/j.1364-3703.2011.00762.x.
Khieu, T. N., Liu, M. J., Nimaichand, S., Quach, N. T., Chu-Ky, S., Phi, Q. T., et al. (2015). Characterization and evaluation of antimicrobial and cytotoxic effects of *Streptomyces* sp. HUST012 isolated from medicinal plant *Dracaena cochinchinensis* Lour. *Front. Microbiol.* 6, 1–9. doi:10.3389/fmicb.2015.00574.
Legrand, F., Picot, A., Cobo-Díaz, J. F., Chen, W., and Le Floch, G. (2017). Challenges facing the biological control strategies for the management of Fusarium head blight of cereals caused by *F. graminearum*. *Biol. Control* 113, 26–38. doi:10.1016/j.biocontrol.2017.06.011.
McCormick, S. P., Stanley, A. M., Stover, N. A., Alexander, N. J., and Pathogens, B. F. (2011). Trichothecenes: from simple to complex mycotoxins. 802–814. doi:10.3390/toxins3070802.
Nourozian, J., Etebarian, H. R., and Khodakaramian, G. (2006). Biological control of *Fusarium graminearum* on wheat by antagonistic bacteria. *Songklanakarin J. Sci. Technol.* 28, 29–38.
O'Donnell, K., Kistler, H. C., Tacke, B. K., and Casper, H. H. (2000). Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proc. Natl. Acad. Sci.* 97, 7905–7910. doi:10.1073/pnas.130193297.
Palazzini, J. M., Ramirez, M. L., Torres, A. M., and Chulze, S. N. (2007). Potential biocontrol agents for Fusarium head blight and deoxynivalenol production in wheat. *Crop Prot.* 26,

1702–1710. doi:10.1016/j.cropro.2007.03.004.
Ponts, N., Pinson-Gadais, L., Boutigny, A.-L., Barreau, C., and Richard-Forget, F. (2011). Cinnamic-derived acids significantly affect *Fusarium graminearum* growth and in *vitro* synthesis of type B trichothecenes. *Phytopathology* 101, 929–934. doi:10.1094/phyto-09-10-0230.
R Core Team (2019). R: A language and environment for statistical computing. Available at: https://www.r-project.org/.
Rey, T., and Dumas, B. (2017). Plenty is no plague: *Streptomyces* symbiosis with crops. *Trends Plant Sci.* 22, 30–37. doi:10.1016/j.tplants.2016.10.008.
Sakuda, S. (2010). Mycotoxin production inhibitors from natural products. *Mycotoxins* 60, 79–86. doi:10.2520/myco.60.79.
Sultan, Y., and Magan, N. (2011). Impact of a *Streptomyces* (AS1) strain and its metabolites on control of *Aspergillus flavus* and aflatoxin B1 contamination *in vitro* and in stored peanuts. *Biocontrol Sci. Technol.* 21, 1437–1455. doi:10.1080/09583157.2011.632078.
van der Meij, A., Worsley, S. F., Hutchings, M. I., and van Wezel, G. P. (2017). Chemical ecology of antibiotic production by actinomycetes. *FEMS Microbiol. Rev.* 41, 392–416. doi:10.1093/femsre/fux005.
Zhao, Y., Selvaraj, J. N., Xing, F., Zhou, L., Wang, Y., Song, H., et al. (2014). Antagonistic action of *Bacillus subtilis* strain SG6 on *Fusarium graminearum*. *PLoS One* 9, e92486. doi:10.1371/journal.pone.0092486.

Chapter 3: Evaluation of biocontrol streptomycetes to limit disease severity in wheat and DON production *in vitro*

- Colombo, E.M., Pizzatti, C., Kunova, A., Saracchi, M., Cortesi, P., and Pasquali, M. (2019). Selection of an endophytic *Streptomyces* sp. strain DEF09 from wheat roots as a biocontrol agent against *Fusarium graminearum*. *Frontiers in Microbiology.* 10, 2356. doi:10.3389/fmicb.2019.02356.

- Colombo, E.M., Gardana, C., Kunova, A., Pizzatti, C., Simonetti, P., Cortesi, P., Saracchi M., Pasquali M. (2019). *Fusarium*, *Streptomyces* and wheat grains: studying the interaction and the effect of inoculation timing on deoxynivalenol accumulation *in vitro*.

Selection of an endophytic *Streptomyces* sp. strain DEF09 from wheat roots as a biocontrol agent against *Fusarium graminearum*

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Abstract

Selection of biological control agents (BCA) profits from an integrated study of the tripartite interactions occurring among the BCA, the plant and the pathogen. The environment plays a crucial role in the efficacy of BCA, therefore, the selection process shall utmost mimic naturally occurring conditions. To identify effective biocontrol strains against *Fusarium graminearum*, the major cause of Fusarium head blight (FHB) in wheat and deoxynivalenol (DON) accumulation in grains, a workflow consisting of *in vitro* and *in vivo* assays was set up. Twenty-one *Streptomyces* strains, 16 of which were endophytes of different plants, were analyzed. *In vitro* and *in vivo* tests characterized their plant growth promotion (PGP) traits. Biocontrol activity against *F. graminearum* was firstly assessed with a dual culture assay. An *in vivo* germination blotter assay measured Fusarium foot rot and root rot symptoms (FFR-FRR) reduction as well as growth parameters of the plant treated with the *Streptomyces* strains. A selected subset of *Streptomyces* spp. strains was then assessed in a growth chamber measuring FFR symptoms and growth parameters of the wheat plant. The approach led to the identification of an effective *Streptomyces* sp. strain, DEF09, able to inhibit FHB on wheat in controlled conditions by blocking the spread of the pathogen at the infection site. The results were further confirmed in field conditions on both bread and durum wheat, where DEF09 decreased disease severity up to 60%. This work confirms that FRR and FFR pathosystems can be used to identify BCA effective against FHB.

Keywords: endophytes, cereal, BCA, toxigenic fungi, PGP, Fusarium head blight, Fusarium root rot, Fusarium foot rot

Introduction

Fusarium graminearum is a major threat to wheat, leading to Fusarium foot rot (FFR) and Fusarium root rot (FRR) (Smiley and Patterson 1996), as well as Fusarium head blight (FHB), the major cause of wheat losses (Goswami and Kistler, 2004). Losses are aggravated by the accumulation of deoxynivalenol (DON), an internationally regulated mycotoxin (Wegulo et al., 2015). The pathogenic behavior of the fungus has been widely studied at the spike level both from a molecular point of view (Ilgen et al., 2009; Lysøe et al., 2011) and from a physiopathological point of view (Boenish and Schäfer, 2011). The pathogen, similarly to other known foot and root rot pathogens of wheat, such as *F. culmorum* (Scherm et al., 2013) and *F. pseudograminearum* (Chakraborty et al., 2006), has a specific pathway of infection and spread via roots (Wang et al., 2015a). Surprisingly, head blight, root, and foot rot caused by *F. graminearum* share most of the developmental steps of pathogenicity (Wang et al., 2018), including the DON synthesis (Covarelli et al., 2012).

Streptomyces spp. are well known Gram-positive bacterial symbionts of living organisms (Seipke et al., 2012), and can establish tight interactions with inner plant tissues (Coombs and Franco, 2003). They can act as plant growth promoters by producing phytohormones, facilitating nutrient uptake and inhibiting plant pathogens (Viaene et al., 2016, Vurukonda et al., 2018). They have been extensively investigated as a source of bioactive molecules (Watve et al., 2001) and *Streptomyces*-derived commercial products have been successfully applied for crop protection (Newitt et al., 2019). Indeed, several *Streptomyces* strains have been proposed as potential biocontrol agents against toxigenic fungi, including numerous *Fusarium* spp. causing diseases and mycotoxin accumulation in cereals (Nourozian et al., 2006; Palazzini et al., 2007; Yekkour et al., 2012; Jung et al., 2013).

Previous studies of *Streptomyces* strains effective against *F. graminearum* (Jung et al., 2013; Palazzini et al., 2007, 2017, 2018) did not assess their

effect on the plant, despite the large arsenal of metabolites they produce may affect plant development. Moreover, often only *in vitro* tests are used to assess plant growth promoting traits for strain characterization, and rarely BCA and PGP traits are evaluated in the presence of the host plant (Anwar et al., 2016).

One of the main limitations of historical biocontrol studies is that often the selection of strains is solely performed *in vitro,* which can result in the lack of activity in field conditions (Burr et al., 1996; Milus and Rothrock, 1997).

In an effort to set up a solid selection procedure of *Streptomyces* strains active against Fusarium head blight pathogens, the goal of this work was to characterize *Streptomyces* strains for both their PGP associated traits and their biocontrol activity, considering also tripartite interactions (plant, microorganism, pathogen) under different environmental conditions. To achieve this, the laboratory amenable pathosystems of FRR and FFR were used. This procedure proved successful in the identification of a *Streptomyces* sp. able to significantly limit FHB losses in field conditions.

Material and methods

Streptomyces *used in the study*

The collection of *Streptomyces* spp. maintained in the laboratory of Plant Pathology at the Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan (Italy), hosts endophytic isolates from roots of different plants (Sardi et al., 1992) as well as from different sources (Table 1).

The twelve most active strains able to significantly inhibit FFR or FRR caused by *F. graminearum* (activity above 40%), identified in a comparative work of *in vitro* screening methods (Colombo et al., 2019), were selected for this study together with new isolates of diverse origin identified in this work (Table 1). Overall, twenty-one strains were used.

Table 1 Streptomyces *strains used in the study.*

Strain code	Source of isolation	Environment of sample collection	Place of sample collection	Year of sample collection	Closest match as similarity % in EzBioCloud database	Completeness (%)	GenBank accession number
DEF06	*Poa annua*	*Golf course*	*Monticello (LC, Italy)*	1989	99.50:*Streptomyces geysiriensis*	99.9	MK463961
DEF07*	*Camellia japonica*	*Greenhouse*	*Arona (NO, Italy)*	1988	99.36:*Streptomyces venetus*	100	MK412001
DEF08	*Polyporus* sp.	*Plane tree*	*Monza (Italy)*	1980	100:*Streptomyces coelicoflavus*	99.9	MK463962
DEF09*	*Triticum aestivum*	*Botanic garden*	*Milano (Italy)*	1989	99.93:*Streptomyces fulvissimus*	100	MK412002
DEF13*	*Polyporus* sp.	*Plane tree*	*Monza (Italy)*	1980	100:*Streptomyces coelicoflavus*	100	MK412004
DEF14*	*Arundo* sp.	*Lake shores*	*Ansedonia (GR, Italy)*	1996	99.93:*Streptomyces fulvissimus*	100	MK412005
DEF15*	*Secale cereale*	*Botanic garden*	*Milano (Italy)*	1989	100:*Streptomyces setonii*	100	MK412006

DEF16*	*Zea mays*	Cultivated field	Cantù (CO, Italy)	1985	99.71:*Streptomyces albidoflavus*	100	MK412007
DEF17	*Hordeum vulgare*	Botanic garden	Milano (Italy)	1989	99.50:*Streptomyces tanashiensis*	100	MK463963
DEF18	*Triticum aestivum*	Botanic garden	Milano (Italy)	1989	100:*Streptomyces setonii*	100	MK463964
DEF19*	*Camellia japonica*	Greenhouse	Arona (NO, Italy)	1988	99.37:*Streptomyces venetus*	100	MK412008
DEF20*	*Carex* sp.	Lake shores	Mergozzo (NO, Italy)	1989	99.37:*Streptomyces venetus*	100	MK412009
DEF21	*Zea mays*	Cultivated field	Cantù (CO, Italy)	1985	100:*Streptomyces setonii*	100	MK463965
DEF31	*Homo sapiens*	Crypt	S. Fruttuoso (GE, Italy)	1960	100:*Streptomyces calvus*	100	MK463966
DEF33	unknown plant	Natural environment (savanna)	Canaima (Venezuela)	1993	99.57:*Streptomyces corchorusii*	100	MK463967
DEF39*	*Secale cereale*	Botanic garden	Milano (Italy)	1989	100:*Streptomyces setonii*	100	MK412014

DEF40	*Secale cereale*	Botanic garden	Milano (Italy)	1989	100:*Streptomyces costaricanus*	100	MK463968
DEF41*	unknown plant	Natural environment (savanna)	Canaima (Venezuela)	1993	100:*Streptomyces costaricanus*	100	MK412015
DEF46	*Homo sapiens*	Crypt	S. Fruttuoso (GE, Italy)	1960	100:*Streptomyces calvus*	100	MK463969
DEF47*	unknown plant	Natural environment (savanna)	Canaima (Venezuela)	1993	100:*Streptomyces costaricanus*	100	MK412018
DEF48*	*Zea mays*	Cultivated field	Cantù (CO, Italy)	1985	99.36:*Streptomyces venetus*	100	MK412019

*Strains identified in Colombo et al., 2019. Underlined strains were not originally isolated as endophytes.

Streptomyces *identification*

Bacterial isolates DEF07, DEF09, DEF13, DEF14, DEF15, DEF16, DEF19, DEF20, DEF39, DEF41, DEF47, and DEF48 were identified in Colombo et al. (2019).

DNA from isolates DEF06, DEF08, DEF17, DEF18, DEF21, DEF31, DEF33, DEF40, and DEF46 was extracted following the method described by Sun et al. (2014). Briefly, a single bacterial colony was transferred to a sterile 1.5 mL tube containing 27 µL Tris (10 mM)-EDTA (1 mM) (pH 7.6); then, 3 µL KOH (0.4M) - EDTA (10 mM) were added and incubated at 70°C for 5 min. Next, 3 µL Tris-HCl (10 mM) (pH 4.0) were added to adjust the pH of the lysate. The lysate was used directly as a DNA template for the PCR amplification. 16S rRNA primers (Turner et al., 1999) used were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3'). PCR was performed in a total volume of 50 µL, which contained 0.3 µL of GoTaq® DNA Polymerase 5 U/µL (Promega, USA), 10 µL of Green GoTaq® Reaction Buffer 5X (Promega, USA), 1 µL of 10 mM dNTP (Promega, USA), 1 µL of 10 µM primer forward, 1 µL of 10 µM primer reverse, 1 µL of template DNA and nuclease free water. The reaction conditions were initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 56°C for 30 s and extension at 72 °C for 90 s. A final extension was performed at 72 °C for 7 min. Reaction products were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized under UV light. The PCR products were sequenced in both directions (Eurofins Genomics, Germany) using 27F and rP2 primers and two internal primers 16s_p692f (5'-AATTCCTGGTGTAGCGGT-3') and 16s_p782r (5'-ACCAGGGTATCTAATCCTGT-3'). Assembled sequences were obtained with Geneious Prime 2019 (Biomatters, USA). EzBioCloud database was used to identify the strains based on 16S rRNA sequences (Yoon et al., 2017).

Preparation of bacterial inoculum

Spores were collected after 2 weeks of incubation at 24°C on Czapek Yeast Extract medium (CZY: 35 g/L czapek dox broth, Difco Laboratories, USA; 2 g/L yeast extract, Difco Laboratories, USA; 15 g/L agar, Amresco, USA; pH 6.5) scraping the surface of the colonies with a sterile loop and 5 mL of 10% sterile glycerol (ICN Biomedicals, USA) + 0.01 % Tween20 solution (Sigma-Aldrich, USA). The concentration was determined using a haemocytometer and adjusted to 10^7 spores/mL. Small aliquots were then stored at - 20°C.

Antibiosis assay

The antibiosis assay was performed using one medium and 22 treatments (21 *Streptomyces* strains + one water control). Three replicates were prepared. Briefly, 10 µL of *Streptomyces* spp. agar-spore suspension (1 x 10^6 spores/mL) or sterile water were inoculated on a Petri plate containing Wheat Meal Agar (WMA; Colombo et al., 2019). After three days, a plug of agar-mycelium (6 mm diameter) was taken from the edge of an actively growing colony of *F. graminearum* Fg8/1 (Boenisch and Schäfer, 2011) and inoculated upside down in the center of the plate at 25 mm distance from the bacterial strain. After a period of incubation (3 days at 24 °C in the dark), the antagonistic activity was assessed measuring the mycelial radial growth of the pathogen in the control (R1) and in the presence of the antagonist (R2). The percentage of mycelium growth inhibition compared to the control was calculated according to the Eq. (1):

(1)

$$\frac{(R1 - R2)}{R1} \times 100$$

Screening for PGP traits in vitro

The twenty-one strains involved in the study were screened for PGP characteristics. Indole acetic acid (IAA) production, tricalcium phosphate solubilization, siderophore and chitinase production, starch hydrolysis, nitrate

reduction and growth in presence of salt were determined following the procedures described below.

The IAA production was evaluated following the method described in Bano and Musarrat (2003). Ten µL of *Streptomyces* strain spore suspension (1 x 10^6 spores/mL) were inoculated in 5 mL of CZY broth (35 g/L czapek dox broth, Sigma-Aldrich, USA; 2 g/L yeast extract, Difco Laboratories, USA; pH 6.5) adding 500 µg/mL of tryptophan (Sigma-Aldrich, USA). Three replicates were prepared. After a period of incubation (24°C for 10 days at 125 rpm), the liquid cultures were centrifuged (10000 rpm, 10 min, 15°C) and 2 mL of supernatant were collected and mixed with 100 µL of 10 mM orthophosphoric acid (Carlo Erba Reagents, Italy) and 4 mL of Salkowski reagent (1 mL 0.5 M $FeCl_3$, Sigma-Aldrich, USA; 49 mL 35% $HClO_4$, Sigma-Aldrich, USA). The samples were incubated at room temperature for 20 min in the dark. The development of pink color indicated the IAA production. The absorbance of the samples was measured with a spectrophotometer (Perkin-Elmer Lambda 20, USA) at 530 nm. The concentration of IAA produced was calculated based on a standard curve of IAA obtained in the range of 1-50 µg/mL.

The ability to solubilize tricalcium phosphate was assessed in Petri plates (90 mm diameter) containing National Botanical Research Institute's Phosphate growth medium (10 g/L glucose, Sigma-Aldrich, USA; 5 g/L $Ca_3(PO_4)_2$, Sigma-Aldrich, USA; 5 g/L $MgCl_2 \times 6H_2O$, Carlo Erba Reagents, Italy; 0.25 g/L $MgSO_4 \times 7H_2O$, Carlo Erba Reagents, Italy; 0.2 g/L KCl, Merck, Germany; 0.1 g/L $(NH_4)_2SO_4$, Carlo Erba Reagents, Italy; 15 g agar, Amresco, USA; pH 7) as described in Nautiyal (1999), inoculated with 10 µL of spore suspension (1 x 10^6). Three replicates were prepared. After an incubation period (24°C for 14 days), the halo was visually assessed. The halo width of 1 mm was marked with +, lack of halo was marked with -.

Siderophore production was observed using Chrome Azurol S agar overlay method (O-CAS) as described by Pérez-Miranda et al. (2007). The strains were grown on a modified CZY medium without iron (pH 6.5) for siderophore production. Ten µL of agar-spore suspension (10 µL of spore suspension in

90 µL of 0.2% water agar) were inoculated in the center of a Petri plate (90 mm diameter) and kept for 14 days at 24°C. Subsequently, 15 mL of Chrome Azurol S agar were cast upon culture agar plates (CAS agar: 60.5 mg/L Chrome Azurol S, Sigma-Aldrich, USA; 72.9 mg/L hexadecyltrimethyl ammonium bromide, Honeywell Fluka, Buchs, Switzerland; 30.24 g/L piperazine-1,4-bis(2-ethanesulfonic acid), Sigma-Aldrich, USA; 10 mL 1 mM $FeCl_3$ x $6H_2O$ in 10 mM HCl, Sigma-Aldrich, USA; 9 g/L agar, Amresco, USA). Three replicates were prepared. In addition, a negative control was prepared using normal CZY medium. After 1 day of incubation at room temperature in the dark, the change of color around the colony (from blue to orange) indicated the siderophore production. The orange halo (D) and colony (d) diameters were measured and the production of siderophores was calculated according to the Eq. (2):

(2)

$$\frac{(D-d)}{2}$$

Chitinase production (Kuddus and Ahmad, 2013) was assessed using chitin medium (6 g/L Na_2HPO_4, Carlo Erba Reagents, Italy; 3 g/L KH_2PO_4, Carlo Erba Reagents, Italy; 1 g/L NH_4Cl, Carlo Erba Reagents, Italy; 0.5 g/L NaCl, Carlo Erba Reagents, Italy; 0.05 g/L yeast extract, Difco Laboratories, USA; 1% (w/v) colloidal chitin; 15 g/L agar, Amresco, USA; pH 6.5). Colloidal chitin was prepared adding 20 g of chitin (Sigma-Aldrich, USA) to 300 mL of 37% HCl (Merck, Germany). The chitin-HCl solution was kept for 60 min at 30°C in continuous stirring and then precipitated adding 1 L of cold water. In order to allow the precipitation of the colloidal particles, the material was kept at 4°C overnight and then collected by filtration on filter paper, washing with deionized water to bring up the pH at 6. Petri dishes (45 mm diameter) containing chitin medium were inoculated with 10 µL of agar-*Streptomyces* spore suspension (1 x 10^6). Three replicates were prepared. After an incubation period (24°C for 14 days), the halo (D) and the colony (d) diameters

were measured and the capacity to degrade chitin was expressed using Eq. (2).

The ability to hydrolyze starch (Shirling and Gottlieb, 1966) was evaluated streaking a single colony of each *Streptomyces* strain on Petri dishes (90 mm diameter) containing ISP Medium 4 (Difco Laboratories, USA, pH 7.2) added with 10 g/L of soluble starch (Difco Laboratories, USA) and 1 mL of trace salts solution (per 100 ml: 100 mg $FeSO_4 \times 7H_2O$ Merck, Germany; 100 mg $MnCl_2 \times 4H_2O$ Carlo Erba Reagents, Italy; 100 mg $ZnSO_4 \times 7H_2O$ Carlo Erba Reagents, Italy). Three replicates were prepared. After a period of incubation (24°C for 14 days), the presence of the hydrolysis halo around the colonies determined the amylase activity.

The nitrogen reduction capability (Shirling and Gottlieb, 1966) was assessed by inoculating glass tubes containing 5 mL Bacto-Nitrate medium (13 g/L nutrient broth, Oxoid, Italy; 2 g/L KNO_3, Carlo Erba Reagents, Italy; 2 g/L bacto agar, Difco Laboratories, USA; pH 6.5) with a single colony of each strain. Three replicates were prepared. After an incubation period (24°C for 14 days), 200 µL of nitrate reagent A (α-naphthylamine, Sigma-Aldrich, USA) and B (sulfanilic acid, Sigma-Aldrich, USA) were added in each tube. The presence of nitrite was confirmed by the development of a red color after the formation of a diazonium salt caused by the reaction between the reagents A and B.

High salt concentration growth was evaluated streaking single colonies on Bennet's agar medium (Jones, 1949) (1 g/L yeast extract, Difco Laboratories, USA; 0.8 g/L, lab-lemco Oxoid, Italy; 10 g/L glucose, Sigma-Aldrich, USA; 2 g/L casitone, Difco Laboratories, USA; 15 g/L agar, Amresco, USA; pH 6.5) added with 3.5% or 7% (w/v) of NaCl (Carlo Erba Reagents, Italy). Three replicates were prepared. After 14 days of incubation at 24°C, the growth of the strains in Petri dishes (45 mm diameter) was evaluated in comparison with control plates (0% NaCl).

Seed treatments and blotter assay germination

The twenty-one strains were tested for their potential growth promoting and biocontrol activities against FRR and FFR. Seeds of *Triticum aestivum* L. cv. Bandera were surface-sterilized in 0.7% sodium hypochlorite for 5 minutes and then rinsed 3 times in sterile water. In sterile Petri dishes, seeds (N = 40) were inoculated with 1 mL of *Streptomyces* strain spore suspension (10^7 spores/mL) and dried under the laminar flow hood. Control seeds were treated with 1 mL of deionized sterile water. For biocontrol experiments, seeds (N = 40) were treated in the same way. After 4 days, the seedlings were inoculated with an agar-mycelium plug (6 mm diameter) taken from the edge of an actively growing *F. graminearum* Fg8/1 colony and inoculated upside down on the roots at a 10 mm distance from the seed. The assay took place in sterile glass dishes as seed trays (diameter 150 mm). In each dish, a filter paper was placed and soaked with 10 mL deionized sterile water before sowing. For each condition, four glass dishes containing 10 seeds arranged in three rows were prepared. The germination of the seeds followed the conditions described in Covarelli et al. (2013). Briefly, the dishes were placed at 5°C in the dark for 24 hours simulating a period of vernalization and then moved at 20°C in the dark. Three days after seed bacterization, dishes were placed in a growth chamber (21°C, 16 h photoperiod using fluora lamp osram L36W/77). Seedlings were watered with sterile deionized water every 2 days.

Evaluation of PGP and biocontrol effects in germination blotter assay

Germination was assessed after 2 days of incubation in the growth chamber, when seeds were still in the dark to simulate normal germination process, while root and seedling length as well as root number were assessed after 3 and 10 days. At the 10^{th} day, seedlings were dried and the root and shoot dry weight was assessed.

The biocontrol potential of the *Streptomyces* spp. against Fusarium root rot (FRR) and foot rot (FFR) was evaluated using *F. graminearum* Fg8/1 infected seedlings. Four days after pathogen inoculation the FRR was measured on

20 roots as necrosis development. FRR data were reported as millimeters of necrosis extension. Percentages of necrosis inhibition were calculated using measurements of necrosis on the control (CN) and on the treated seedlings (TN) using the Eq. (3):

(3)

$$\frac{(CN - TN)}{CN} \times 100$$

Six days after seed bacterization, FFR was evaluated by scoring the symptoms at the crown level on 20 seedlings (Covarelli et al., 2013) with a 0-4 scale (0 = symptomless; 1 = slightly necrotic; 2 = moderately necrotic; 3 = severely necrotic; 4 = completely necrotic) (Colombo et al., 2019). The FFR disease severity was calculated for each treatment using the Eq. (4):

(4)

$$\left[\frac{\sum(Disease\ grade \times Number\ of\ plants\ in\ each\ grade)}{(Total\ number\ of\ plants) \times (Highest\ disease\ grade)}\right] \times 100$$

The ability of the antagonists to reduce symptom development was assessed with the Eq. (5): (5)

$$\frac{(DC - DT)}{DC} \times 100$$

DC and DT were the disease severity in the control and the treated seedlings, respectively.

In addition, shoots from infected and control seedlings were dried and their weight was measured.

Streptomyces *biocontrol activity against FFR in soil substrate*

Seed bacterization with *Streptomyces* spp. spore suspension was carried out as described above with strains that showed promising BCA features *in vitro* (DEF07, DEF09, DEF19, DEF20, DEF39, DEF47, and DEF48). DEF08 was used as a negative control, as it showed no FFR inhibition in the previous test. Conidia of *F. graminearum* Fg8/1 were produced in CMC medium (15 g/L

carboxymethyl-cellulose, Sigma-Aldrich, USA; 1 g/L NH_4NO_3, Carlo Erba Reagents, Italy; 1 g/L KH_2PO_4, Carlo Erba Reagents, Italy; 0.5 g/L $MgSO_4$ x $7H_2O$, Carlo Erba Reagents, Italy; 1 g/L yeast extract, Difco Laboratories, USA; pH 6.5). Conidia were collected as described in Breakspear et al. (2011) after 5 days of incubation by filtering cultures through one layer of Miracloth (Calbiochem, USA) and centrifuging the filtrate for 10 min at 3000 rpm. Supernatant was discarded and the pelleted conidia were washed twice with sterile water (centrifuge 10 min, 3000 rpm).

Twenty inoculated seeds for each treatment (water or *Streptomyces* spp. spore suspension) were placed in sterile glass dishes (diameter 150 mm) to allow their germination at room temperature. After 6 days, wheat seedlings were transplanted in polystyrene seed trays (32x52x5.5 cm) containing sterile substrate (1:1 ratio of Irish peat and sand, pH 6.5, EC 0.2 dS/m, density 340 kg/m^3, porosity 89% v/v, Vigorplant, Italy) watered with tap water. After that, roots were inoculated with one agar-mycelium plug (6 mm diameter) taken from a colony of *F. graminearum* Fg8/1 grown on V8 medium (Spanu et al., 2012). In addition, 1 mL of *F. graminearum* Fg8/1 conidia (1 x 10^6 conidia/mL) or a mixture of *F. graminearum* Fg8/1 (1 x 10^6 conidia /mL) + *Streptomyces* (5x10^6 spores/mL) was added to control plants or already bacterized plants, respectively (Simpson et al., 2000). Seedlings inoculated only with *Streptomyces* strains, as well as non-inoculated ones (water-only) to be used as controls were prepared.

Plants were grown in a growth chamber (Conviron, Winnipeg, Canada) at 24 °C, 55 % relative humidity and 15 h photoperiod, watered with tap water every two days. After 20 days, FFR disease symptoms were visually evaluated using a 0-4 scale (0 = symptomless; 1 = slightly necrotic; 2 = moderately necrotic; 3 = severely necrotic; 4 = completely necrotic) (Supplementary file 1). The FFR disease severity and protection level were calculated for each treatment using the Eq. (4) and (5), respectively. Dried shoot weight of the infected seedlings was also assessed.

Streptomyces *spp. re-isolation from inner root tissues and evaluation of PGP effect in soil substrate assay*

Control plants and plants inoculated only with *Streptomyces* (no-*Fusarium* inoculation) from the previous test were harvested 20 days after transplant and washed in sterile water to remove the excess soil. Shoot length and dried weight of wheat plants were assessed for each treatment.

For inner root tissue analysis, 10 seedlings for each treatment were selected and cut at the base. The roots were washed and surface sterilized with propylene oxide (Sigma-Aldrich, USA) for one hour (Sardi et al., 1992). Subsequently, 10 or 15 root pieces were cut in sterile conditions and placed on water agar medium (WA) containing 15 g/L agar (Amresco, Italia), 25 mg/L nalidixic acid (Sigma-Aldrich, USA), 50 mg/L nystatin (Sigma-Aldrich, USA), and 50 mg/L cycloheximide (Sigma-Aldrich, USA). Plates were incubated for 7 days at 24°C. Growth of *Streptomyces* spp. colonies on the plate was visually observed using a microscope. Morphological examination was carried out to confirm the re-isolation. Roots not inoculated with *Streptomyces* strains were used as negative control and subjected to the same procedure to check the presence of *Streptomyces* spp.

Evaluation of biocontrol activity against FHB in growth chamber

Spring wheat (*Triticum aestivum* L.) cv. Bandera was cultivated in growth chamber following the procedure described in Watson et al. (2018) to speed up the plant development to reach anthesis in approximately two months. Briefly, seeds were sterilized as described before and placed in sterile glass dishes (diameter 150 mm) to allow their germination. After 3 days at 4°C they were placed at room temperature for another period before sowing them in pots (21x13x15.5 cm, five seeds per pot) containing non-sterilized Irish and Baltic peat-based growth substrate (pH 6, EC 0.25 dS/m, density 120 kg/m^3, porosity 90% v/v, Vigorplant, Italy). The lighting was set to 12h light/ 12h dark cycle for four weeks and then increased to an 18h light/ 6h dark photoperiod using fluora lamp osram L36W/77 until complete spike development. The

temperature of the growth chamber was set at 18 °C. *F. graminearum* strain PH1 (Seong et al., 2009) was used to inoculate wheat heads. Bacterial spores of DEF09, which showed consistent biocontrol efficacy under all tested conditions, were prepared in CZY as described previously and *F. graminearum* conidia were prepared in CMC medium. The day of the treatment spores and conidia were collected and mixed with 0.01% Tween 20 (Sigma-Aldrich, USA) immediately before head inoculation. The final concentration of the mixture was 1 x 10^7 spore/mL for DEF09 and 1 x 10^6 conidia/mL for PH1. Ten µL of this mixture was used to inoculate the fifth centrally located spikelet from the bottom at anthesis. Three replicates were prepared for each treatment and arranged in a randomized block design. Three head treatments were performed: 1*) F. graminearum*, 2) *F. graminearum* + *Streptomyces* sp. DEF09, 3) Control (sterile distilled water + 0.01% Tween 20). Each spike was sealed in a plastic bag for 3 days. The FHB severity was visually estimated using a 0–100% scale 7 days after the treatment (Stack and McMullen, 1998). The average of FHB infection level was scored and the protection level calculated using the Eq. (5).

Evaluation of biocontrol activity against FHB in field conditions

In order to further verify the biocontrol effect of DEF09 against FHB under complex environmental conditions, a field trial was performed. Field trial was conducted in Travacò Siccomario, Pavia (45°08'50.1''N 9°09'20.0''E, Italy), during the growth season 2019. The spring wheat (*Triticum aestivum* L.) cv. Bandera and the durum wheat (*Triticum turgidum* L. ssp. *durum*) cv. Claudio (both susceptible to *F. graminearum*) were sown with a 200 kg/ha density at the end of October 2018 on a loamy soil (Sand 31.2%, Silt 47.5%, Clay 21.3, cation exchange capacity 21.3 $cmol^+$ kg DM^{-1}, total organic carbon 1.51% DM, soil organic matter 2.60% DM, total Kjeldal nitrogen 0.19% DM, C/N ratio 7.95, P_2O_5 Olsen 87 mg kg DM^{-1}, where DM stands for dry matter) with neutral pH (7.1). The field was previously cultivated with soybeans. Nitrogen fertilization was 30 kg/ha at the sowing and 50 kg/ha before booting. Weeding was carried

out with Arianne II (Corteva, Italy) the 15/03/2019 at a dose of 3.5 l/ha. DEF09 spores and *F. graminearum* PH1 conidia were freshly produced in the laboratory as described above and collected at the day of field inoculation. Biocontrol assays started at wheat anthesis stage (beginning of May). Flowering period of the two cultivars differed by 7 days. Spores were kept on ice (max 2 hours) until inoculation. Thirty plants at anthesis stage were selected for each treatment. Controls included: a) conidia of *F. graminearum* PH1 $2x10^6$ conidia/mL, b) spores of *Streptomyces* DEF09 $2x10^7$ spores/mL; c) sterile distilled water + 0.01% Tween 20 (Sigma-Aldrich, USA). The treatment consisted of bacterial suspension and conidia + 0.01% of Tween 20 (Sigma-Aldrich, USA), mixed before head inoculation. The final concentration of the mixture was $2x10^7$ spores/mL for DEF09 and $2x10^6$ conidia/mL for PH1. Ten µL of spore suspension per treatment were used to inoculate a single, centrally located spikelet at anthesis. Inoculation was arranged in a randomized block design. Wheat heads were evaluated after 30 days. The infected spikelets were counted and FHB disease severity was visually estimated using a 0–100% scale (Stack and McMullen, 1998) for both wheat cultivars. Protection level of DEF09 treatment was assessed using the Eq. (5).

Statistical analysis

Statistical analyses were performed using R software, version 3.5.1 (R Core Team 2018), unless stated otherwise. To understand the effect of *Streptomyces* treatments on plant development and on FRR a Kruskal-Wallis test was applied, followed by a Dunn's test with Bonferroni's correction of the *P*-values to control the experiment-wise error rate (R package "dunn.test", Dinno, 2017). Unless stated otherwise $P < 0.05$ was considered significant.

In order to identify treatments able to protect seedlings from FFR symptom development in comparison to the untreated control, a Fisher's test was performed pooling the FFR symptoms in two groups (asymptomatic (class 0) or symptomatic (classes 1-4)). Moreover, to assess also differences within the range of symptomatic seedlings, an additional Fisher's test was carried out

comparing the group of mild symptomatic (class 1-2) with the severely diseased group (classes 3-4). $P < 0.01$ for both tests was considered significant.

CORREL function [CORREL(x, y)] in Microsoft Excel was used to determine the correlation coefficient between the results of the dual culture assay and chitinase activity, FRR protection and FFR protection. The equation (6) for correlation coefficient is:

(6)

$$Correl(X,Y) = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2 \sum (y-\bar{y})^2}}$$

For field trials, a two-group analysis (Mann-Whitney test) using Estimation stats (Ho et al., 2018) was conducted for each cultivar on the number of diseased spikelets of control (PH1) and treatment (PH1+DEF09). The results are presented on a Gardner-Altman estimation plots.

Results

Screening for Streptomyces *biocontrol and PGP activities* in vitro

Identification of the nine isolates not identified in a previous study (Colombo et al., 2019) by 16S rRNA confirmed that all 21 strains belong to *Streptomyces* spp. (Table 1).

Results of *in vitro* tests for physiological and biochemical features directly or indirectly involved in plant growth promotion are reported in Table 2. Chitinase activity is widespread among all strains, but it is not correlated with the ability to reduce *F. graminearum* mycelium development (r = 0.22). Low amount of IAA production was recorded at the tested conditions, except for DEF09 and DEF33 that produced 2.50 ± 0.04 and 7.51 ± 0.00 µg/mL of IAA, respectively. Siderophore production was observed for DEF06, DEF17, DEF18 and DEF46. The radius of the halo ranged from 3 to 36 mm and DEF46 showed the widest halo of siderophore production on CAS agar. Only DEF06, DEF17, and DEF21 were able to solubilize tricalcium phosphate on NBRIP medium. Starch

hydrolysis was common among the strains except for DEF09, DEF13, DEF20, and DEF41. Eleven strains reduced nitrate at the tested conditions (Table 2). All strains except DEF33 were able to grow at 3.5 % salt in the medium and 71 % grew even at 7 % salt concentration. The antifungal activity of the *Streptomyces* strains against *F. graminearum* Fg8/1 in dual culture assay varied from 41% inhibition for DEF31 to 70% inhibition for DEF07, DEF19, DEF20, and DEF48 (Table 3).

Evaluation of PGP effects in germination blotter assay

Under soilless conditions, none of the tested *Streptomyces* strains significantly altered the germination percentage compared to the control plants, which had a germination percentage of around 99 (Table 2). A slight but significant reduction of the germination after seed bacterization was observed only for DEF17. Some strains inhibited the shoot and seminal root length three days after the seed bacterization (Supplementary file 2). After 10 days of incubation, an overall attenuation of these negative effects was observed (Table 2) except for DEF41, DEF46, DEF47, and DEF48, which still negatively affected both shoot and seminal root elongation (Table 2).

Ten days after seed bacterization, root number was not significantly different from the control with the exception of DEF41 and DEF09, which showed significantly lower number of roots compared to the control (4 versus 5) (Supplementary file 2).

To assess the potential gain/loss in biomass, root and shoot dry weight were also assessed after 10 days of growth (Table 2). Overall, the effect was minimal and a significantly lower weight was obtained only for shoots in plants treated with DEF17, while root dry weight was not significantly affected.

Evaluation of biocontrol activity in germination blotter assay

FRR was assessed 8 days after the antagonist inoculation. The results confirmed the biocontrol activity observed *in vitro* (Table 3 and Supplementary file 3) with a correlation coefficient of r = 0.5. DEF07, DEF09, DEF16, DEF19,

DEF20, DEF21, DEF31, DEF39, DEF41, and DEF48 significantly reduced the necrosis development on wheat roots in comparison with the untreated control ($P < 0.05$), showing up to 46% inhibition of necrosis.

The Fisher's test analysis of the FFR scores grouped in asymptomatic (0) and symptomatic (1-2-3-4) showed the ability of DEF09 and DEF47 to maintain the seedlings healthy in comparison to the untreated control ($P = 2.57e-08$, $P = 3.24e-05$). Interestingly, seedlings treated with DEF08 showed more severe necrosis (45.45%) at the crown in comparison to the untreated control ($P = 7.00e-04$), (Table 3 and Supplementary file 4). All *P*-values of Fisher's test analyses are reported in Supplementary file 5.

The strains with a capacity to reduce FRR did not reduce FFR symptoms in the same manner. The best performing strain against FRR was DEF19 (46.74%), while the best performing strain against FFR was DEF47 with protection percentages of 87.50%. Only DEF09 was able to control both symptoms of *F. graminearum* infection with high level of efficiency, resulting in approximately 80% inhibition of FFR development and >40% in FRR development.

None of the non-endophytic strains showed the ability to effectively reduce the disease severity *in planta* with the exception of DEF31, which showed a partial efficacy against FRR only (29.14% reduction of necrosis extension).

In order to analyze if the BCA treatments were able to counteract the biomass loss following the infection, the shoots from infected seedlings were dried and weighed. Only DEF06 and DEF07 increased significantly the shoot weight compared to the *Fusarium*-treated control (Table 3).

Biocontrol and PGP activities in soil substrate

To further verify whether the biocontrol and the PGP activities were consistent in a more complex environment - soil, and over a longer period of cultivation, 26 days - FFR, stem shoot length and dried weight were evaluated for strains showing interesting biocontrol activities: DEF07, DEF09, DEF19, DEF20, DEF39, DEF47, and DEF48. DEF08 was used as negative control.

First, the colonization of inner root tissues by selected *Streptomyces* strains was verified. All root pieces (10/10) of wheat seedlings were extensively colonized by the tested *Streptomyces* strains on WA plates. They showed the ability to move in soil and internally colonize the plant, including DEF08 that was not originally isolated as endophyte.

The use of soil and the longer cultivation period until disease symptom evaluation and PGP analysis led to decreased BCA activity of most of the strains (Table 3, Supplementary file 6), with the exception of DEF07 and DEF09 which were able to significantly reduce FFR (61% and 46% level of protection, respectively) (Supplementary file 7 for Fisher's test *P*-values). Plant growth promotion of non-infected plants colonized by the *Streptomyces* spp. strains was not significant for the two parameters analyzed (Table 2). Shoot dried weight of *Fusarium*-infected plants was, however, affected by some strains: DEF07, DEF09, DEF39 and DEF47 lowered the dried biomass in comparison with the *Fusarium*-treated control (Table 3).

Table 2 The screening of plant growth promotion traits in vitro *and* in planta *(germination blotter or soil substrate assays).*

	***In vitro* assays**								**Germination blotter assay**					**Soil substrate assay**	
Treatment	**IAA production (µg/mL) ± SD**	**Phosphate solubilization activity**	**Siderophore production (mm) ± SD**	**Chitinase activity (mm) ± SD**	**Starch hydrolysis**	**Nitrate reduction**	**Growth at high salt concentration**		**Germinated seeds (%) per blotter ± SD**	**Root length (mm) per plant ± SD**	**Shoot length (mm) per plant ± SD**	**Root dried weight (mg) per plant ± SD**	**Shoot dried weight (mg) per plant ± SD**	**Shoot length (mm) per plant ± SD**	**Shoot dried weight (mg) per plant ± SD**
							3.5%	**7%**							
Water control	/	/	/	/	/	/	/	/	98.13±3.96	176.50±32.51	135.43±17.57	25.95±6.23	12.45±3.79	285.95±32.79	57.50±14.05
DEF06	0.00±0.07	+	10.75±3.18	1.00±0.00	+	+	+	-	93.75±5.17	193.17±30.66	127.78±26.58	23.09±5.90	12.19±2.12	nt	nt
DEF07	1.36±0.29	-	-	6.00±1.75	+	-	+	+	95.00±7.56	173.88±22.31	136.06±12.54	22.86±8.22	11.04±2.23	287.13±27.03	51.44±10.29
DEF08	0.00±0.09	-	-	0.5±0.71	+	+	+	+	91.25±11.26	174.33±27.89	125.60±10.19	24.80±7.05	21.24±12.22	309.40±28.10	44.28±12.70
DEF09	2.50±0.04	-	-	1.58±0.80	-	+	+	+	97.50±4.63	118.13±42.97*	132.63±13.62	25.29±6.29	14.08±4.40	307.10±21.20	62.20±7.91
DEF13	0.00±1.74	-	-	5.58±2.65	-	+	+	+	87.50±11.65	199.00±14.67	131.56±15.50	28.35±6.67	9.27±4.05	nt	nt
DEF14	0.47±0.65	-	-	2.17±1.04	+	+	+	+	93.75±7.44	168.93±22.21	130.00±21.84	23.61±6.36	10.51±2.39	nt	nt
DEF15	0.85±1.86	-	-	1.08±0.95	+	+	+	+	97.50±4.63	169.22±21.17	122.00±27.86	25.85±5.54	10.73±1.85	nt	nt
DEF16	0.71±0.86	-	-	1.25±1.09	+	-	+	+	96.25±5.17	143.00±28.40	125.30±17.42	25.44±5.86	10.76±1.95	nt	nt

DEF17	0.00±0.32	+	3.50±0.70	3.58±0.52	+	+	+	-	90.00±5.34	143.42±52.81	131.79±16.79	25.92±6.55	9.54±1.80*	nt	nt
DEF18	0.00±0.03	-	4±0.70	2.12±0.53	+	+	+	+	97.50±4.63	166.06±39.08	122.72±25.31	31.03±6.44	12.45±1.60	nt	nt
DEF19	0.96±0.48	-	-	6.41±1.38	+	-	+	+	100.00±0.00	161.65±23.05	119.76±15.88	26.91±5.81	9.76±2.07	301.47±23.92	52.39±10.42
DEF20	0.00±0.21	-	-	3.42±2.45	-	-	+	-	100.00±0.00	155.39±18.76	128.28±16.60	22.09±5.48	10.08±2.06	269.00±31.86	46.49±8.62
DEF21	1.03±1.01	+	-	5.67±0.63	+	+	+	+	97.50±4.63	152.17±38.13	123.83±22.56	30.01±5.84	11.75±2.89	nt	nt
DEF31	1.17±0.73	-	-	1.00±0.00	+	-	+	+	98.75±3.53	169.00±21.05	132.79±9.99	23.56±5.31	10.36±1.84	nt	nt
DEF33	7.51±0.00	-	-	3.17±0.58	+	-	-	-	98.75±3.53	151.47±25.31	133.24±14.57	24.86±6.39	10.87±2.23	nt	nt
DEF39	1.60±0.04	-	-	1.00±0.00	+	+	+	+	100.00±0.00	147.12±30.20	112.53±19.69*	19.71±7.00	9.93±1.93	302.57±14.11	68.21±10.96
DEF40	0.00±0.13	-	-	1.12±0.18	+	+	+	+	98.75±3.53	142.74±32.36	110.58±20.13*	22.51±4.75	10.02±2.37	nt	nt
DEF41	0.00±0.03	-	-	1.33±0.58	-	-	+	+	100.00±0.00	126.94±21.77*	114.78±23.84*	30.86±3.58	12.75±1.31	nt	nt
DEF46	1.12±0.10	-	36.50±0.00	2.66±2.02	+	-	+	-	98.75±3.53	139.78±31.31*	105.50±21.61*	28.45±7.13	10.45±1.75	nt	nt
DEF47	0.00±0.13	-	-	1.83±0.63	+	-	+	+	93.75±14.08	105.00±17.13*	108.75±10.88*	25.38±8.31	10.04±2.94	312.13±23.32	53.04±11.69
DEF48	1.70±0.02	-	-	7.33±1.89	+	-	+	+	100.00±0.00	133.90±40.73*	109.60±22.38*	23.13±4.75	11.70±2.60	311.55±24.61	48.84±9.65

Underlined are the strains that were not originally isolated as endophytes. * Indicates significant difference ($P < 0.05$) from the control assessed with Dunn's test and Bonferroni correction for multiple comparison. nt Not tested. SD Standard Deviation. +: halo width 1 mm; -: not active. The growth at high salt concentration were compared to control plates (+ grown like control plates; - not grown).

Table 3 Screening tests for biocontrol activity against F. graminearum in vitro *and* in planta *(germination blotter and soil substrate assays).*

Treatment	Dual culture assay	Germination blotter assay			Soil substrate assay	
	Mean of growth inhibition (%)	FRR protection (%) per plant	FFR protection (%) per treatment	Infected shoot dried weight (mg) per plant	FFR protection (%) per treatment	Infected shoot dried weight (mg) per plant
Water control	-	-	-	10,69±3,15	-	75,14±16,47
DEF06	66.66±6.41	12,14±23,38	17.61	17,71±4,49*	nt	nt
DEF07	74.07±3.70^	38,20±21,41*^	24.24^	16,35±4,78*	61.28*	57,30±11,65*
DEF08	60.49±4.27	15,20±15,12	-45.45**	10,81±4,70	-1.12	64,47±13,80
DEF09	59.26±3.70^	42,66±15,21*^	80.86*^	13,89±3,63	46.38*	59,07±12,01*
DEF13	32.1±10.69^	7,87±19,35^	20.45^	11,88±4,90	nt	nt
DEF14	49.38±2.14^	6,57±19,96^	41.18*^	11,66±5,84	nt	nt
DEF15	37.25±3.92^	18,59±22,84^	62.41*^	11,29±2,42	nt	nt
DEF16	56.79±4.28^	38,45±27,18*^	43.61*^	9,61±2,23	nt	nt
DEF17	39.50±5.66	-7,22±13,51	28.57*	10,57±2,54	nt	nt
DEF18	61.73±2.14	18,48±24,57	15.97	7,49±3,01	nt	nt
DEF19	76.54±2.14^	46,74±17,43*^	25.93^	11,08±2,51	39.55	63,19±12,93

DEF20	77.78±0^	27,26±24,45*^	41.23^	11,23±2,02	10.64	59,58±12,42
DEF21	45.68±9.32	35,22±25,73*	12.55	11,86±2,08	nt	nt
DEF31	41.98±19	29,14±20,43*	-14.81	10,98±1,64	nt	nt
DEF33	55.56±0	7,67±21,09	17.36	11,29±1,84	nt	nt
DEF39	64.20±2.14^	39,77±15,00*^	43.75*^	13,40±3,13	-24.47	44,55±13,36*
DEF40	43.21±2.14	24,13±23,23	38.82	8,61±1,53	nt	nt
DEF41	60.49±4.27^	35,96±18,90*^	54.17*^	10,19±2,47	nt	nt
DEF46	39.50±8.55	21,07±25,69	45.00	8,90±2,33	nt	nt
DEF47	54.32±4.28^	24,79±23,31^	87.50*^	9,81±2,63	-7.23	54,93±14,24*
DEF48	70.37±3.70^	32,28±27,81*^	55.88*^	10,69±1,77	29.04	61,37±11,00

Underlined strains were not originally isolated as endophytes. ^ Experimental data obtained from Colombo et al., 2019. nt Not tested. FRR Fusarium root rot. FFR Fusarium foot rot

The average of mycelium growth inhibitions recorded in dual culture assay against *F. graminearum* strain Fg8/1 is reported. *In planta* results of germination blotter assay or using soil are displayed as FRR protection (*$P < 0.05$ is considered significant) and FFR protection (Fisher's test analysis: *$P < 0.01$ treatment considered significantly able to maintain the seedling asymptomatic, ** $P < 0.01$ treatment considered significantly able to increase disease severity). The average of infected shoot weight (mg) for each treatment assessed after 10 or 20 days after seed bacterization or transplant is also reported.

Biocontrol effects against FHB severity in growth chamber and field conditions

The strain DEF09 showed the best performance against both FRR and FFR diseases, with consistent results in all the assays. Being an endophyte obtained from wheat, its efficacy against FHB disease was first assessed in fully controlled environment (growth chamber). The strain, co-inoculated with the pathogenic strain PH1, stopped the spreading of the disease at the first infected spikelet in all plants (Figure 1). High level of protection (75%) was reached under controlled conditions (Supplementary file 8).

In order to assess whether the strain could be effective also in field conditions, where different biotic and abiotic interactions occur, a field trial was carried out on bread and durum wheat. The *P*-value of the Mann-Whitney test was 2.47e-05 for Bandera and 1.35e-08 for Claudio. The presence of DEF09 reduced the number of diseased spikelets in comparison to the untreated control (Figure 2), decreasing FHB severity up to 60% and 45% on cv. Bandera and Claudio, respectively (Supplementary file 9).

Figure 1 Example of Fusarium head blight symptoms on wheat spikes grown in growth chamber. The red arrow indicates the spikelet of infection. Examples of water inoculated control (A), Fusarium *inoculated control (B) and* Fusarium+*DEF09 treatment (C) are shown.*

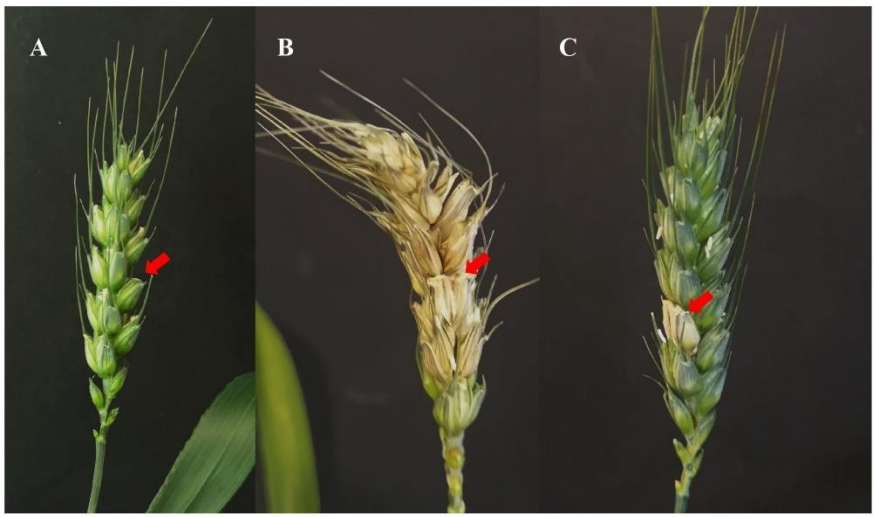
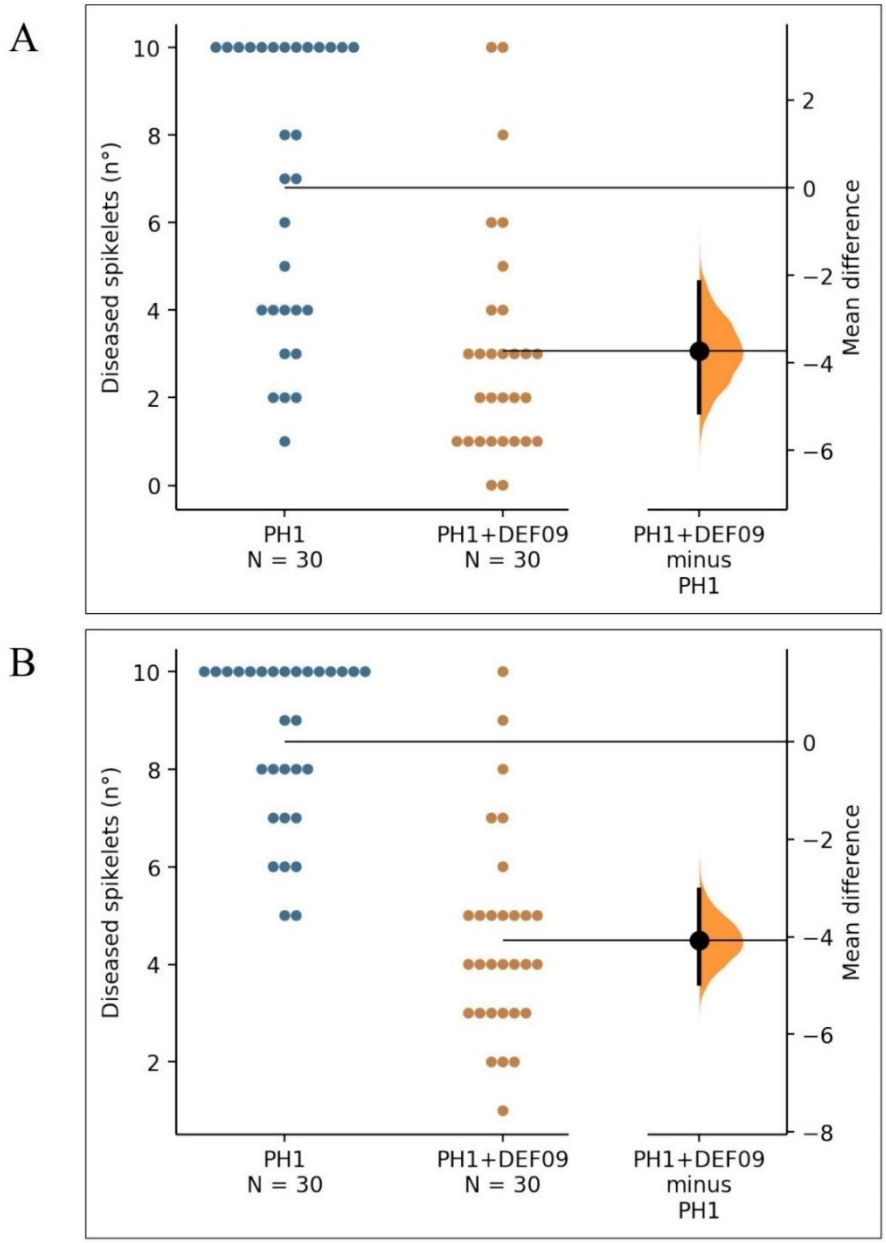


Figure 2 The mean difference between PH1 and PH1+DEF09 for diseased spikelets (n°) of cultivars "Bandera" (A) and "Claudio" (B) is shown in the Gardner-Altman estimation plot. The unpaired mean difference of data obtained between PH1 and PH1+DEF09 is -3.73 (95.0% CI -5.13, -2.17) and -4.07 (95.0% CI -4.97, -3.03) for cv. Bandera and cv. Claudio, respectively. Both groups are plotted on the left axes; the mean difference is plotted on floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot; the 95% confidence interval is indicated by the vertical error bar.



Discussion

Comprehensive observation of different parameters, including the physiological characteristics of *Streptomyces* strains and their interaction with the plant (Colombo et al., 2019), is essential for successful selection and characterization of bioactive strains able to adapt to complex environmental conditions and microbiomes (Winter et al., 2019).

In this study, *Streptomyces* strains were extensively characterized for their plant growth associated features, together with detailed examination of their activity on germinating wheat and on wheat infected with *F. graminearum.* The combination of *in vitro* and *in vivo* laboratory assays led to the identification of an effective strain, DEF09, which also showed promising results in field trials on both durum and bread wheat. The use of FRR and FFR pathosystems for selecting a strain effective against FHB proved successful. This study is in accordance with the observation by Wang et al. (2015b), who showed a good correlation between FFR and FHB biocontrol activities for a diverse set of bacterial strains. It also confirms functional analyses of genes from wheat-infecting *Fusarium* species. Different genes were reported to be equally involved in the pathogenic mechanisms of both FHB and FFR (Spanu et al., 2012, 2018; Pasquali et al., 2013). From a physiopathological point of view, *F. graminearum* shows a common infection process during both root- and head infection (Wang et al., 2015a, 2018). Our work therefore supports the idea that the use of FRR and FFR pathosystems, being more manageable laboratory models than the FHB pathosystem, is suitable for selection of BCA strains effective against FHB. In this work it was not possible to include strains previously selected as BCA in other scientific works, therefore, it is not possible to have a direct comparison of the activity of the strain DEF09 with other *Streptomyces* strains, given that results depend on the complex interactions occurring in the environment (Vurukonda et al., 2018). Nonetheless, based on the reported efficacy of the different microorganisms, the level of protection achieved by the strain DEF09 was comparable to that obtained in field trials using *Bacillus* sp. and *Cryptococcus* sp. (Schisler et al.,

2002) and slighty higher than those achieved with other *Streptomyces* strains in field trials on bread wheat (Palazzini et al., 2017, Jung et al., 2013) and durum wheat (Palazzini et al., 2018). It is plausible that the inoculation method may affect the level of protection. Interestingly, Jung et al. (2013) reported significant protection against FHB by the BN1 *Streptomyces* strain only when the strain was sprayed on spikes but not when it was co-inoculated. In our case, the high level of protection, comparable with fungicide treatments (Giraud et al., 2011), was obtained with co-inoculation. Other inoculation methods will need to be tested to better compare the level of protection obtained by DEF09 in different environmental conditions with that of previously studied strains. Novel approaches are also needed to explore the efficacy of the strains in large scale field trials.

The combination of the methods used to assess the bioactivity of the strains examined in our study allowed us to gain insight into their possible mechanisms of activity. For example, the *in vitro* assays carried out on DEF09 suggest that this strain blocks the growth of the fungus with specific antifungal molecules, as shown by the dual culture inhibition assay. Chitinase production has been identified as the main biocontrol mechanism in some studies (Herrera-Estrella and Chet 1999). DEF09 is a chitinase producer, but the lack of correlation ($r = 0.22$) between the chitinase production in different strains and the growth inhibition of *F. graminearum* indicates that chitin degradation may not be the unique factor responsible for the observed bioactivity of the strains. Likely, the inhibition of fungal growth might be the result of a synergistic effect of different lytic enzymes and metabolites (Zhang and Yuen, 2000; Zhao et al., 2013). DEF09 directly affects wheat plant growth, modifying root development by way of seminal root elongation, as seen in the germination blotter assay after 10 days, and impacting overall plant growth (shoot dried weight) after pathogen infection. Interestingly, morphological changes of roots have been associated with the induction of systemic resistance (Zamioudis et al., 2013). Moreover, DEF09 was among the best IAA producers in the pool. Indeed, IAA is known to play a role in plant

morphology as well as in disease modulation, stimulating plant defense (Pieterse et al., 2009) and therefore may contribute to protection against the pathogen. From all these data we may infer that DEF09 possesses multiple mechanisms leading to limitation of FHB on wheat. Metabolic profiling coupled with functional genomics of DEF09 will likely allow for the delineation of the mechanisms of action for the strain (Chen et al., 2018). A large set of potentially bioactive strains was identified in this study. Strains able to significantly interfere with pathogen development also transiently affected plant growth, suggesting that a complex set of molecules is produced during the tripartite interaction (Mayo-Prieto et al., 2019). Our future goal will be to identify the determinants of these specific interactions occurring among the BCA, the fungus, and the host, as detailed knowledge of their interaction, is essential for developing novel plant protection strategies (Berendsen et al., 2012).

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References

Anwar, S., Ali, B., Sajid, I. (2016). Screening of rhizospheric actinomycetes for various *in vitro* and *in vivo* plant growth promoting (PGP) traits and for agroactive compounds. *Front. Microbiol*. 7, 1–11. doi: 10.3389/fmicb.2016.01334

Bano, N., and Musarrat, J. (2003). Characterization of a new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. *Curr. Microbiol.* 46, 324–328. doi: 10.1007/s00284-002-3857-8

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science* 17, 478–486. doi:10.1016/j.tplants.2012.04.001.

Boenisch, M.J., and Schäfer, W. (2011). *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. *BMC Plant Biol.* 11:110. doi:10.1186/1471-2229-11-110.

Breakspear, A., Pasquali, M., Broz, K., Dong, Y., and Corby Kistler, H. (2011). Npc1 is involved in sterol trafficking in the filamentous fungus *Fusarium graminearum*. *Fungal Genet. Biol.* 48, 725–730. doi:10.1016/j.fgb.2011.03.001.

Burr, T. J., Matteson, M. C., Smith, C. A., Corral-Garcia, M. R., and Huang, T.-C. (1996). Effectiveness of bacteria and yeasts from apple orchards as biological control agents of apple scab. *Biol. Control* 6, 151–157. doi:10.1006/BCON.1996.0019.

Chakraborty, S., Liu, C.J., Mitter, V., Scott, J.B., Akinsanmi, O.A., Ali, S., et al. (2006). Pathogen population structure and epidemiology are keys to wheat crown rot and *Fusarium* head blight management. *Australasian Plant Pathology*, 35, 643-655. doi:10.1071/AP06068

Chen, Y., Wang, J., Yang, N., Wen, Z., Sun, X., Chai, Y., et al. (2018). Wheat microbiome bacteria can reduce virulence of a plant pathogenic fungus by altering histone acetylation. *Nature Communications* 9, 3429. doi:10.1038/s41467-018-05683-7.

Colombo, E. M., Pizzatti, C., Kunova, A., Gardana, C., Saracchi, M., Cortesi, P., Pasquali, M. (2019). Evaluation of in-vitro methods to select effective streptomycetes against toxigenic fusaria. *PeerJ* 7, e6905. doi:10.7717/peerj.6905.

Coombs, J. T., and Franco, C. M. M. (2003). Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. *Appl. Environ. Microbiol.* 58, 2691–2693. doi:10.1128/aem.67.10.4414-4425.2001

Covarelli, L., Beccari, G., Steed, A., and Nicholson, P. (2012). Colonization of soft wheat following infection of the stem base by *Fusarium culmorum* and translocation of deoxynivalenol to the head. *Plant Pathology*, 61, 1121-1129. doi:10.1111/j.1365-3059.2012.02600.x.

Covarelli, L., Gardiner, D., Beccari, G., Nicholson, P. (2013). *Fusarium* virulence assay on wheat and barley seedlings. *Bio-protocol* 3:e446. doi:10.21769/BioProtoc.446

Dinno A. (2017). dunn.test: Dunn's Test of multiple comparisons using rank sums. R package version 1.3.5. https://CRAN.R-project.org/package=dunn.test

Giraud, F., Pasquali, M., Jarroudi, M. El, Cocco, M., Delfosse, P., Hoffmann, L., and Bohn T. (2011). Timely fungicide application: a strategy to minimize fusarium head blight and associated mycotoxin production in winter wheat. *J. Plant Pathol.* 93, S15-S18. doi:10.2307/41998924.

Goswami, R.S., Kistler, H.C. (2004). Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol*. 5, 515–525. doi: 10.1111/j.1364-3703.2004.00252.x

Herrera-Estrella, A., and Chet, I. (1999). Chitinases in biological control. *EXS-BASEL* 87, 171–184.

Ho, J., Tumkaya, T., Aryal, S., Choi, H., and Claridge-Chang, A. (2018). Moving beyond P values: everyday data analysis with estimation plots. *Nature Methods* 1548-7105. doi: 10.1038/s41592-019-0470-3

Ilgen, P., Hadeler, B., Maier, F. J., and Schäfer, W. (2009). Developing kernel and rachis node induce the trichothecene pathway of *Fusarium graminearum* during wheat head infection. *Mol. Plant-Microbe Interact.* 22, 899–908. doi:10.1094/MPMI-22-8-0899.

Jones, K. L. (1949). Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. *J. Bact.* 57*,* 141.

Jung, B., Park, S. Y., Lee, Y. W., and Lee, J. (2013). Biological efficacy of *Streptomyces* sp. strain BN1 against the cereal head blight pathogen *Fusarium graminearum*. *Plant Pathol. J.* 29, 52–58. doi:10.5423/PPJ.OA.07.2012.0113.

Kuddus, M., and Ahmad, I.Z. (2013). Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. *J. Gen. Eng. Biotechnol.* 11, 39-46. doi: 10.1016/j.jgeb.2013.03.001

Lysøe, E., Seong, K.-Y., and Kistler, H. C. (2011). The transcriptome of *Fusarium graminearum* during the infection of wheat. *Mol. Plant-Microbe Interact.* 24, 995–1000. doi:10.1094/MPMI-02-11-0038.

Mayo-Prieto, S., Marra, R., Vinale, F., Rodríguez-González, Á., Woo, S. L., Lorito, M., et al. (2019). Effect of *Trichoderma velutinum* and *Rhizoctonia solani* on the metabolome of bean plants (*Phaseolus vulgaris* L.). *Int J Mol Sci* 20. doi:10.3390/ijms20030549.

Milus, E. A., and Rothrock, C. S. (1997). Efficacy of bacterial seed treatments for controlling *Pythium* root rot of winter wheat. *Plant Dis.* 81, 180–184. doi:10.1094/PDIS.1997.81.2.180.

Nautiyal Shekhar C. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett*. 170, 265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x

Newitt, J.T., Prudence, S.M. M., Hutchings, M.I., and Worsley, S.F., (2019). Biocontrol of cereal crop diseases using streptomycetes. *Pathogens* 8, 78. doi:10.3390/pathogens8020078.

Nourozian, J., Etebarian, H. R., and Khodakaramian, G. (2006). Biological control of *Fusarium graminearum* on wheat by antagonistic bacteria. *Songklanakarin J. Sci. Technol.* 28, 29–38.

Palazzini, J. M., Ramirez, M. L., Torres, A. M., and Chulze, S. N. (2007). Potential biocontrol agents for *Fusarium* head blight and deoxynivalenol production in wheat. *Crop Prot.* 26, 1702–1710. doi:10.1016/j.cropro.2007.03.004.

Palazzini, J.M., Yerkovich, N., Alberione, E., Chiotta, M., and Chulze, S.N. (2017). An integrated dual strategy to control *Fusarium graminearum sensu stricto* by the biocontrol agent *Streptomyces* sp. RC 87B under field conditions. *Plant Gene* 9, 13–18. doi:10.1016/j.plgene.2016.11.005.

Palazzini, J., Roncallo, P., Cantoro, R., Chiotta, M., Yerkovich, N., Palacios, S., et al. (2018). Biocontrol of *Fusarium graminearum sensu stricto*, reduction of deoxynivalenol accumulation and phytohormone induction by two selected antagonists. *Toxins (Basel)* 10. doi:10.3390/toxins10020088.

Pasquali, M., Spanu, F., Scherm, B., Balmas, V., Hoffmann, L., Hammond-Kosack, K. E., et al. (2013). FcStuA from *Fusarium culmorum* controls wheat foot and root rot in a toxin dispensable manner. *PLoS ONE* 8, e57429. doi:10.1371/journal.pone.0057429.

Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L. S., and Fernández, F. J. (2007). O-CAS, a fast and universal method for siderophore detection. *J. Microbiol. Methods* 70, 127–131. doi:10.1016/j.mimet.2007.03.023.

Pieterse, C.M., Leon-Reyes, A., Van der Ent, S., Van Wees, S.C. (2009). Networking by small-molecule hormones in plant immunity. *Nature chemical biology*. 5, 308-316. doi:10.1038/nchembio.164.

R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at https://www.R-project.org/ (Accessed February 2019)

Sardi, P., Saracchi, M., Quaroni, S., Petrolini, B., Borgonovi, G. E., and Merli, S. (1992). Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. *App. Environ. Microbiol.* 58, 2691-2693.

Scherm, B., Balmas, V., Spanu, F., Pani, G., Delogu, G., Pasquali, M., and Migheli, Q. (2013). *Fusarium culmorum*: causal agent of foot and root rot and head blight on wheat. *Mol. Plant Pathol*. 14, 323–341. doi: 10.1111/mpp.12011.

Schisler, D. A., Khan, N. I., Boehm, M. J., and Slininger, P. J. (2002). Greenhouse and field evaluation of biological control of *Fusarium* head blight on durum wheat. *Plant Disease* 86, 1350–1356. doi:10.1094/PDIS.2002.86.12.1350.

Seipke, R. F., Kaltenpoth, M., and Hutchings, M. I. (2012). *Streptomyces* as symbionts: an emerging and widespread theme? *FEMS Microbiol. Rev.* 36, 862–876. doi:10.1111/j.1574-6976.2011.00313.x

Seong, K.-Y., Pasquali, M., Zhou, X., Song, J., Hilburn, K., McCormick, S., et al. (2009). Global gene regulation by *Fusarium* transcription factors *Tri6* and *Tri10* reveals adaptations for toxin biosynthesis. *Mol. Microbiol.* 72, 354–367. doi:10.1111/j.1365-2958.2009.06649.x.

Shirling, E. T., and Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bact.*, 16, 313-340.

Simpson, D.R., Rezanoor, H.N., Parry, D.W., and Nicholson, P. (2000). Evidence for differential host preference in *Microdochium nivale* var. *majus* and *Microdochium nivale* var. *nivale*. *Plant Pathol.* 49, 261-268. doi:10.1046/j.1365-3059.2000.00453.x

Smiley, R. W., and Patterson, L. M. (1996). Pathogenic fungi associated with *Fusarium* foot rot of winter wheat in the semiarid Pacific Northwest. *Plant Disease* 80, 944–949. doi:10.1094/PD-80-0944.

Spanu, F., Pasquali, M., Scherm, B., Balmas, V., Marcello, A., Ortu, G., et al. (2012). Transposition of the miniature inverted-repeat transposable element mimp1 in the wheat pathogen *Fusarium culmorum*. *Mol. Plant Pathol.* 13, 1149-1155. doi:10.1111/J.1364-3703.2012.00823.X.

Spanu, F., Scherm, B., Camboni, I., Balmas, V., Pani, G., Oufensou, S., et al. (2018). FcRav2, a gene with a ROGDI domain involved in *Fusarium* head blight and crown rot on durum wheat caused by *Fusarium culmorum*. *Mol. Plant Pathol. Molecular Plant Pathology* 19, 677–688. doi:10.1111/mpp.12551.

Stack, R.W., and McMullen, M.P. (1998). A visual scale to estimate severity of *Fusarium* head blight in wheat. *N.D. State Univ. Ext. Serv. Bull.* 1095.

Sun, Z., Huang, Y., Wang, Y., Zhao, Y., Cui, Z. (2014). Potassium hydroxide-ethylene diamine tetraacetic acid method for the rapid preparation of small-scale PCR template DNA from actinobacteria. *Mol. Genet. Microbiol. Virol*. 29, 42-46. doi:10.3103/S089141681401008X.

Turner, S., Pryer, K. M., Miao, V. P., and Palmer, J. D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J. Eukaryot. Microbiol*. 46, 327–338.

Viaene, T., Langendries, S., Beirinckx, S., Maes, M., and Goormachtig, S. (2016). *Streptomyces* as a plant's best friend? *FEMS Microbiol. Ecol*. 92:8 doi:10.1093/femsec/fiw119.

Vurukonda, S. S. K. P., Giovanardi, D., and Stefani, E. (2018). Plant growth promoting and biocontrol activity of *Streptomyces* spp. as endophytes. *Int. J. Mol. Sci.* 19, 952. doi:10.3390/ijms19040952.

Wang, Q., Vera Buxa, S., Furch, A., Friedt, W., and Gottwald, S. (2015a). Insights into *Triticum aestivum* seedling root rot caused by *Fusarium graminearum*. *Mol. Plant-Microbe Interact.* 28, 1288–1303. doi:10.1094/MPMI-07-15-0144-R.

Wang, L.-Y., Xie, Y.-S., Cui, Y.-Y., Xu, J., He, W., Chen, H.-G., et al. (2015b). Conjunctively screening of biocontrol agents (BCAs) against *Fusarium* root rot and *Fusarium* head blight caused by *Fusarium graminearum*. *Microbiological Research* 177, 34–42. doi:10.1016/j.micres.2015.05.005.

Wang, Q., Shao, B., Shaikh, F. I., Friedt, W., and Gottwald, S. (2018). Wheat resistances to *Fusarium* root rot and head blight are both associated with deoxynivalenol- and jasmonate-related gene expression. *Phytopathology* 108, 602–616. doi:10.1094/PHYTO-05-17-0172-R.

Watson, A., Ghosh, S., Williams, M. J., Cuddy, W. S., Simmonds, J., Rey, M.-D., et al. (2018). Speed breeding is a powerful tool to accelerate crop research and breeding. *Nat. Plants* 4, 23–29. doi:10.1038/s41477-017-0083-8.

Watve, M., Tickoo, R., Jog, M., and Bhole, B. (2001). How many antibiotics are produced by the genus *Streptomyces* ? *Arch. Microbiol.* 176, 386–390. doi:10.1007/s002030100345.

Wegulo, S. N., Baenziger, P. S., Hernandez Nopsa, J., Bockus, W. W., and Hallen-Adams, H. (2015). Management of *Fusarium* head blight of wheat and barley. *Crop Prot.* 73, 100–107. doi:10.1016/J.CROPRO.2015.02.025.

Winter, M., Samuels, P.L., Otto-Hanson, L.K., Dill-Macky, R., and Kinkel, L. (2019). Biocontrol of *Fusarium* crown and root rot of wheat by *Streptomyces* isolates–it's complicated. *Phytobiomes*, 3, 52-60. doi:10.1094/PBIOMES-11-18-0052-R

Yekkour, A., Sabaou, N., Zitouni, A., Errakhi, R., Mathieu, F., and Lebrihi, A. (2012). Characterization and antagonistic properties of *Streptomyces* strains isolated from Saharan soils, and evaluation of their ability to control seedling blight of barley caused by *Fusarium culmorum*. *Lett. Appl. Microbiol.* 55, 427–435. doi:10.1111/j.1472-765x.2012.03312.x.

Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA and whole genome assemblies. *Int. J. Syst. Evol. Microbiol.* 67, 1613-1617. doi: 10.1099/ijsem.0.001755

Zamioudis, C., Mastranesti, P., Dhonukshe, P., Blilou, I., Pieterse, C. M. J., and Genetics, M. (2013). Unraveling root developmental programs initiated by beneficial *Pseudomonas* spp. Bacteria *Plant Physiol.* 162, 304-318. doi:10.1104/pp.112.212597.

Zhang, Z., and Yuen, G.Y. (2000). The role of chitinase production by *Stenotrophomonas maltophilia* strain C3 in biological control of *Bipolaris sorokiniana*. *Phytopathology.* 90, 384–389. doi:10.1094/PHYTO.2000.90.4.384.

Zhao, J., Xue, Q.H., Niu, G.G., Xue, L., Shen, G.H., and Du, J.Z. (2013). Extracellular enzyme production and fungal mycelia degradation of antagonistic *Streptomyces* induced by fungal mycelia preparation of cucurbit plant pathogens. *Ann. Microbiol*. 63: 809–812. doi: 10.1007/s13213-012-0507-7.

Fusarium, *Streptomyces* and wheat grains: studying the interaction and the effect of inoculation timing on deoxynivalenol accumulation *in vitro*

Abstract

Streptomyces spp. have been exploited as biocontrol agents (BCAs) against plant pathogens for their ability to produce different bioactive compounds. They can be used against *Fusarium graminearum,* the main causal agent of Fusarium head blight (FHB) as well as against the contamination of grains with deoxynivalenol (DON). In the present research, the effect of four *Streptomyces* strains on fungal growth and mycotoxin production in microsilage conditions has been evaluated, testing the efficacy of two inoculation timings of the selected BCAs. In addition, the impact of the pathogen presence on the BCAs development was assessed. Quantitative real-time PCR detection of the two targets (*Fusarium* and *Streptomyces* spp.) and chemical extraction and quantification of DON and ergosterol have been used for these purposes. The results indicate that the highest level of DON inhibition (99%) as well as a strong reduction of fungal biomass can be achieved following the simultaneous inoculation of BCA and pathogen. This research enabled studying the biocontrol efficacy of the tested *Streptomyces* strains and monitoring their development in microsilage conditions. The diversity of effects manifested by the different *Streptomyces* strains confirms the importance of studying the interactions between the grains, pathogen, and BCA.

Introduction

Fusarium head blight (FHB) is one of the most devastating cereal diseases, especially for wheat and barley. It is caused by a complex group of *Fusarium* spp., in which *Fusarium graminearum* is the main causal agent (Osborne and Stein, 2007; Pasquali et al., 2016a). Severe yield losses occur in the field, together with a variable level of grain contamination with mycotoxins

belonging to the group of type B trichothecenes, such as deoxynivalenol (DON) (Salgado et al., 2015). The fungal contamination in harvested grains can be kept under control during the storage period, but the mycotoxin incidence in feed and food products often increases dramatically, becoming a threat for food and feed safety (Beattie et al., 1998; Yuan et al., 2018). The risks to human and animal health have forced organizations worldwide to establish standards for maximum allowable levels in products for human consumption (Lee and Ryu, 2017). The toxic effects of DON include the alteration of intestinal, nervous, and immune systems due to the inhibition of protein synthesis and induction of apoptosis (Maresca, 2013).

Several control strategies to manage FHB incidence and consequent DON contamination in harvested grains have been exploited in field, such as the development of resistant varieties, application of fungicides, and crop rotation (Miedaner et al., 2017). Nevertheless, some agricultural practices can promote *Fusarium* development or enhance mycotoxin accumulation under favorable environmental conditions (Vogelgsang et al., 2019). For this reason, research on biocontrol agents (BCAs) has been receiving increased attention as integrative approaches to manage FHB are urgently needed (Gilbert and Haber, 2013). Biological control is an environmentally friendly approach to fight plant pathogens using microbial antagonists. The complex epidemiology of FHB results in the possibility of applying antagonists to seeds, crop residues, or to the spikes (Legrand et al., 2017), as well as during post-harvest (Magan et al., 2010).

Streptomyces spp. are Gram-positive bacteria belonging to the phylum Actinobacteria which are ubiquitous in soil and commonly exploited for antibiotic production in human and veterinary medicine. They grow through a combination of tip extension and branching of hyphae, forming a vegetative mycelium. Later, in response to nutrient depletion and other signals, they form an aerial mycelium carrying spores (Elliot et al., 2008; Flärdh and Buttner, 2009). In correspondence with these morphological changes, they produce a variety of secondary metabolites active against possible competitors present

in their niche (Chater et al., 2010). Recently, *Streptomyces* have also been found to be established in symbiotic interactions with plants and other eukaryotes (Seipke et al., 2012). Therefore, they have the potential to become key players for developing novel strategies against plant pathogens and to limit toxin contamination thanks to the discovery of promising metabolites for crop protection (Rey and Dumas, 2017). Indeed, recent studies confirmed their ability to reduce toxigenic *Fusarium* spp. growth and disease severity (Jung et al., 2013; Nourozian et al., 2006; Winter et al., 2019), as well as DON production *in vitro* and *in planta* (Palazzini et al., 2007, 2017). Two commercial products based on live *Streptomyces* spp. are available on the market (Mycostop® and Actinovate®). However, none of them was specifically registered for FHB management (Newitt et al., 2019).

The lack of appropriate screening procedures and studies on product formulation as well as complex procedures for microbial antagonist registration still represent the major reasons for the low number of biocontrol agents available on the market (Fravel, 2005).

In an effort to select novel *Streptomyces* strains able to counteract fungal and mycotoxin contamination in a wheat–*Fusarium* spp. pathosystem, their survival on wheat grains and the potential inhibitory effect of co-culture with the pathogen needs to be assessed.

Ergosterol quantification is routinely used to determine the microbiological status of grains and feeds (Ng et al., 2008; Rao et al., 1989; Tangni and Pussemier, 2006) and can be exploited to evaluate the effect of a fungicide or a natural product on fungal pathogen growth (da Silva Bomfim et al., 2015; Shah et al., 2015). This fungal marker can also be used to normalize mycotoxin levels based on fungal development (Bluhm and Woloshuk, 2005; Zhao et al., 2014). Nevertheless, in recent years, quantitative real-time PCR (qPCR) has been recognized as a rapid and highly sensitive technique to accurately quantify fungal biomass in a wide range of food and grain samples, a parameter which can be easily correlated with the level of disease observed in field as well as mycotoxin contamination (Bilska et al., 2018; Zhang et al.,

2009). Moreover, as qPCR is able to detect specific strains, it has been increasingly used for biocontrol agent monitoring in the target substrate (Gimeno et al., 2019), enabling the assessment of BCA survival under specific abiotic and biotic conditions as well as how the time and application method influence this survival (Sanzani et al., 2014).

In order to study the mechanism of action of four promising *Streptomyces* strains (Colombo et al., 2019a, 2019b) against *F. graminearum* growth and toxin production (DON) in vitro, specific qPCR methods to monitor fungal and BCA strains were developed. These analyses were combined with ergosterol quantification to confirm the qPCR-based quantification and to normalize DON content in flour samples.

The goals of the paper were to test whether (1) fungal growth is affected by the tested *Streptomyces* strains; (2) toxin production is affected by the tested *Streptomyces* strains; (3) BCA development is affected by pathogen presence; and (4) the timing of BCA inoculation is an important determinant of their efficacy in microsilage conditions.

Materials and methods

Microorganisms

The four *Streptomyces* strains (DEF09, DEF20, DEF39, and DEF48) used in this work were part of a collection of isolates maintained in the laboratory of Plant Pathology at the Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Italy. They were originally isolated from the inner root tissues of graminaceous plants (Sardi et al., 1992): DEF09 from wheat, DEF20 from *Carex* sp., DEF39 from rye, and DEF48 from corn (Colombo et al., 2019a). These strains showed promising biocontrol features *in vitro* and *in planta* against *F. graminearum* in previous works, being able to limit the growth of various *Fusarium* strains in dual culture by over 40% (Colombo et al., 2019a, 2019b). They were grown on Czapek yeast extract medium (CZY: 35 g/L Czapek dox broth, Difco Laboratories, USA; 2 g/L yeast

extract, Difco Laboratories, USA; 15 g/L agar; Amresco, USA) for 14 days at 24 °C. Spores were collected by adding 5 mL of 10% sterile glycerol (ICN Biomedicals, USA) + 0.01% Tween 20 solution (Sigma-Aldrich, USA) to the plate and scraping the surface of the colonies with a sterile loop. The concentration was determined using a hemocytometer and adjusted to 2×10^7 spores/mL. Small aliquots were then stored at −20 °C.

The toxigenic *Fusarium* strain used in this study was *F. graminearum* CS3005 (Gardiner et al., 2014). The strain was grown on V8 medium (200 mL/L V8 juice, Campbell's, USA; 2 g/L $CaCO_3$, Sigma-Aldrich, USA; 15 g/L Agar, Amresco, USA) at 24 °C.

BCA treatments on wheat grains

Wheat grains of *Triticum aestivum* cv. Bandera (20 g) were placed in 100 mL flasks, soaked with 20 mL of deionized water, and autoclaved for 20 min at 120 °C. The treatments consisted of four *Streptomyces* strains, one type of fungus inoculation (*F. graminearum* CS3005), and two different times of antagonist inoculation.

Grains were treated with 500 µL of *Streptomyces* strain spore suspension (2×10^7 spore/mL) and six agar–mycelium plugs (6 mm in diameter) taken from a five-day-old *Fusarium* colony grown on V8 medium. BCAs were applied as follows: 0 DPI (*Streptomyces* spp. treatments were carried out at the same time as pathogen inoculation); 3 DPI (*Streptomyces* spp. treatments were performed 3 days post-pathogen inoculation). Each combination of treatments was performed in quadruplicate. Three controls were included. Blank samples (grain without any treatment) only inoculated with 500 µL of 10% sterile glycerol were prepared to define the background levels for each quantification, the *Streptomcyes*-control, in which 500 µL of *Streptomyces* spore suspension (2×10^7 spore/mL) was inoculated, and the *Fusarium*-control, in which six agar-mycelium plugs of *F. graminearum* CS3005 were added to the sterilized grains. The incubation was performed at 24 °C for 11 days in the dark. Flasks were monitored and shaken daily. Wheat seeds were lyophilized (model Heto-

EPD3, Thermo Scientific, San Jose, CA) for 24 h and ground to a fine powder. Samples were kept at −80 °C for subsequent extraction.

Chemicals

Standards of ergosterol and mycotoxins (DON and 3ADON) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents were purchased from Sigma-Aldrich unless otherwise (St. Louis, MO, USA). Water was supplied by a Milli-Q apparatus (Millipore, Milford, MA).

Ergosterol extraction and determination by liquid chromatography-diode array detection

Ergosterol extraction was performed following the procedure described in a previous research (Bluhm and Woloshuk, 2005). Briefly, samples were prepared as follows: 400 mg of flour was weighed and extracted overnight using 10 mL of a $CHCl_3$/MeOH (2:1 (v/v)) solution. After centrifugation at 9000 rpm for 10 min, the supernatant was collected, and the pellet was extracted again with 5 mL of the same solvent solution. The two extracts were combined, and the volume was made up to 20 mL using $CHCl_3$/MeOH (2:1 (v/v)). The resulting solution was diluted 1:2 prior to injection and chromatographic analysis. Ergosterol levels were used to normalize DON content per fungal mass.

The used HPLC system was an Alliance 2695 (Waters, Milford, MA, USA) equipped with a model 2998 photodiode array detector (Waters). A 5 µm Hypersyl C_{18} column (250 mm × 4.6 mm, Thermo Scientific, San Jose, CA) maintained at 30 °C carried out the separation in isocratic mode. The flow rate was 1.0 mL/min, and the eluent was methanol. Samples were maintained at 20 °C. Chromatographic data were acquired from 195 to 350 nm and integrated at 282 nm. Ergosterol stock solution (0.26 mg/mL) was prepared in MeOH and stored at −20 °C. Working solutions (n = 7) were prepared in the range of 1.3–130 µg/mL, and 50 µL was injected into the chromatographic system. Each analysis was carried out in duplicate.

DON extraction and determination

To determine the amount of mycotoxins, the flour (1 g) was extracted with 10 mL of a water/CH_3CN (20:80 (v/v)) solution under sonication for 30 min. Then, the mixture was centrifuged at 1600*g* for 10 min, and the supernatant was transferred into a 10 mL flask where the volumes were adjusted using a water/CH_3CN (20:80 (v/v)) solution. The residues were extracted again as described above, and the two extracts were analyzed separately. Mycotoxin determination was carried out using a UHPLC model Acquity (Waters) coupled with an HR Fourier transform Orbitrap mass spectrometer (model Exactive, Thermo Scientific, San Jose, CA), equipped with a HESI-II probe for ESI (electrospray ionization) and a collision cell (HCD). A Hypersil Gold C_{18} column (100 mm × 2.1 mm, 1.9 µm, Thermo Scientific, San Jose, CA) was used for the separation. The MS data were processed using Xcalibur software (Thermo Scientific, San Jose, CA). The used operative conditions as well as the elution gradient have been previously described (Colombo et al., 2019a).

DNA extraction

The total DNA from flour samples was extracted using a DNA extraction kit (DNeasy *mericon* Food Kit, Qiagen, Germany). Briefly, 100 mg of the sample was weighed and processed following the manufacturer's instructions with one minor modification whereby 5 µL of 20 mg/mL RNase A (Invitrogen, Fisher Scientific, USA) was added during the first incubation step at 60 °C. Quantification and verification of the 260/280 ratio of the extracted DNA was carried out with a Take3 Micro-Volume plate in a microplate reader (Synergy H1, Biotek, USA). Spectrophotometric quantification was confirmed by fluorometric quantification. DNA samples were stored in Elution buffer (EB) (Qiagen, Germany) supplied by the DNA extraction kit at 4 °C.

Primers

Details of the primers used in this study are listed in Table 1. In order to obtain *recA* gene sequences of *Streptomyces* strains involved in this study, DNA was extracted as described in (Sun et al., 2014) and amplified using primers

*recA*PF and *recA*PR (Guo et al., 2008). Briefly, PCR was performed in a total volume of 25 µL which contained 0.25 µL of GoTaq® DNA Polymerase 5 U/ µL (Promega, Madison, WI, USA), 5 µL of Green GoTaq® Reaction Buffer 5× (Promega, Madison, WI, USA), 1 µL of 10 mM dNTP (Promega, Madison, WI, USA), 1 µL of 10 mM forward primer, 1 µL of 10 mM reverse primer, and 1 µL of template DNA in nuclease-free water. The reaction conditions were initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s. A final extension was performed at 72 °C for 7 min. Reaction products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized under UV light. The PCR products were sequenced (Eurofins Genomics, Germany) using *recA*F primer (Guo et al., 2008). Specific primers were designed for *Streptomyces* quantification based on *recA* gene polymorphisms. Sequences of the *recA* gene of DEF09, DEF20, DEF39, and DEF48 (NCBI accession numbers MN207071- MN207074) were aligned using Geneious version R11.1.4 (Figure S1). Primers for *F. graminearum* quantification were designed based on *TRI12* gene sequence (NC_026475). In both cases, primer specificity was tested using NCBI Primer-BLAST with default parameters against the nr database to assess the level of specificity and identify potential mistargets. Bioinformatic analysis confirmed the specificity of the designed primers for *Streptomyces* spp. *recA* (File S1). Similarly, a novel primer set was designed based on a specific region of *TRI12* which proved specific for *Fusarium* spp. (File S2).

Hor1f and Hor2r primers (Nicolaisen et al., 2009), based on the plant *EF1α* gene, were used as an internal control for each sample and to normalize fungal and bacterial DNA quantities. *Streptomyces* spp. and *F. graminearum* quantities were calculated as the copy number of target DNA/copy number of wheat DNA using the formula described in File S3 (Pasquali et al., 2006).

Table 1. Primers used in the study for the amplification and sequencing of the recA *gene together with those used to quantify the three targets (*Streptomyces *spp.,* F. graminearum*, and wheat).*

Primer name	Assay target	Primer sequence	Melting temp.	GC %	Reference
***recA*PF**	*Streptomyces* spp.	CCGCRCTCGCACAGAT TGAACGSCAATTC	70.2	56.9	(Guo et al., 2008)
***recA*PR**	*Streptomyces* spp.	GCSAGGTCGGGGTTG TCCTTSAGGAAGTTGC G	74.6	56.9	(Guo et al., 2008)
***recA*F**	*Streptomyces* spp.	ACAGATTGAACGGCAA TTCG	55.3	45	(Guo et al., 2008)
***recA*R**	*Streptomyces* spp.	ACCTTGTTCTTGACCA CCTT	55.3	45	(Guo et al., 2008)
qstrepto REcAF	*Streptomyces* spp.	AAGATCACCAGTGCGC TCAA	59.96	50	This study
qstrepto REcAR	*Streptomyces* spp.	GAGCTGGTTGATGAAG ATCGC	59.40	52	This study
TRI12QF	*F. graminearum*	ATCTCAGCCAGACGAC AGGT	59.87	55	This study
TRI12DR	*F. graminearum*	CGAGGCGAGGTGTAAT ATCC	59.55	55	This study
Hor1f	Wheat	TCTCTGGGTTTGAGGG TGAC	62	55	(Nicolaisen et al., 2009)
Hor2r	Wheat	GGCCCTTGTACCAGTC AAGGT	51	57	(Nicolaisen et al., 2009)

qPCR analysis

Quantitative real-time PCR (qPCR) was performed in order to evaluate the amount of *Fusarium*, *Streptomyces*, and wheat DNA in milled grain samples following The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Prior to experimentation, the primers, PCR protocol specifications, and thermocycling parameters were adapted to the available reaction mixes and laboratory devices. All sample dilutions were prepared simultaneously using DEPC-

treated and nuclease-free water (Fischer Scientific, USA), and stored dilutions were kept at 4 °C during the week of the experiments. Primers were stored in aliquots at −20 °C. All samples and primers were stored using only low adsorption tubes (Eppendorf, Germany).

In addition, the possible interference of wheat DNA in *Streptomyces* and *Fusarium* quantification was checked adding 5 ng of wheat DNA extracted from untreated samples as previously described. Three replicates of each dilution (5, 0.5, 0.05, 0.005, and 0.0005 ng) of fungal and bacterial DNA were prepared and used to build the respective standard curves. Two non-template controls (NTCs) were used: water-only and background DNA-only samples. The reaction efficiency and determination coefficient (R^2) were calculated based on the obtained Cq values. As there was no influence on Cq values due to the presence of wheat DNA (data not shown), all the experiments were carried out with standard curves that were freshly prepared with diethylpyrocarbonate (DEPC)-treated and nuclease-free water (Fischer Scientific, USA).

The qPCR reactions were carried out using an Applied Biosystems QuantStudio 3 PCR Systems (Thermo Fisher Scientific, USA) in standard mode. The amplification mix consisted of 2× PowerSYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, USA), 0.3 µM of each primer, 5 µL template DNA (1 ng/µL) in DEPC-treated and nuclease-free water (Fischer Scientific, USA) in a total volume of 20 µL. The adopted amplification protocol was 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles with 15 s at 95 °C for denaturation and 60 s at 60 °C for primer annealing, extension, and data collection. After the amplification reaction, a melting curve analysis was used to determine the specificity of the amplification products by incubating them for 15 s at 95 °C and 60 s at 60 °C and then reading the fluorescence at 0.15 °C increments from 60 to 95 °C. Primer specificity was verified by the presence of a single peak in the melting curve. Analysis of each DNA target (*Fusarium*, *Streptomyces*, or wheat) was conducted in 96-well optical reaction plates (Applied Biosystems, Thermo Fisher Scientific, USA)

covered with Microamp optical adhesive film (Applied Biosystems, Thermo Fisher Scientific, USA), and included a standard curve for quantification that was prepared for each pair of primers with *F. graminearum* PH1 genomic DNA (Cuomo et al., 2007), *Streptomyces* spp. DEF09 genomic DNA, and wheat genomic DNA. The first concentration of the standard curve was 10 ng, then six dilutions 1:10 (5 ng to 0.00005 ng) were selected based on previous experiments. Three replicates were performed to check the linearity of the assay and efficiencies were calculated for each plate. Each sample was also amplified in triplicate and NTCs were added to each plate in triplicate.

Cq values were obtained by using QuantStudio™ Design and Analysis Software version 1.5.0 (Thermo Fisher Scientific, USA) and exporting the amplification results into Excel format. The threshold for the Cq analysis was manually adjusted for each amplified target. Outliers among the three technical replicates were discarded if the standard error of Cq was higher than 0.2. PCR efficiency was calculated from the standard curves using the formula based on regression slopes, where the efficiency (E) of the different assays was $E = 10^{-1/slope}$. Normalization of the curves was obtained using the standard curves obtained for each plate as calibrator. All the amplification results and calculations are available in File S3.

The amount (ng) of fungal, bacterial, and plant DNA was calculated using the adjusted Cq and the selected standard curve. After the transformation of the DNA amount in copy numbers, *Streptomyces* and *Fusarium* values were normalized with the copy number of wheat DNA for each sample.

Data analysis

All the statistical analyses were performed using R software, version R 3.5.3 (R Core Team, 2019), unless otherwise stated. $P < 0.05$ was considered significant.

The whole study was divided into multiple experiments in order to manage the analysis. Therefore, each set of experiment required separate control replicates (no-BCA treatment).

The efficacy of the bacterial antagonist treatment against fungal growth and DON production was assessed as the difference between controls and treated samples. Therefore, ergosterol quantification data, *Fusarium* copy number (expressed as *Fusarium* copy number/wheat copy number), and DON level (expressed as µg DON/mg ergosterol) were subjected to ANOVA followed by a Tukey HSD post hoc test for multiple comparison.

In addition, the ratio between fungal quantity in control samples and treated samples was evaluated for ergosterol and qPCR data. In particular, from the qPCR data for each sample, the average of the ng of fungal DNA in control samples was divided by the ng of fungal DNA in the treated samples. Ergosterol ratios were calculated as the average of the µg of ergosterol in control treatments divided by the µg of ergosterol in the treated samples. The correlation between the ergosterol and qPCR ratios was then estimated.

In order to understand whether *Streptomyces* strains developed in the same way with treatment at 0 and 3 DPI, the *Streptomyces* copy number data (expressed as *Streptomyces* copy number/wheat copy number) from qPCR were submitted to ANOVA, followed by a Tukey HSD post hoc test for multiple comparison. In addition, the growth of *Streptomyces* strains at 0 DPI treatment was evaluated, comparing *Streptomyces* copy number in control samples (no-*Fusarium* spp.) and those obtained from co-cultured samples (*Fusarium* spp. + *Streptomyces* spp.). Data were subject to comparison using a *t*-test.

Results

Streptomyces *influence on fungal growth*

Four *Streptomyces* strains (DEF09, DEF20, DEF39, and DEF48) were selected based on their promising biocontrol features against *F. graminearum* obtained *in vitro* and *in planta* as assessed in previous studies (Colombo et al., 2019a, 2019b). Wheat grains were treated with *F. graminearum* CS3005 and independently with each *Streptomyces* spore suspension and incubated for 11 days as described in the Materials and methods section. The effect of

the *Streptomyces* strains on fungal growth in flour samples was evaluated at two different inoculation times (0 or 3 days post-pathogen inoculation; DPI). The time point of 3 days post-inoculation was selected as this corresponds with toxin synthesis by the fungus (Ponts et al., 2006). Two methods for fungal quantification were evaluated (ergosterol and qPCR). Ergosterol concentration was evaluated by chemical extraction and HPLC analysis. The qPCR-based *Fusarium* quantification was carried out based on *TRI12*, a gene commonly exploited to quantify trichothecene producers in samples. No significant quantities of ergosterol or qPCR amplifications were detected in blank samples (no-BCA or fungal treatment), confirming that the used seeds were free of *F. graminearum* (Table S1). In addition, the ratios of the fungal amount in the control to that in the treated samples were calculated for both the ergosterol and the qPCR method in order to assess the correlation between the two quantification methods, and a correlation value of 0.82 was obtained (Figure S2).

The inoculation of DEF09, DEF20, DEF39, and DEF48 strains at 3 days after fungal inoculation (3 DPI) were conducted to simulate a treatment of an already established *Fusarium* infection, and did not result in any antifungal effect as measured in terms of ergosterol amounts as well as DNA copy number (Figures 1, 2). On the contrary, when co-cultured with *Streptomyces* strains from the first day of inoculation (0 DPI), the fungal growth was clearly inhibited by all *Streptomyces* spp. strains ($P < 0.05$). All *P*-values from the ANOVA analysis are listed in Table S2.

Figure 1 Ergosterol content (µg/g) quantified in flour samples treated with Streptomyces *spp. DEF09, DEF39, DEF20, and DEF48 at 0 days post inoculation (0 DPI) (A,B) and 3 DPI (C,D) after 11 days of incubation. The presence of different control replicates (no-biocontrol agent (BCA) treatment) is due to multiple experiments being performed. Means of four replicates were subjected to ANOVA and separated according to Tukey's HSD (honestly significant difference) test (P < 0.05).*

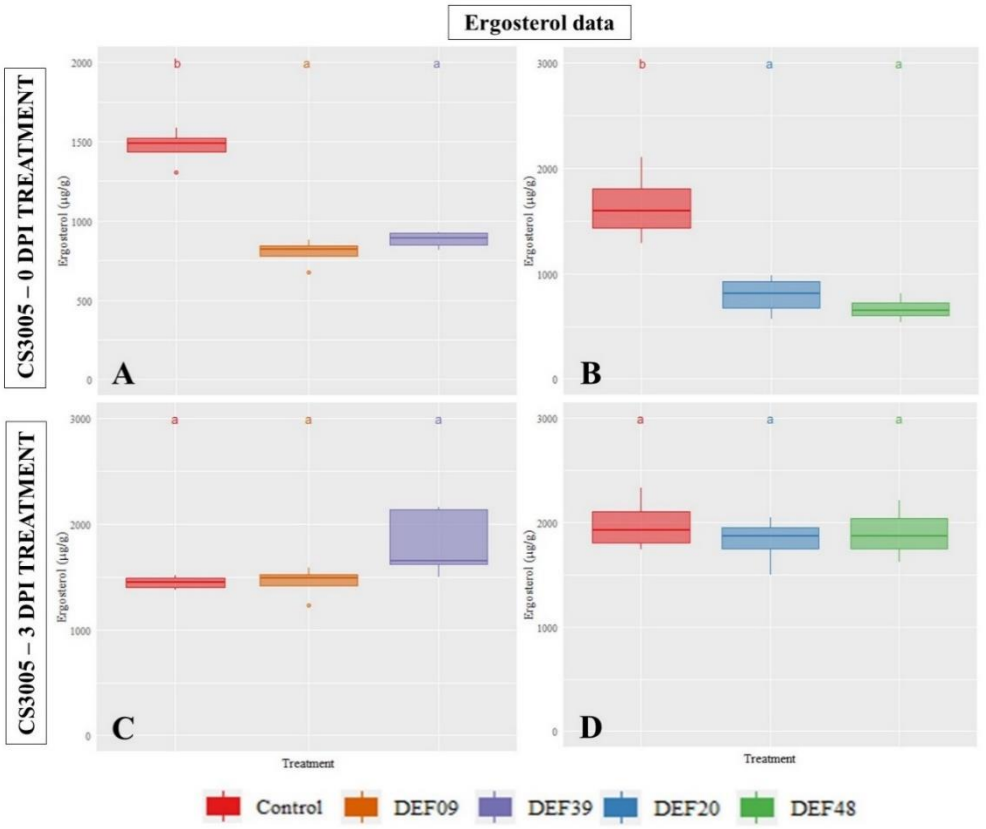
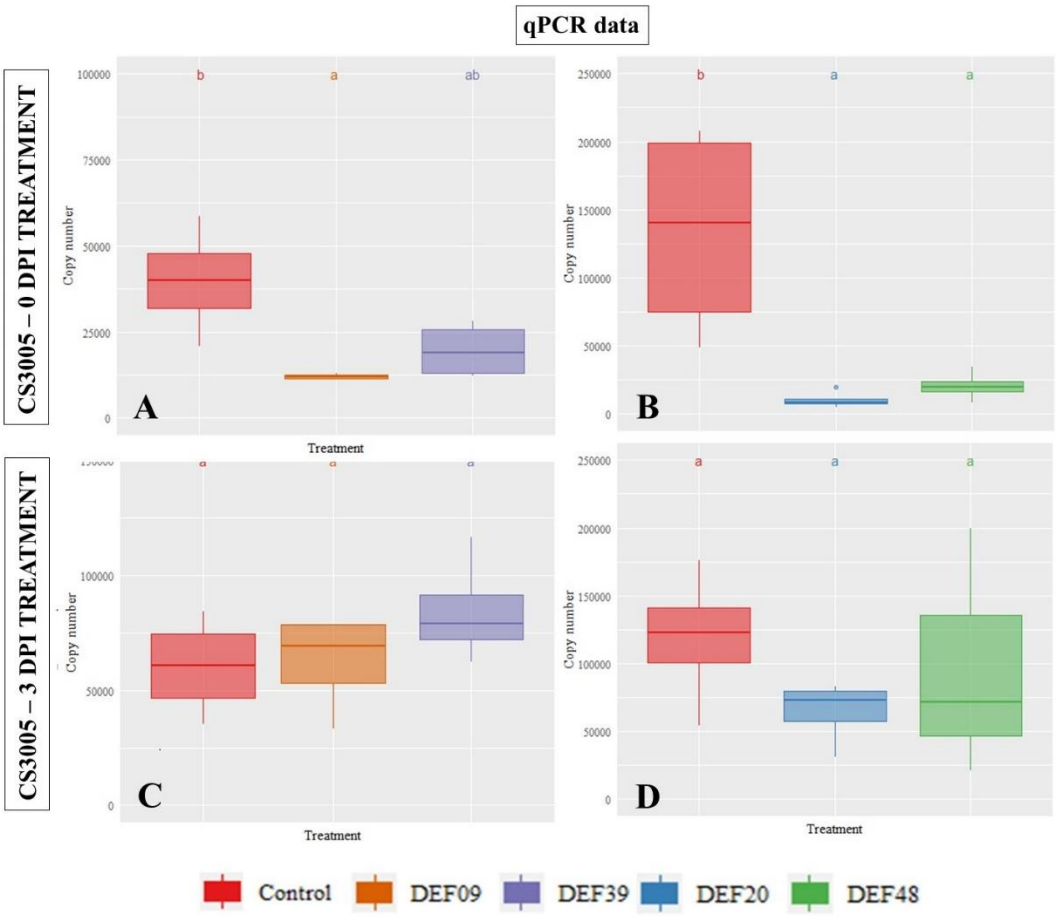


Figure 2 Fusarium *copy number normalized to wheat copy number in flour samples treated with* Streptomyces *spp. DEF09, DEF39, DEF20, and DEF48 at 0 DPI (A,B) and 3 DPI (C,D) after 11 days of incubation. The presence of different control replicates (no-BCA treatment) is due to multiple experiments being performed. The means of four replicates were subjected to ANOVA and separated according to Tukey's HSD test (P < 0.05).*



The BCAs were able to limit ergosterol content up to 45%, 52%, 40%, and 60% for DEF09, DEF20, DEF39, and DEF48, respectively. *Fusarium* copy number reduction reached up to 70%, 92%, 50%, and 85% for DEF09, DEF20, DEF39, and DEF48, respectively. The highest *Fusarium* inhibition was recorded for DEF20 and DEF48 treatments, suggesting they were the most effective strains, and this conclusion was consistent for both the qPCR and the ergosterol analyses (Figure S3).

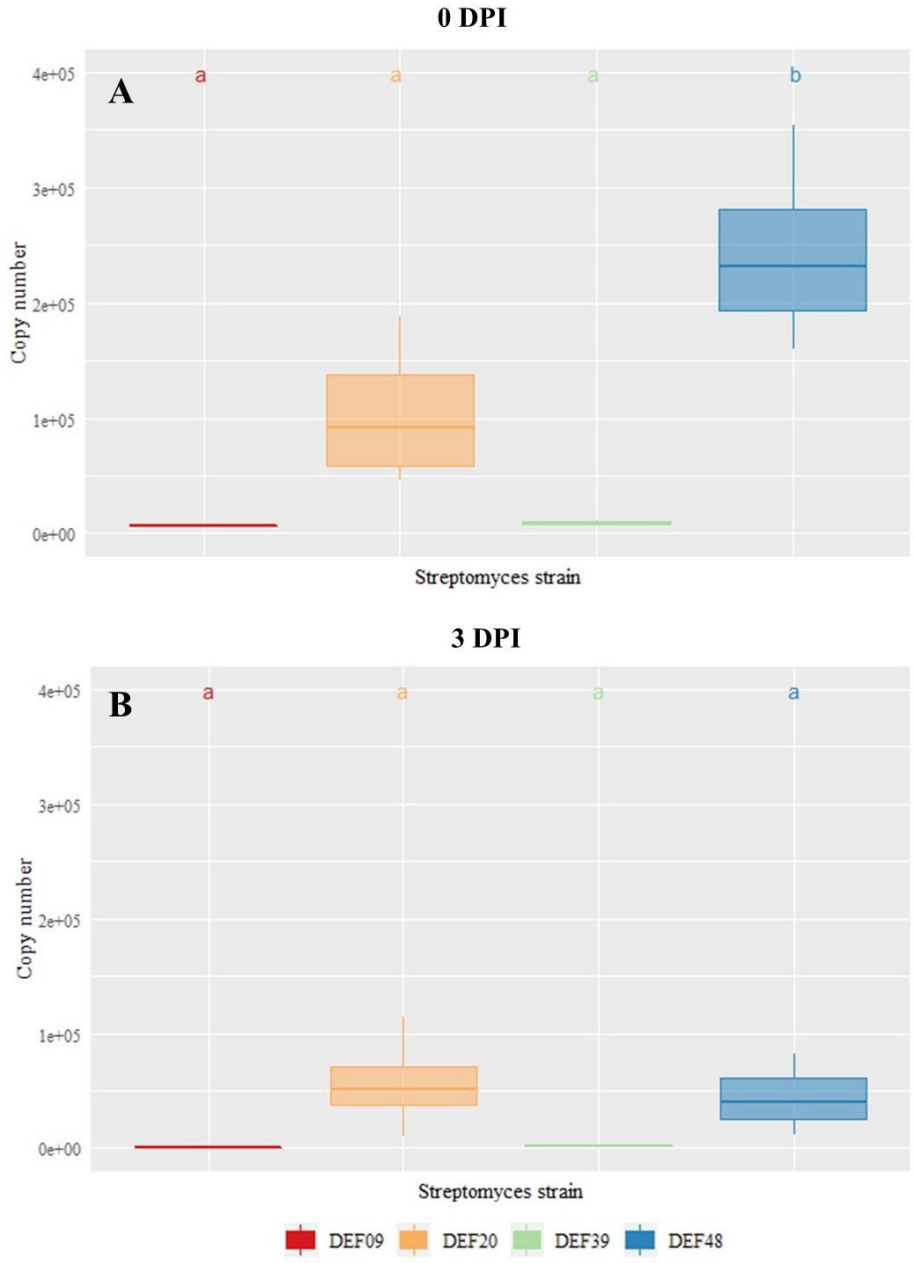
Streptomyces *strain fitness in microsilage*

To assess the effect of *Fusarium* presence in the contaminated seeds on the growth of the four *Streptomyces* spp. used in this study, a qPCR analysis targeting the *recA* gene region of the strains was performed.

In the 0 DPI treatment (Figure 3A), DEF20 and DEF48 showed increased growth in the presence of *F. graminearum* which, in the case of DEF48, was statistically significant in comparison to the other strains ($P = 9.79 \times 10^{-5}$).

However, the growth of all *Streptomyces* strains was reduced when applied 3 days after *F. graminearum* inoculation. Although the growth of DEF20 and DEF48 was higher than that of the other two *Streptomyces* strains, these differences were not statistically significant (Figure 3B).

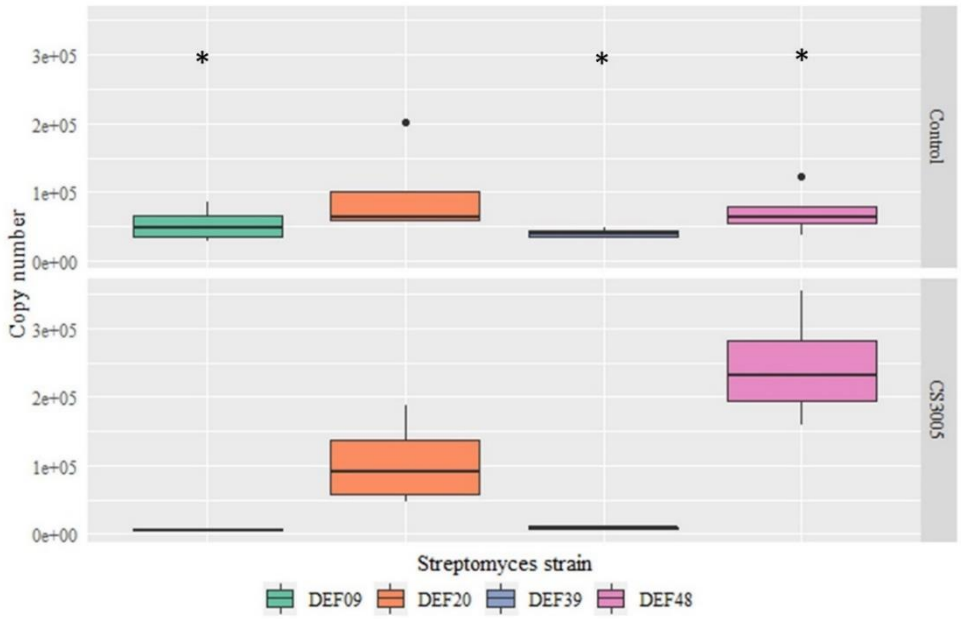
Figure 3 Copy number of Streptomyces *spp. normalized to wheat copy number in the (A) 0 DPI or (B) 3 DPI treatments. Means of four replicates were subjected to ANOVA and separated according to Tukey's HSD test (P < 0.05).*



The fungal quantification confirmed that the antagonists affect pathogen development in microsilage conditions when co-inoculated with fungus. Since the fungal pathogen might also reciprocally affect the development of the

antagonist, we used qPCR to evaluate if the growth of *Streptomyces* strains was comparable to that on grains without fungal contamination after 11 days of incubation at 24 °C. The development of almost all the *Streptomyces* strains after 11 days was clearly influenced by the presence of the pathogen (Figure 4). The *t*-test comparison highlighted the differences among BCA development in co-culture with CS3005 or when cultured alone (Figure 4).

Figure 4 Streptomyces *spp. copy number normalized to wheat copy number in flour samples after 11 days of incubation. *P < 0.05 was considered significant in the t-test comparison between control replicates (*Streptomyces *alone) and 0 DPI treatment (*Streptomyces *strain +* F. graminearum *CS3005).*



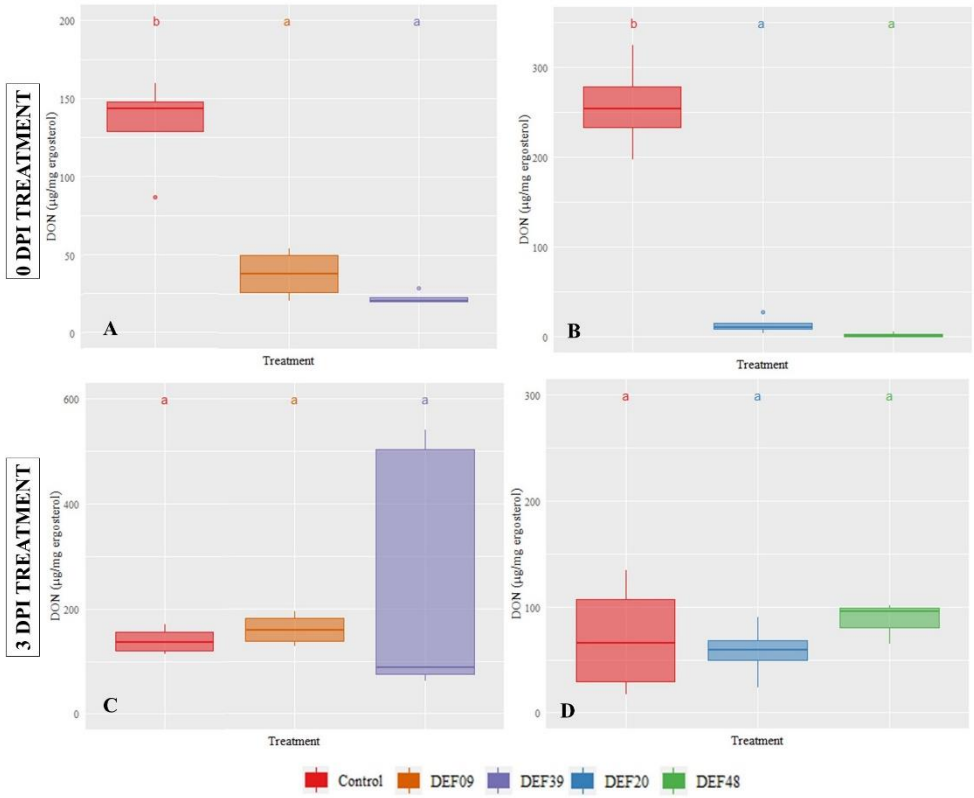
DEF09 and DEF39 were clearly inhibited by the presence of the fungus (*P* = 0.03 and *P* = 0.00). Despite their reduced growth, they were still able to exert antifungal activity against the pathogen (Figures 1A, 2A). DEF20 was not influenced by the co-culture with the *Fusarium* strain (*P* = 0.87). Indeed, it showed the same development as in control flasks (*Streptomyces* alone). Surprisingly, DEF48 growth increased in the presence of *F. graminearum* CS3005 in comparison with the control treatment (*P* = 0.03).

Therefore, DEF20 and DEF48 demonstrated a great ability to colonize the substrate and to exhibit a strong antifungal effect in the tested conditions (Figure 1B, 2B).

Influence of the BCA treatment on mycotoxin production in microsilage conditions

To verify the ability of the biocontrol *Streptomyces* spp. to limit toxin production, DON amounts (µg/mg ergosterol) were measured in all the experiments. Ergosterol content in flour samples were used to normalize mycotoxin production to fungal mass (Seong et al., 2009). Similarly to what observed for fungal quantification (Figure 1, 2), when co-cultured (0 DPI), all four BCAs significantly reduced mycotoxin production (Figure 5A-B). No significant decrease in toxin amount was recorded when *Streptomyces* spp. were applied 3 days after pathogen inoculation (3 DPI, Figure 5C-D).

Figure 5 Deoxynivalenol (DON) amount (µg/mg ergosterol) quantified in flour samples treated with Streptomyces *spp. DEF09, DEF39, DEF20, and DEF48 at 0 DPI (A,B) and 3 DPI (C,D) treatment after 11 days of incubation. The presence of different control replicates (no-BCA treatment) is due to multiple experiments being performed. Means of four replicates were subjected to ANOVA and separated according to Tukey's HSD test (P < 0.05).*



Therefore, all strains were effectively able to reduce DON (µg/mg ergosterol) at 0 DPI treatment according to the following percentages: 71%, 94%, 83%, and 99% for DEF09, DEF20, DEF39, and DEF48, respectively (Figure S4).

Discussion

Our study contributes to deciphering the interactions occurring between *Streptomyces* strains and a toxigenic strain of *F. graminearum* in a controlled environment. Direct antifungal activity was observed only when the two microorganisms were co-inoculated (0 DPI treatment). Therefore, these results have important implications on the development of BCAs that are effective in controlling mycotoxin accumulation in grains.

The fact that the antimycotoxigenic activity is most probably a consequence of the antifungal activity suggests that the BCA needs to colonize the seed before the fungus becomes established, and no curative effects can be expected as confirmed by absence of inhibition of the fungal growth and mycotoxin production following the treatment at 3 DPI. This might be linked to the *Streptomyces* developmental program, which requires colonization of the substrate with the formation of vegetative mycelium, and only in response to nutrient depletion and other signals, can morphological differentiation and the production of bioactive compounds occur (Flärdh and Buttner, 2009). Our results also confirm previous observations where a higher level of mycotoxin reduction was obtained after treating peanut grains with a *Streptomyces* strain in a preventive way (24 h before pathogen inoculation) (Zucchi et al., 2008). The appropriate strategy of BCA application can substantially improve their efficacy under complex environmental conditions; indeed, several reports have highlighted the importance of helping the *Streptomyces* spp. to get pre-established in the target substrate before pathogen infections (Newitt et al., 2019).

Indeed, the different BCA strains had different developments at 0 DPI. Although DEF09 and DEF39 showed reduced growth in the presence of *Fusarium* in comparison with the control without fungal inoculation, they were still able to reduce mycotoxin production and fungal growth. DEF20 growth was not affected by co-inoculation with the pathogen. Surprisingly, DEF48 biomass increased in response to co-culture with CS3005 and, moreover, it showed the highest inhibition percentage of ergosterol and mycotoxin levels. Indeed, some strains can also benefit from fungal metabolite production (Mille-Lindblom et al., 2006) and, during this interaction, the production of antifungal metabolites can be elicited (Elleuch et al., 2013; Fguira et al., 2008; Zhao et al., 2013). Therefore, further studies are needed on the interaction with multiple fungi causing DON accumulation (Pasquali and Migheli, 2014) to decipher the specific mechanism of activity and the reliability of the BCA in the environment.

A key aspect in the selection of BCAs against toxigenic *Fusarium* spp. is the evaluation of their ability to counteract mycotoxin production (Martinez Tuppia et al., 2017). Indeed, the microbial reduction of pathogen development does not always correspond to a reduction in toxin level (Dalié et al., 2012; He et al., 2009). In the present research, treated samples at 0 DPI not only had inhibited *Fusarium* growth but also reduced mycotoxin levels in comparison to control treatments. A strong level of DON inhibition (µg/mg ergosterol) of above 94% was recorded for DEF20 and DEF48 in parallel with a strong reduction of fungal biomass. Indeed, high chitinase activity was already described for these two strains in a previous study (Colombo et al., 2019b), but we cannot exclude the contemporary production of other bioactive metabolites able to suppress pathogen development, a common trait characterizing the *Streptomyces* genus (Palaniyandi et al., 2013). Interestingly, DEF39 showed a remarkable decrease in toxin production (83%) and the lowest inhibition of fungal biomass (40% of ergosterol inhibition). Chitinase activity was weak for this strain (Colombo et al., 2019b), suggesting that within its arsenal of secondary metabolites it also harbors the potential to regulate toxin production via specific mechanisms that are not linked to antibiosis alone. Several microbial metabolites have been characterized for their specific inhibitory activity against aflatoxins produced by *Aspergillus parasiticus*, such as dioctatin A, blasticidin A, and aflastatin A (Kondo et al., 2001; Sakuda et al., 2000; Yoshinari et al., 2010). Future studies will characterize the metabolites of the strain DEF39 to decipher their bioactivity.
Despite the fact that the DEF09 strain was effective against FHB development in field conditions (Colombo et al., 2019b), it exhibited a lower antifungal activity in comparison to DEF20 and DEF48 in this study. It will be of interest to understand the biocontrol mechanisms elicited by this strain *in planta*. It is likely that plant defense mechanisms are also triggered and can be investigated based on known hormone signaling defense signals (Palazzini et al., 2018). Moreover, the behavior of the other analyzed strains should be assessed under complex environmental conditions, such as in field studies,

where multiple environmental factors can influence the fitness and the activity of the strains.

Identifying the molecules involved in the specific interactions between the grains and pathogen will be important for deciphering the mode of action and to identify the determinants of the antifungal and antimycotoxigenic activity of these *Streptomyces* strains, as has been done in other cases (Wang et al., 2013).

To conclude, the selection of novel BCAs against toxigenic *Fusarium* spp. cannot be carried out without taking into account their activity against mycotoxin production and testing their ability to grow on mycotoxin-contaminated substrates (Dalié et al., 2012; He et al., 2009; Martinez Tuppia et al., 2017). Our preliminary results, despite the interesting features of the strains as potential BCAs against *F. graminearum*, indicate that they are active only before pathogen establishment. Therefore, it will be necessary to test the optimal method of application to achieve toxin reduction under complex environmental conditions. Future integration of functional genome analysis of the strains may lead to the identification of effective molecules able to block fungal growth and modulate toxin biosynthesis (Pasquali et al., 2016b), a promising approach to developing novel targeted strategies to limit the damage of toxin accumulation in food and feed (Pani et al., 2016).

Supplemental information for this article can be found online at https://drive.google.com/drive/folders/13h35atx2IKSJiXGm4bjUDMotQUMnEETm?usp=sharing

References

Beattie, S., Schwarz, P. B., Horsley, R., Barr, J., and Casper, H. H. (1998). The effect of grain storage conditions on the viability of *Fusarium* and deoxynivalenol production in infested malting barley. *J. Food Prot.* 61, 103–106.

Bilska, K., Kulik, T., Ostrowska-Kołodziejczak, A., Buśko, M., Pasquali, M., Beyer, M., et al. (2018). Development of a highly sensitive FcMito qPCR assay for the quantification of the toxigenic fungal plant pathogen *Fusarium culmorum*. *Toxins (Basel).* 10, 211. doi:10.3390/toxins10050211.

Bluhm, B. H., and Woloshuk, C. P. (2005). Amylopectin induces fumonisin B1 production by *Fusarium verticillioides* during colonization of maize kernels. *Mol. plant-microbe Interact.* 18, 1333–1339. doi:10.1094/MPMI-18-1333.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–622. doi:10.1373/clinchem.2008.112797.

Chater, K. F., Biró, S., Lee, K. J., Palmer, T., and Schrempf, H. (2010). The complex extracellular biology of *Streptomyces*. *FEMS Microbiol. Rev.* 34, 171–198. doi:10.1111/j.1574-6976.2009.00206.x.

Colombo, E. M., Pizzatti, C., Kunova, A., Gardana, C., Saracchi, M., Cortesi, P., et al. (2019a). Evaluation of in-vitro methods to select effective streptomycetes against toxigenic fusaria. *PeerJ* 7, e6905. doi:10.7717/peerj.6905.

Colombo, E. M., Pizzatti, C., Kunova, A., Saracchi, M., Cortesi, P., and Pasquali, M. (2019b). Selection of an endophytic *Streptomyces* sp. strain DEF09 from wheat roots as a biocontrol agent against *Fusarium graminearum*. *Front. Microbiol.* 10, 2356. doi:10.3389/fmicb.2019.02356.

Cuomo, C. A., Güldener, U., Xu, J.-R., Trail, F., Turgeon, B. G., Di Pietro, A., et al. (2007). The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. *Science (80-.).* 317, 1400–1402. doi:10.1126/science.1143708.

da Silva Bomfim, N., Nakassugi, L. P., Faggion Pinheiro Oliveira, J., Kohiyama, C. Y., Mossini, S. A. G., Grespan, R., et al. (2015). Antifungal activity and inhibition of fumonisin production by *Rosmarinus officinalis* L. essential oil in *Fusarium verticillioides* (Sacc.) Nirenberg. *Food Chem.* 166, 330–336. doi:10.1016/J.FOODCHEM.2014.06.019.

Dalié, D., Pinson-Gadais, L., Atanasova-Penichon, V., Marchegay, G., Barreau, C., Deschamps, A., et al. (2012). Impact of *Pediococcus pentosaceus* strain L006 and its metabolites on fumonisin biosynthesis by *Fusarium verticillioides*. *Food Control* 23, 405–411. doi:10.1016/J.FOODCONT.2011.08.008.

Elleuch, L., Smaoui, S., Najah, S., Chakchouk, A., Sellem, I., Karray-Rebai, I., et al. (2013). Production of diketopiperazine derivative cyclo (L-Leu-L-Arg) by *Streptomyces* sp. TN262 after exposure to heat-killed fungus *Fusarium* sp. *J. Chem. Soc. Pakistan* 35, 1530–1534.

Elliot, M. A., Buttner, M. J., and Nodwell, J. R. (2008). “Multicellular development in *Streptomyces*,” in *Myxobacteria: Multicellularity and Differentiation*, ed. D. E. Whitworth (ASM Press, Washington, D.C.), 419–437.

Fguira, L. F. B., Smaoui, S., Karray-Rebai, I., Bejar, S., and Mellouli, L. (2008). The antifungal activity of the terrestrial *Streptomyces* US80 strain is induced by heat-killed fungi. *Biotechnol. J.* 3, 1058–1066. doi:10.1002/biot.200700155.

Flärdh, K., and Buttner, M. J. (2009). *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nat. Rev. Microbiol.* 7, 36. doi:10.1038/nrmicro1968.

Fravel, D. R. (2005). Commercialization and implementation of biocontrol. *Annu. Rev. Phytopathol.* 43, 337–359. doi:10.1146/annurev.phyto.43.032904.092924.

Gardiner, D. M., Stiller, J., and Kazan, K. (2014). Genome sequence of *Fusarium graminearum* isolate CS3005. *Genome Announc.* 2, e00227-14. doi:10.1128/genomeA.00227-14.

Gilbert, J., and Haber, S. (2013). Overview of some recent research developments in fusarium head blight of wheat. *Can. J. Plant Pathol.* 35, 149–174. doi:10.1080/07060661.2013.772921.

Gimeno, A., Sohlberg, E., Pakula, T., Limnell, J., Keller, B., Laitila, A., et al. (2019). TaqMan qPCR for quantification of *Clonostachys rosea* used as a biological control agent against *Fusarium graminearum*. *Front. Microbiol.* 10, 1627. doi:10.3389/fmicb.2019.01627.

Guo, Y. P., Zheng, W., Rong, X. Y., and Huang, Y. (2008). A multilocus phylogeny of the

Streptomyces griseus 16S rRNA gene clade: use of multilocus sequence analysis for streptomycete systematics. *Int. J. Syst. Evol. Microbiol.* 58, 149–159. doi:10.1099/ijs.0.65224-0.

He, J., Boland, G. J., and Zhou, T. (2009). Concurrent selection for microbial suppression of *Fusarium graminearum*, Fusarium head blight and deoxynivalenol in wheat. *J. Appl. Microbiol.* 106, 1805–1817. doi:10.1111/j.1365-2672.2009.04147.x.

Jung, B., Park, S. Y., Lee, Y. W., and Lee, J. (2013). Biological efficacy of *Streptomyces* sp. strain BN1 against the cereal head blight pathogen *Fusarium graminearum*. *Plant Pathol. J.* 29, 52–58. doi:10.5423/PPJ.OA.07.2012.0113.

Kondo, T., Sakurada, M., Okamoto, S., Ono, M., Tsukigi, H., Suzuki, A., et al. (2001). Effects of aflastatin A, an inhibitor of aflatoxin production, on aflatoxin biosynthetic pathway and glucose metabolism in *Aspergillus parasiticus*. *J. Antibiot. (Tokyo).* 54, 650–657.

Lee, H. J., and Ryu, D. (2017). Worldwide occurrence of mycotoxins in cereals and cereal-derived food products: public health perspectives of their co-occurrence. *J. Agric. Food Chem.* 65, 7034–7051. doi:10.1021/acs.jafc.6b04847.

Legrand, F., Picot, A., Cobo-Díaz, J. F., Chen, W., and Le Floch, G. (2017). Challenges facing the biological control strategies for the management of Fusarium head blight of cereals caused by *F. graminearum*. *Biol. Control* 113, 26–38. doi:10.1016/j.biocontrol.2017.06.011.

Magan, N., Aldred, D., Mylona, K., and Lambert, R. J. W. (2010). Limiting mycotoxins in stored wheat. *Food Addit. Contam. Part A* 27, 644–650. doi:10.1080/19440040903514523.

Maresca, M. (2013). From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. *Toxins (Basel).* 5, 784–820. doi:10.3390/toxins5040784.

Martinez Tuppia, C., Atanasova-Penichon, V., Chéreau, S., Ferrer, N., Marchegay, G., Savoie, J.-M., et al. (2017). Yeast and bacteria from ensiled high moisture maize grains as potential mitigation agents of fumonisin B 1. *J. Sci. Food Agric.* 97, 2443–2452. doi:10.1002/jsfa.8058.

Miedaner, T., Gwiazdowska, D., and Waśkiewicz, A. (2017). Management of *Fusarium* species and their mycotoxins in cereal food and feed. *Front. Microbiol.* 8, 1543. doi:10.3389/fmicb.2017.01543.

Mille-Lindblom, C., Fischer, H., and J. Tranvik, L. (2006). Antagonism between bacteria and fungi: substrate competition and a possible tradeoff between fungal growth and tolerance towards bacteria. *Oikos* 113, 233–242. doi:10.1111/j.2006.0030-1299.14337.x.

Newitt, J. T., Prudence, S. M. M., Hutchings, M. I., Worsley, S. F., Newitt, J. T., Prudence, S. M. M., et al. (2019). Biocontrol of cereal crop diseases using streptomycetes. *Pathogens* 8, 78. doi:10.3390/pathogens8020078.

Ng, H. E., Raj, S. S. A., Wong, S. H., Tey, D., and Tan, H. M. (2008). Estimation of fungal growth using the ergosterol assay: a rapid tool in assessing the microbiological status of grains and feeds. *Lett. Appl. Microbiol.* 46, 113–118. doi:10.1111/j.1472-765X.2007.02279.x.

Nicolaisen, M., Suproniene, S., Nielsen, L. K., Lazzaro, I., Spliid, N. H., and Justesen, A. F. (2009). Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *J. Microbiol. Methods* 76, 234–240. doi:10.1016/j.mimet.2008.10.016.

Nourozian, J., Etebarian, H. R., and Khodakaramian, G. (2006). Biological control of *Fusarium graminearum* on wheat by antagonistic bacteria. *Songklanakarin J. Sci. Technol.* 28, 29–38.

Osborne, L. E., and Stein, J. M. (2007). Epidemiology of Fusarium head blight on small-grain cereals. *Int. J. Food Microbiol.*, 103–108. doi:10.1016/j.ijfoodmicro.2007.07.032.

Palaniyandi, S. A., Yang, S. H., Zhang, L., and Suh, J. W. (2013). Effects of actinobacteria on plant disease suppression and growth promotion. *Appl. Microbiol. Biotechnol.* 97, 9621–9636. doi:10.1007/s00253-013-5206-1.

Palazzini, J. M., Ramirez, M. L., Torres, A. M., and Chulze, S. N. (2007). Potential biocontrol agents for Fusarium head blight and deoxynivalenol production in wheat. *Crop Prot.* 26, 1702–1710. doi:10.1016/j.cropro.2007.03.004.

Palazzini, J. M., Yerkovich, N., Alberione, E., Chiotta, M., and Chulze, S. N. (2017). An integrated dual strategy to control *Fusarium graminearum sensu stricto* by the biocontrol agent *Streptomyces* sp. RC 87B under field conditions. *Plant Gene* 9, 13–18. doi:10.1016/j.plgene.2016.11.005.

Palazzini, J., Roncallo, P., Cantoro, R., Chiotta, M., Yerkovich, N., Palacios, S., et al. (2018). Biocontrol of *Fusarium graminearum sensu stricto*, reduction of deoxynivalenol accumulation and phytohormone induction by two selected antagonists. *Toxins (Basel).* 10, 88. doi:10.3390/toxins10020088.

Pani, G., Dessì, A., Dallocchio, R., Scherm, B., Azara, E., Delogu, G., et al. (2016). Natural phenolic inhibitors of trichothecene biosynthesis by the wheat fungal pathogen *Fusarium culmorum*: a computational insight into the structure-activity relationship. *PLoS One* 11. doi:10.1371/journal.pone.0157316.

Pasquali, M., Beyer, M., Logrieco, A., Audenaert, K., Balmas, V., Basler, R., et al. (2016a). A European database of *Fusarium graminearum* and *F. culmorum* trichothecene genotypes. *Front. Microbiol.* 7, 406. doi:10.3389/fmicb.2016.00406.

Pasquali, M., Cocco, E., Leclercq, C. C., Planchon, S., Guignard, C., Renaut, J., et al. (2016b). A *Fusarium graminearum* strain-comparative proteomic approach identifies regulatory changes triggered by agmatine. *J. Proteomics* 137, 107–116. doi:10.1016/J.JPROT.2015.11.010.

Pasquali, M., and Migheli, Q. (2014). Genetic approaches to chemotype determination in type B-trichothecene producing Fusaria. *Int. J. Food Microbiol.* 189, 164–182. doi:10.1016/j.ijfoodmicro.2014.08.011.

Pasquali, M., Piatti, P., Gullino, M. L., and Garibaldi, A. (2006). Development of a real-time polymerase chain reaction for the detection of *Fusarium oxysporum* f. sp. *basilici* from basil seed and roots. *J. Phytopathol.* 154, 632–636. doi:10.1111/j.1439-0434.2006.01160.x.

Ponts, N., Pinson-Gadais, L., Verdal-Bonnin, M. N., Barreau, C., and Richard-Forget, F. (2006). Accumulation of deoxynivalenol and its 15-acetylated form is significantly modulated by oxidative stress in liquid cultures of *Fusarium graminearum*. *FEMS Microbiol. Lett.* 258, 102–107. doi:10.1111/j.1574-6968.2006.00200.x.

R Core Team (2019). R: A language and environment for statistical computing. Available at: https://www.r-project.org/.

Rao, B. S., Rao, V. S., Ramakrishna, Y., and Bhat, R. V. (1989). Rapid and specific method for screening ergosterol as an index of fungal contamination in cereal grains. *Food Chem.* 31, 51–56. doi:10.1016/0308-8146(89)90150-7.

Rey, T., and Dumas, B. (2017). Plenty is no plague: *Streptomyces* symbiosis with crops. *Trends Plant Sci.* 22, 30–37. doi:10.1016/j.tplants.2016.10.008.

Sakuda, S., Ono, M., and Ikeda, H. (2000). Blasticidin A as an inhibitor of aflatoxin production by *Aspergillus parasiticus* of aflatoxin. *J. Antibiot. (Tokyo).* 53, 1265–1271.

Salgado, J. D., Madden, L. V, and Paul, P. A. (2015). Quantifying the effects of Fusarium head

blight on grain yield and test weight in soft red winter wheat. *Phytopathology* 105, 295–306.

Sanzani, S. M., Li Destri Nicosia, M. G., Faedda, R., Cacciola, S. O., and Schena, L. (2014). Use of quantitative PCR detection methods to study biocontrol agents and phytopathogenic fungi and oomycetes in environmental samples. *J. Phytopathol.* 162, 1–13. doi:10.1111/jph.12147.

Sardi, P., Saracchi, M., Quaroni, S., Petrolini, B., Borgonovi, G. E., and Merli, S. (1992). Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. *Appl. Environ. Microbiol.* 58, 2691–2693.

Seipke, R. F., Kaltenpoth, M., and Hutchings, M. I. (2012). *Streptomyces* as symbionts: an emerging and widespread theme? *FEMS Microbiol. Rev.* 36, 862–876. doi:10.1111/j.1574-6976.2011.00313.x.

Seong, K. Y., Pasquali, M., Zhou, X., Song, J., Hilburn, K., McCormick, S., et al. (2009). Global gene regulation by *Fusarium* transcription factors Tri6 and Tri10 reveals adaptations for toxin biosynthesis. *Mol. Microbiol.* 72, 354–367. doi:10.1111/j.1365-2958.2009.06649.x.

Shah, J. J., Khedkar, V., Coutinho, E. C., and Mohanraj, K. (2015). Design, synthesis and evaluation of benzotriazole derivatives as novel antifungal agents. *Bioorg. Med. Chem. Lett.* 25, 3730–3737. doi:10.1016/J.BMCL.2015.06.025.

Sun, Z., Huang, Y., Wang, Y., Zhao, Y., and Cui, Z. (2014). Potassium hydroxide-ethylene diamine tetraacetic acid method for the rapid preparation of small-scale PCR template DNA from actinobacteria. *Mol. Genet. Microbiol. Virol.* 29, 42–46. doi:10.3103/S089141681401008X.

Tangni, E. K., and Pussemier, L. (2006). Ochratoxin A and citrinin loads in stored wheat grains: impact of grain dust and possible prediction using ergosterol measurement. *Food Addit. Contam.* 23, 181–189. doi:10.1080/02652030500391911.

Vogelgsang, S., Beyer, M., Pasquali, M., Jenny, E., Musa, T., Bucheli, T. D., et al. (2019). An eight-year survey of wheat shows distinctive effects of cropping factors on different *Fusarium* species and associated mycotoxins. *Eur. J. Agron.* 105, 62–77. doi:10.1016/j.eja.2019.01.002.

Wang, C., Wang, Z., Qiao, X., Li, Z., Li, F., Chen, M., et al. (2013). Antifungal activity of volatile organic compounds from *Streptomyces alboflavus* TD-1. *FEMS Microbiol. Lett.* 341, 45–51. doi:10.1111/1574-6968.12088.

Winter, M., Samuels, P. L., Otto-hanson, L. K., Dill-macky, R., and Linda, L. (2019). Biocontrol of Fusarium crown and root rot of wheat by *Streptomyces* isolates – it's complicated. *Phytobiomes* 3, 52–60.

Yoshinari, T., Noda, Y., Yoda, K., Sezaki, H., Nagasawa, H., and Sakuda, S. (2010). Inhibitory activity of blasticidin A, a strong aflatoxin production inhibitor, on protein synthesis of yeast: selective inhibition of aflatoxin production by protein synthesis inhibitors. *J. Antibiot. (Tokyo).* 63, 309–314. doi:10.1038/ja.2010.36.

Yuan, Q.-S., Yang, P., Wu, A.-B., Zuo, D.-Y., He, W.-J., Guo, M.-W., et al. (2018). Variation in the microbiome, trichothecenes, and aflatoxins in stored wheat grains in Wuhan, China. *Toxins (Basel).* 10, 171. doi:10.3390/toxins10050171.

Zhang, Y. J., Fan, P. S., Zhang, X., Chen, C. J., and Zhou, M. G. (2009). Quantification of *Fusarium graminearum* in harvested grain by real-time polymerase chain reaction to assess efficacies of fungicides on Fusarium head blight, deoxynivalenol contamination, and yield of winter wheat. *Phytopathology* 99, 95–100. doi:10.1094/PHYTO-99-1-0095.

Zhao, J., Xue, Q. H., Niu, G. G., Xue, L., Shen, G. H., and Du, J. Z. (2013). Extracellular enzyme production and fungal mycelia degradation of antagonistic *Streptomyces* induced by

fungal mycelia preparation of cucurbit plant pathogens. *Ann. Microbiol.* 63, 809–812. doi:10.1007/s13213-012-0507-7.

Zhao, Y., Selvaraj, J. N., Xing, F., Zhou, L., Wang, Y., Song, H., et al. (2014). Antagonistic action of *Bacillus subtilis* strain SG6 on *Fusarium graminearum*. *PLoS One* 9, e92486. doi:10.1371/journal.pone.0092486.

Zucchi, T. D., De Moraes, L. A. B., and De Melo, I. S. (2008). *Streptomyces* sp. ASBV-1 reduces aflatoxin accumulation by *Aspergillus parasiticus* in peanut grains. *J. Appl. Microbiol.* 105, 2153–2160. doi:10.1111/j.1365-2672.2008.03940.x.

Chapter 4: Investigation of specialized metabolites from antifungal streptomycetes

The importance of natural products isolated from streptomycetes is not restricted to human and veterinary medicine. Indeed, these bacteria have been evaluated also in agricultural field as plant growth promoting bacteria (PGPB) for their ability to establish symbiosis with plants (Seipke et al., 2012). Therefore, a wide range of compounds are produced in order to win the competition with other microorganisms present in the rhizosphere and to better colonize the host tissues (Compant et al., 2010). Several metabolites have been identified to be involved in this interactions and for biocontrol purposes, such as chitinases (Baharlouei et al., 2010), volatile organic compounds (Wang et al., 2013) and antifungal agents (Mizuhara et al., 2011). The genes responsible for this secondary metabolites production are organized in biosynthetic gene clusters (Bentley et al., 2003; Ikeda et al., 2003). Antifungal metabolites produced by streptomycetes belong mainly to the class of polyene macrolides, a particular group of polyketides (Zotchev, 2012). Important polyene macrolides isolated from streptomycetes are the tetraene nystatin, the heptaene candicidin, trichomycin, ascosin, and amphotericin B (Waksman et al., 1965). The presence in these molecules of a series of conjugated double bound facilitate their identification by the particular light-absorption spectra (Oroshnik et al., 1955). In addition, the presence of a glycosylic residue attached to the macrocyclic lactone complete the chemical structure of these natural products, which facilitate the interaction with fungal membranes, making them permeable to ions and small molecules. Other macrolides acting as ionophore antibiotics belongs to the class of the macrotetrolides, which exhibit antimicrobial and insecticidal effects (Žižka, 1998).

The isolation of natural products from microorganisms or plants relies mainly on different chemical extraction techniques coupled with HPLC-UV and LC-MS analysis of the crude extracts (Bucar et al., 2013). Quality assessment is

conducted with the bioassay-guided isolation technique, which help to check the bioactivity of the crude extracts or fractions against the target organism and speed up the identification of metabolite of interest (Zhang et al., 2007). In the last twenty years, genome sequencing and annotation for secondary metabolic gene clusters became useful for natural product discovery, allowing the structure prediction and biosynthetic pathway characterization of novel and already known metabolites (Nett et al., 2009). In this way, the combination of chemical analysis and genome information helped to develop high quality natural product library with high structural diversity and interesting bioactivity The characterization of *Streptomyces* strains active against *F. graminearum* and *F. culmorum* carried out during this PhD project led to the selection of three potential biocontrol streptomycetes producing bioactive compounds. DEF20 and DEF48 were able to limit mycelial development and DEF39 reduced deoxynivalenol (DON) production in microplate bioassay. Therefore, their antifungal secondary metabolites produced *in vitro* were investigated in collaboration with the research group of Professor Barrie Wilkinson at the Department of Molecular Microbiology (John Innes Centre, Norwich, UK). Bioassay guided isolation technique was used to identify the metabolites by evaluating the activity of fractions obtained from agar plate extraction against the target pathogen. In addition, Illumina sequencing of their genomes was performed. Crude extracts from co-culture with the fungal strains or alone on three different agar-media was obtained. By checking their activity against the target pathogen, we selected the medium used to obtain extracts exhibiting the highest bioactivity and therefore with the ability to trigger the production of bioactive molecules. Interestingly, we observed that the presence of the fungus was not essential for the metabolite production.

Afterwards we further purified the compounds from all the three streptomycetes. The integration of the antiSMASH bioinformatic analysis to identify secondary metabolite biosynthetic gene clusters (Blin et al., 2007), literature searches for antifungal compound composition, analysis of the active fractions allowed us to identify bioactive molecules produced by three

strains. Further molecule characterization will be needed to confirm their role and bioactivity. This work allowed to identify putative new undescribed molecules and molecules with new bioactivities which can lead to patenting.

References

Baharlouei, A., Sharifi-Sirchi, G. R., and Shahidi Bonjar, G. H. (2010). Identification of an antifungal chitinase from a potential biocontrol agent, *Streptomyces plicatus* strain 101, and its new antagonistic spectrum of activity. *Philipp. Agric. Sci.* 93, 439–445.

Bentley, S. D., Chater, K. F., Cerdeño-Tárraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., et al. (2003). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 417, 141–147. doi:10.1038/417141a.

Blin, K., Wolf, T., Chevrette, M. G., Lu, X., Schwalen, C. J., Kautsar, S. A., et al. (2017). antiSMASH 4.0—improvements in chemistry prediction and gene cluster boundary identification. *Nucleic acids res.* 45, W36-W41. doi: 10.1093/nar/gkx319

Bucar, F., Wube, A., and Schmid, M. (2013). Natural product isolation – how to get from biological material to pure compounds. *Nat. Prod. Rep.* 30, 525–545. doi:10.1039/c3np20106f.

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42, 669–678. doi:10.1016/j.soilbio.2009.11.024.

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., et al. (2003). Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nat. Biotechnol.* 21, 526–531. doi:10.1038/nbt820.

Mizuhara, N., Kuroda, M., Ogita, A., Tanaka, T., Usuki, Y., and Fujita, K. I. (2011). Antifungal thiopeptide cyclothiazomycin B1 exhibits growth inhibition accompanying morphological changes via binding to fungal cell wall chitin. *Bioorganic Med. Chem.* 19, 5300–5310. doi:10.1016/j.bmc.2011.08.010.

Nett, M., Ikeda, H., and Moore, B. S. (2009). Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat. Prod. Rep.* 26, 1353–1508. doi:10.1039/b817069j.

Oroshnik, W., Vining, L. C., Mebane, A. D., and Taber, W. A. (1955). Polyene antibiotics. *Science (80-.).* 121, 147–149. doi:10.2307/1682745.

Seipke, R. F., Kaltenpoth, M., and Hutchings, M. I. (2012). *Streptomyces* as symbionts: an emerging and widespread theme? *FEMS Microbiol. Rev.* 36, 862–876. doi:10.1111/j.1574-6976.2011.00313.x.

Waksman, S. A., Lechevalier, H. A., and Schaffner, C. P. (1965). Candicidin and other polyenic antifungal antibiotics. *Bull. World Health Organ.* 33, 219–226.

Wang, C., Wang, Z., Qiao, X., Li, Z., Li, F., Chen, M., et al. (2013). Antifungal activity of volatile organic compounds from *Streptomyces alboflavus* TD-1. *FEMS Microbiol. Lett.* 341, 45–51. doi:10.1111/1574-6968.12088.

Zhang, L., Yan, K., Zhang, Y., Huang, R., Bian, J., Zheng, C., et al. (2007). High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of fungal infections. *Proc. Natl. Acad. Sci.* 104, 4606–4611. doi:10.1073/pnas.0609370104.

Žižka, Z. (1998). Biological effects of macrotetrolide antibiotics and nonactic acids. *Folia Microbiol. (Praha).* 43, 7–14. doi:10.1007/BF02815533.

Zotchev, S. (2012). Polyene macrolide antibiotics and their applications in human therapy. *Curr. Med. Chem.* 10, 211–223. doi:10.2174/0929867033368448.

General conclusion

Fusarium spp. causal agents of Fusarium root rot (FRR), foot rot (FFR) and head blight (FHB) in wheat and barley reduce yield quality and quantity, but also contaminate grains with mycotoxins, in particular deoxynivalenol, nivalenol and their acetylated derivatives. The management of these pathogens in field is already carried out using different control strategies (e.g. resistant varieties, crop rotation) due to the lack of a complete efficacy of the use of chemicals. Indeed, the common agricultural practices together with favourable environmental conditions can influence *Fusarium* development, enhancing mycotoxin accumulation. Therefore, biocontrol strategies can be a feasible option within an integrative approach to manage toxigenic *Fusarium* spp. One of the major obstacles in biocontrol research is the lack of consistency and efficacy of biocontrol agents (BCAs), in particular when the selected strains *in vitro* are tested under complex environmental conditions. In addition, strains able to limit pathogen development are not always successful in limiting mycotoxin contamination. Given the different type of toxins produced by strains of the same *Fusarium* species leading to different virulence and toxigenic effects, it is worth exploring the efficacy of biocontrol agents against a subset of *Fusarium* strains representing the toxigenic variety existing worldwide. *Streptomyces* spp. are soil dwelling bacteria, which have been exploited mainly for antibiotic production in human and veterinary medicine, but they are becoming important also within the agricultural framework due their efficacy to promote plant growth and protect from pathogens. Recently, many streptomycetes have been considered valuable as BCAs against toxigenic *Fusarium* spp., but rarely their specific ability to minimize/detoxify deoxynivalenol production has been considered. In addition, in the majority of cases, the screening for biocontrol activity is conducted *in vitro* or *in planta* under controlled conditions. Therefore, finding novel approaches to screen and select microorganisms able to counteract toxigenic fungi is necessary to better mimic the abiotic and biotic interactions

occurring in natural environment, but also to take into account the effect on deoxynivalenol production.

This PhD thesis presents our efforts to select and characterize, with novel approaches, streptomycete strains taking into account the tripartite interactions occurring within wheat-*Fusarium* spp. pathosystem.

A pool of *Streptomyces* spp. belonging to the collection of Plant Pathology laboratory of DeFENS (University of Milan, Italy) have been tested for their ability to act as biofungicides, limiting *Fusarium* development reducing disease severity *in planta* and deoxynivalenol production. Since we were searching for microorganisms against cereal pathogens, we used *Streptomyces* strains showing endophytic habitus in graminaceous plants together with strains isolated from other substrates as outliers.

The first experimental step of the work has been focused on the evaluation of the influence of different culture media and *Fusarium* strain diversity in a common procedure for screening antifungal agents (dual culture assay). In addition, we assessed which media used for *in vitro* selection predict the level of antifungal activity assessed *in planta* against FRR and FFR. Dual culture results have been affected by the adopted parameters: media, fungal strain and their interactions had an effect on the level of antifungal activity exhibited by the pool of streptomycetes. Interestingly we observed that the use of common media for *in vitro* screening reduced the level of antifungal activity showed by the strains. In addition, there was a lack of correlation between results obtaining with media used in dual culture and the antagonisms observed *in planta* confirming the inability of common screening procedure to select effective BCAs. However, we tested a medium based on wheat grains, which seems to be the most effective one to elicit antifungals production and give the higher correlation with the observed FRR level of inhibition.

In an effort to improve the selection of biocontrol agents (BCAs) against toxigenic fusaria, the evaluation of their influence on mycotoxin production is required. In order to get a high-throughput screening of novel biocontrol agents (BCAs) or natural products able to inhibit toxin induction and possibly

its biosynthesis we developed a microplate bioassay exploiting the use of fluorescence measured over time and a transgenic strain as a predictor of DON production in *F. graminearum* under highly inductive conditions (a particular nitrogen sources). This method avoids the use of chemical extraction and quantification analysis of the samples which are time consuming and expensive. In this way, we could identify a promising strain (DEF39) which was able, under the tested conditions, to completely suppress the *TRI5::GFP* protein expression without affecting fungal development. Further studies are needed to identify the specific secondary metabolite/s responsible for the observed inhibition.

A second step of the research focused on the evaluation of the plant growth promotion and biocontrol features *in planta* of a group of strains against FRR. Performing germination blotter and soil substrate assays under controlled environmental conditions we observed that none of the strains were able to enhance plant germination parameters but one (DEF09) exhibited consistent efficacy to limit FRR-FFR symptoms severity (protection level > 40%). Therefore, we tested the strains against FHB and we obtained up to 60% of protection under field conditions.

On the basis of the obtained results with *in vitro* and *in planta* screening we tested the ability of four streptomycetes (DEF09, DEF20, DEF39 and DEF48) to reduce deoxynivalenol production in microsilage conditions. We tested two timepoints of BCA application 1) contemporary infection 2) 3 days post pathogen inoculation. The antifungal activity against mycelial development and deoxynivalenol production was observed only when both fungal species and streptomycetes were co-inoculated, suggesting that preventive treatments or, worst case contemporary treatments, should be considered for BCAs efficacy. Interestingly, DEF39 showed a remarkable decrease of toxin production (83%) and the lowest inhibition of biomass of the fungus (40% of ergosterol inhibition). This result confirms outcomes obtained in the high-throughput assay and lead to the hypothesis that the strain arbors the potential to regulate toxin production via specific mechanisms that are not linked to

fungal growth antagonism. DEF48 combined instead the ability to colonize massively the grain as growth substrate and inhibit efficiently the fungal biomass together with DON level, acting as biofungicide.

The identification of the antifungal compounds produced by DEF20, DEF48 and DEF39 was carried out in collaboration with the research group of Prof. Barrie Wilkinson at the Department of Molecular Microbiology of the John Innes Centre. Bioassay guided isolation technique coupled with Illumina sequencing of their genomes were performed. The integration of the antiSMASH bioinformatic analysis to identify secondary metabolite biosynthetic gene clusters, literature searches for antifungal compound composition, and the screening of the UV spectra of the active fractions allowed us to identify molecules from DEF20, DEF39 and DEF48. The opportunity for patenting is currently being explored.

Accordingly, to the results obtained in this study, a pool of promising biocontrol agents was selected acting against fungal development or DON production. This research highlighted the complexity of finding efficient screening procedure due to the several interactions occurring in wheat-*Fusarium* spp. pathosystem. However, we think that further studies could confirm the activity *in planta* as well as lead to the identification of the molecules able to inhibit, directly or indirectly, mycotoxin production.

Implications and future directions

The first part of the PhD project addressed the selection of novel *Streptomyces* strains using common as well as innovative screening methods. Dual culture assay is commonly adopted to evaluate antibiosis against the target pathogen. In many studies, it has been considered inappropriate to select BCA active *in planta* (Pliego et al., 2011). However, *in vivo* tests are not always a feasible option, as they can be expensive and time consuming. Therefore, the evaluation of new culture media able to 1) modulate the production of antifungal metabolites, and 2) resemble the plant tissue, is a strategy to improve dual culture assays. Indeed, a medium based on wheat has been proved to better correlate with the antifungal activity observed *in planta* (Colombo et al., 2019).

The selection of antagonists of toxigenic fungi should take into account their ability to reduce mycotoxin contaminations (He et al., 2009). The development of the microplate bioassay presented in Chapter 2 reduces analysis costs and lowers the use of chemicals and consumables in comparison with common chemical analyses. In addition, in the future this bioassay will be applied to increase the speed and capacity to screen a large set of natural products, as well as purified compounds.

The microplate experiments allowed to observe an interesting activity of culture filtrate from DEF39 strain, which suppresses completely the *TRI5::GFP* protein expression and the total DON production at the end of the period of incubation. Future research will address the antimycotoxigenic metabolites involved. Indeed, previous researches observed the ability of different natural products to act on the mycotoxin biosynthetic pathway, without affecting fungal development (Sakuda, 2010). The purified compound will be applied on wheat heads or in post-harvest to test its activity against trichothecenes also *in planta*.

The promising strains evaluated in the manuscripts presented in Chapter 3 showed the ability to reduce head blight and foot rot severity (DEF09) and to

limit fungal contamination in wheat grains (DEF09, DEF20, DEF39 and DEF48). The reduction of fungal biomass was positively correlated with a reduction of mycotoxin accumulation. Therefore, we obtained a pool of promising BCA to further characterize and ideally develop as commercial products. DEF09, a wheat endophyte, has the ability to counteract *F. graminearum in planta*. Induction of plant defense responses could be the mechanism of action involved, due to the observed activity throughout the experiments performed (e.g. the modification of root architecture, low antifungal activity in dual culture). This mechanism has been rarely described in wheat-*Fusarium* spp. pathosystem. However, one strain of non-pathogenic *Pseudomonas fluorescens* elicited host defense responses and protected barley heads against *F. culmorum* infections (Petti et al., 2010). Therefore, repetition of field experiment will be carried out to confirm the FHB protection, as well as transcriptome analysis to determine the effect of the bacterial treatment on the transcriptome of wheat head tissue. For the other strains, bioassay guided fractionation technique and genome analysis of biosynthetic gene clusters were combined in order to identify the produced antifungal metabolites, possibly active against *F. graminearum.* It is well known that *Streptomyces* spp. have evolved the ability to produce chemically different metabolites at the same time in order to succeed the competition in their niche (Challis and Hopwood, 2003). The identification of antifungals produced by the selected *Streptomyces* strains is essential. Indeed, their application should be preferred to living *Streptomyces* applied as BCA, to avoid the dispersion in the environment of the variety of medical antibiotics they produce (Rey and Dumas, 2017).

Based on our knowledge, it is the first time that the isolated compounds have an effect against *F. graminearum*. Additional experiments will evaluate the antimycotoxigenic activity of compounds found to be produced by DEF39.

References

Challis, G. L., and Hopwood, D. A. (2003). Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc. Natl. Acad. Sci.* 100, 14555–14561. doi:10.1073/pnas.1934677100.

Colombo, E. M., Pizzatti, C., Kunova, A., Gardana, C., Saracchi, M., Cortesi, P., et al. (2019). Evaluation of in-vitro methods to select effective streptomycetes against toxigenic fusaria. *PeerJ* 7, e6905. doi:10.7717/peerj.6905.

He, J., Boland, G. J., and Zhou, T. (2009). Concurrent selection for microbial suppression of *Fusarium graminearum*, Fusarium head blight and deoxynivalenol in wheat. *J. Appl. Microbiol.* 106, 1805–1817. doi:10.1111/j.1365-2672.2009.04147.x.

Petti, C., Khan, M., and Doohan, F. (2010). Lipid transfer proteins and protease inhibitors as key factors in the priming of barley responses to Fusarium head blight disease by a biocontrol strain of *Pseudomonas fluorescens*. *Funct. Integr. Genomics* 10, 619–627. doi:10.1007/s10142-010-0177-0.

Pliego, C., Ramos, C., de Vicente, A., and Cazorla, F. M. (2011). Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. *Plant Soil* 340, 505–520. doi:10.1007/s11104-010-0615-8.

Rey, T., and Dumas, B. (2017). Plenty is no plague: *Streptomyces* symbiosis with crops. *Trends Plant Sci.* 22, 30–37. doi:10.1016/j.tplants.2016.10.008.

Sakuda, S. (2010). Mycotoxin production inhibitors from natural products. *Mycotoxins* 60, 79–86. doi:10.2520/myco.60.79.

PhD activities

Collaborations with other research groups

During my PhD I had the chance to work with other research groups. In particular, in order to test the selected biocontrol agents against deoxynivalenol accumulation in cereals, I worked with Dr. Claudio Gardana and research group of Prof. Paolo Simonetti of the Department of Food, Environmental and Nutritional Sciences of the University of Milan (Italy) in order to set up a protocol for mycotoxin extraction and quantification in flour samples.

The isolation of antifungal metabolites from the streptomycetes selected during the first part of the PhD project was carried out in collaboration with the research group of Prof. Barrie Wilkinson at the Department of Molecular Microbiology (John Innes Centre, Norwich, UK) which is one of the major group working with streptomycete natural products. Therefore, I had the chance to be a visiting worker in his group for five months (March-July 2018) and to acquire knowledge about natural product research and genome analysis for biosynthetic gene clusters. The results have been briefly described in the last chapter of the thesis.

Conferences

The results obtained during my PhD project have been presented at national and international conferences.

Oral presentations:

Colombo E.M., Kunova A., Pizzatti C., Saracchi M., Cortesi P., Gardiner D., Pasquali M. - "Development of a *Fusarium graminearum* biosensor assay to monitor the activity of naturally derived products to control trichothecene production", 7th Conference on physiology of yeasts and filamentous fungi, June 24th-28th 2019, Milan, Italy

Colombo E.M., Kunova A., Pizzatti C., Cortesi P., Saracchi M., Pasquali M. - "*In vitro* study of endophytic streptomycetes and their influence on wheat-

Fusarium spp. pathosystem", First International Congress of Biological Control, May 14th-16th 2018, Beijing, China.

Poster presentations:

Colombo E.M., Batey S., Kunova A., Wilkinson B. - "Identification of antifungal metabolites from a *Streptomyces* sp. effective against *Fusarium graminearum* and *F. culmorum",* XXV National congress Italian Phytopathological Society (SIPaV), September 16th-18th 2019, Milan, Italy.

Colombo E.M., Kunova A., Pizzatti C., Saracchi M., Pasquali M. - "Use of *Streptomyces* spp. as biocontrol agents of wheat crown rot caused by fusaria", poster, ICPP2018: Plant health in a global economy, July 29th-August 3rd 2018, Boston, USA.

Colombo E.M., Gardana C., Kunova A., Pizzatti C., Saracchi M., Pasquali M. - "Killing or modulating. Selection strategies of streptomycetes limiting trichothecene type B production in cereals affected by *Fusarium*" European Fusarium Seminar, April 8th-11th 2018, Tulln, Austria.

Colombo E.M., Kunova A., Pizzatti C., Burrone E., Cortesi P., Saracchi M., Pasquali M. - "Streptomycetes against fusaria: limiting toxin production and fungal growth", XXIII National congress Italian Phytopathological Society (SIPaV), October 4th-6th 2017, Piacenza, Italy.

Colombo E.M., Pasquali M. - "Exploring streptomycetes - fusaria interactions to limit toxin production", 22nd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, September 20th-22nd 2017, Bozen, Italy.

Publications

The results obtained during my PhD have been published in:

Colombo E.M., Pizzatti C., Kunova A., Gardana C., Saracchi M., Cortesi P., Pasquali M. (2019) Evaluation of *in-vitro* methods to select effective streptomycetes against toxigenic fusaria. *PeerJ*, e6905. DOI: 10.7717/peerj.6905.

Colombo E.M., Pizzatti C., Kunova A., Saracchi M., Cortesi P., Pasquali M. (2019). Selection of an endophytic *Streptomyces* sp. strain DEF09 from wheat roots as a biocontrol agent against *Fusarium graminearum*. *Frontiers in Microbiology*, 10, 2356. DOI:10.3389/fmicb.2019.02356.

In addition, a review of literature was submitted to the special issue "*Fusarium* Mycotoxins" of the *International Journal of Molecular Sciences* in September 2019:

Colombo E.M., Kunova A., Cortesi P., Saracchi M., and Pasquali M. (2019) Critical assessment of *Streptomyces* spp. able to control toxigenic fusaria in cereals: a literature and patent review.

The manuscripts "Selection of *Streptomyces* strains limiting trichothecene B production using a *TRI5::GFP* biosensor assay" and "*Fusarium*, *Streptomyces* and wheat grains: studying the interaction and the effect of inoculation timing on deoxynivalenol accumulation *in vitro*" are under final evaluation by the authors and will be submitted to *Toxins*.

Advanced course

In order to acquire experience in prokaryotic genome analysis I attended one week of training course "Annotation and analysis of prokaryotic genomes using the Microscope platform" organized by the LABGeM, a bioinformatic team of Genoscope (the French sequencing center), now part of the France Génomique infrastructure. I acquired basic knowledge in functionalities of the Microscope platform and bacterial metabolism analyses, exploration and

annotation. The course has been held in March 2019 at the University of Evry-Val d'Essone (Paris, France).

Award

- Young researcher in training award for registration to SIPaV Congress held in September 2019 in Milan.
- Travel award for young researcher participating to the "First International Congress of Biological Control" held in May 2018 in Beijing (China), granted by the International Organization for Biological Control (IOBC).

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