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EFFECT OF SOME BACTERIAL ANTAGONISTS ON GROWTH AND MYCOTOXIN PRODUCTION OF *FUSARIUM GRAMINEARUM* AND *F.CULMORUM* ISOLATES

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Abstract

The impact of mycotoxin contamination of foodstuffs and feedstuffs on human and animal health is well documented. Trichothecene mycotoxins are metabolic compounds produced by various Fusarium species such as F.graminearum, F. culmorum, F. sporotrichioides, F. poae and F. equiseti on different grains like wheat, oats or maize. The most significant thrichothecenes produced by fusaria are T-2 toxin, HT-2 toxin, diacetoxyscirpenol (type A) and nivalenol, deoxynivalenol, 3- and 15-acetyldeoxynivalenol (type B). Deoxynivalenol (DON), mainly synthesized by F. graminearum and F. culmorum (that produce Fusarium Head Blight disease = FHB), is the most common trichothecene contaminant of wheat, and appears to play an important role in the aggressiveness of both species toward wheat. In order to prevent the contamination of the cereals with mycotoxigenic fungi, various strategies were developed (cultural practices: tillage, crop rotation; use of fungicides, resistant cultivars or biological control agents = BCAs). Because of the low efficacy of fungicides to control members of the Fusarium head blight Complex, the selection of highly efficient antagonists is of great interest for the specialists all over the world. Environmental friendly, the use of BCA is an important additional strategy that can be used as part of an integrated management of FHB.
For this reason, the aim of our work was to select potential microbial antagonists that are able to inhibit the growth of Fusarium graminearum and F.culmorum, and to prevent the presence of DON on wheat. 37 Fusarium spp. strains were used in experiments and the in vitro aggressiveness of these isolates was determined. Twelve fungal strains with differences in the aggressiveness potential and DON biosynthesis were selected and used for interactions with eight bacterial strains isolated from compost or soil. Four out of the eight bacterial strains used were selected based on their high inhibitory activity against all the fungal isolates. Specific microscopically modifications of hyphae were observed at the edge of fungal colonies near the antagonist. The effect of the bacterial antagonists on DON accumulation in specific culture medium was also checked by TLC method. The results showed the possibility to use one or more bacterial strains as a tool for the biological control of FHB agents that survives in crop residues.

Keywords: *biological control agents, mycotoxins, Fusarium.*

INTRODUCTION

Fusarium head blight is the most important disease of wheat (*Triticum aestivum* L.) being found in large regions of the world. Intensive studies were performed for the identification and characterization of the pathogen, and up to 19 species have been associated with the disease (Parry et al., 1995). Among these species, the most prevalent pathogens are *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schweinitz) Petch] and *Fusarium culmorum* (W. G. Smith) Saccardo (Walter et al., 2010). Moreover, in a recent study, Dean et al. (2012) presented a list of the Top 10 most important fungal plant pathogen, where in fourth place is *Fusarium graminearum,* recognized by the specialists as one of the most significant pathogen for cereals. The importance of *Fusarium* species is related not only to the diseases produced to infected plants but also to mycotoxin biosynthesis (trichothecenes, for example) and accumulation in infected plant material. The

impact of mycotoxin contamination of foodstuffs and feedstuffs on human and animal health is well documented (Cano et al., 2012; Iqbal et al., 2014). Trichothecene mycotoxins are metabolic compounds produced by *Fusarium* species such as *F.graminearum, F.culmorum, F.poae, F.sporotrichioides,* and *F.equiseti* on wheat, oats or maize. It was shown that the most significant thrichothecenes produced by fusaria are: T-2 toxin, HT-2 toxin, diacetoxyscirpenol (type A), deoxynivalenol (DON), nivalenol (NIV), 3- and 15-acetyl deoxynivalenol (type B), mainly synthesized by *F. graminearum* and *F. culmorum*. Recent studies (Audenaert et al., 2013) summarizes the arguments related to the significance of mycotoxins like DON in the competition for niches on crop residues and organic matter: DON is an antimicrobial metabolite effective against various eukaryotic soil organisms (Sorocco et al., 2012), and DON can affect the metabolite production of other soil-residing fungi, such as *Trichoderma* sp., that are known for their strong outcompeting capacity by mycoparasitism (Matarese et al., 2012).

Several strategies have been developed for the control of *Fusarium spp.* infections of cereals (cultural practices: tillage, crop rotation; use of fungicides, resistant cultivars or biological control agents = BCAs) (Mesterházy et al., 2012; Leplat et al., 2013; Schrader et al., 2013). Because of the low efficacy of fungicides to control members of the Fusarium head blight Complex (Mesterházy et al., 2011) the selection of highly efficient antagonists is of great interest for the specialists all over the world (Wagacha and Muthomi, 2007; Vujanovic and Goh, 2011). Environmental friendly, the use of BCA is an important additional strategy that can be used as part of an integrated management of FHB (directed against fungal growth and/or mycotoxins biosynthesis/ accumulation).

The aim of this work was to select potential microbial antagonists isolated from natural sources (soil or compost) that are able to inhibit the growth of *Fusarium graminearum* and *F.culmorum*, and to prevent the accumulation of DON.

MATERIALS AND METHODS

Culture media

The media used in the present study were potato dextrose agar (PDA) for fusaria cultivation and Luria-Bertani medium for maintaining bacterial strains. For mycotoxin production, potato sucrose medium (PSA) was used. The potato sucrose agar media consisted of 200 g sucrose and 20 g agar for a liter of potato extract prepared in laboratory from 200 g peeled potato boiled in water. The reason behind choosing this media for the culture of *Fusarium* strains relies in the findings of Jiao et al. (2008) according to whom the expression of important trichothecene biosynthesis genes (*tri4* and *tri5*) were up-regulated in a high sucrose containing media but not in glucose. Also, Vujanovic and Mansour (2011) reported that 15-ADON chemotype of *Fusarium graminearum* produced DON and ZEA mycotoxins only in the presence of high sucrose concentrations (20% and above).

Biological material

Three strains of phytopathogenic *Fusarium* genus used in experiments are presented in table1.

Table 1. Fungi strains used in experiments

Strain designation	Fungal species	Source
Fc46	*Fusarium culmorum*	NARDI Fundulea (Ittu et al, 2010)
G28	*Fusarium culmorum*	NARDI Fundulea (Ittu et al., 2010)
F183	*Fusarium graminearum* DSMZ 4527	RDIPP, Bucharest
Fs	*Fusarium solani*	Isolated from potato tuber, FB Collection, Bucharest*
FORL	*Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM 2407	RDIPP, Bucharest
F54	*Fusarium spp.*	Isolated from potato tuber, FB Collection, Bucharest*

*Collection of the Faculty of Biotechnology Bucharest

Eight bacterial strains were used in experiments: B1-B6 strains were isolated from compost tea and selected according to their inhibitory action against different fungi (data not shown); *Bacillus amyloliquefaciens* BW (Sicuia et al., 2011) and *Bacillus pumilus* BIR isolated from soil.

Antagonism evaluation

Dual-culture assay was used to examine the degree of fungal inhibition by bacterial strains. Mycelia plugs (5mm diameter) cut from the margin of 6 days old culture of each *Fusarium* strain were placed in the middle of the PDA Petri plates. 10 µl of each bacterial culture were placed at 20 mm from the edge of fungal colony situated at the centre of the plate. The plates were incubated at 26ºC. The mycelial growth of *Fusarium* strains, both in control and dual-culture plates, were linearly measured and recorded daily for 5 days. The antagonistic activity of the bacterial strains was calculated according to Cornea et al. (2008).

In order to examine both the fungal growth inhibition and the effect on mycotoxin production of the selected bacterial strains, the interactions were conducted on PSA medium. Four individual bacterial strains (B1, B5, BW and BIR) and a mixture of these strains (equal volumes of each bacterial strain were combined) were used in a dual-culture manner, as previously described. The plates were incubated for seven days at 26^{o}C and examined in comparison with the control (fungal strains grown on PSA medium without bacteria).

Extraction and analysis of mycotoxins

Mycotoxins (trichotecenes) extraction was performed according to Mačkinaitë (2006) with some adaptations. Sample zones in the shape of a disc of 0.5cm diameter located approximately 0.2 cm behind the contact zone between *F.graminearum/ F.culmorum* and selected bacteria were excised and subjected to mycotoxins extraction. Control samples were extracted from the edges of the *F.graminearum / F.culmorum* growth area following the same procedure. The sample plugs were left overnight in a 5ml of chloroform/methanol mixture (2:1) extraction solution and evaporated under vacuum. The sediment was resuspended in chloroform and 20 µl of each sample was spotted onto TLC plates. Deoxynivalenol standard was used at a concentration of 100 µg mL^{-1} (5µl/spot). The extracts were separated on silica gel 60 plates (10x10 cm, 0.20 mm thick; Roth) with toluene/ethyl acetate/formic acid (5:4:1) as mobile phase. The chromatograms were air dried and the fluorescent spots were revealed under UV light (365 nm) after spraying with 20% aluminium chloride in absolute ethanol and drying at 110ºC for 10 minutes (Vujanovic and Mansour, 2011).

RESULTS AND DISCUSSIONS

Evaluation of the inhibitory effect of bacteria on the Fusarium strains

It was reported by several authors that crop residues, like wheat straw, could be the major sources of the fungal inoculum for fusaria due to the ability of pathogenic fungi to survive in infested debris (as saprotrophic mycelium or as resting chlamydospores). However, the surviving of fungi in such residues can be affected by other microorganisms, via competition, parasitism, and predation or by influencing the rate of plant debris decomposition (Sarroco et al, 2012). For these reasons, the identification of new antagonists from soil is of great interest for preventing plant contamination with fungal pathogens.

Therefore, *in vitro* screening was conducted to determine the ability of various bacterial strains previously isolated from soil or compost (data not shown) to suppress strains of *Fusarium spp.* isolated from different plant host (seeds of *Triticum aestivum* or potato tubers)(table 2).

Table 2. Results of preliminary inhibitory assay

Bacterial strains	Inhibition of fungal growth					
	F183	G28	FC46	FORL	F. solani	F54
B1	+++	+++	+++	+++	+++	+++
B2	+	+	+	++	++	+++
B3	++	+	++	++	++	+
B4	-	-	-	-	-	-
B5	+++	+++	+++	+++	+++	+++
B6	+	+	+	++	++	+
BW	++	+++	++	+++	+++	++
BIR	+++	+++	+++	+++	+++	+++

Where: + + + = strong inhibition of the fungal growth; + + = moderate inhibition of the fungal growth; + = slight inhibition of the fungal growth; – = no inhibition of the fungal growth.

Best results, i.e. inhibition against almost all tested fungi were obtained with the new isolates B1 and B5, as well as with BW and BIR strains (Figure 1).

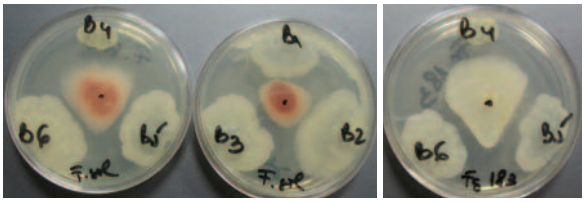

Figure 1. Inhibition of *Fusarium solani* and *F.graminearum F183* by the selected bacterial strains on PDA medium

Based on these results, the selected four strains were used for the inhibition assays on PSA medium, against *F. graminearum* and *F. culmorum* strains, and their inhibitory efficacy was evaluated.

It was shown that the aspect of bacterial cultures was different due to the high sucrose content of the culture medium, but the inhibitory activity was not affected. No clear areas of inhibition between bacteria and fungi were observed on PSA, comparing with the results on PDA; moreover, bacteria tended to cover the edges of fungal colonies (figure 2). The highest inhibition efficacy was detected for B1 strain, and the most reduced, for BW strain (table 3).

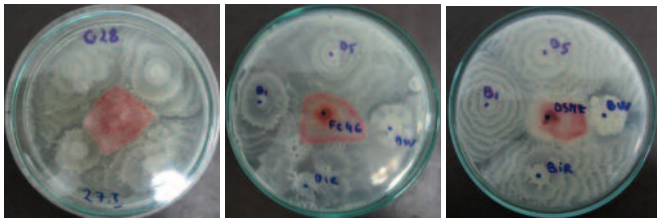

Figure 2. Inhibitory effect of four selected bacterial strains against *F.culmorum* G28*, F.culmorum* Fc46 and *F.graminearum* F183 (DSMZ) on PSA medium

It has been suggested by some authors (Kloepper et al., 2004) that mixing different strains of BCAs can increase efficacy against pathogens, the effect being superior compared to individual strain. In our experiments, when a mixture of all selected bacterial strains (B1, B5, BW and BIR) was tested, the inhibition area was different from that detected for each of the strains when were tested alone (table 3). The highest inhibitory action of the bacterial mixture was observed against *F.graminearum* F183 strain which is highly pathogenic and toxigenic.

Table 3. *In vitro* inhibitory efficacy of the selected bacterial strains against phytopathogenic *Fusarium* spp. strains

Fungal strain	Antifungal efficacy (% of inhibition)				
	B1	B5	BW	BIR	Mixture
FC46	75.6	78.04	53.65	65.85	69
F183	70.1	68.29	43.9	70.7	78
G28	80.24	80.48	58.5	60.9	70.85

The inhibition efficacy of the bacterial strains mixture against the other two fungal strains, Fc46 and G28 (figure 3), was comparable with that of individual bacteria, except BW. These results suggested that the efficacy of the inhibition could be influenced not only by the specific interactions between bacteria but by target fungal pathogen (the natural variability of pathogenic fungi could be an important factor of limiting the *in vivo* efficacy of bacteria based biopreparates). In order to improve the effectiveness of selected bacteria, the optimizing the proportion between bacterial strains in the mixtures is necessary.

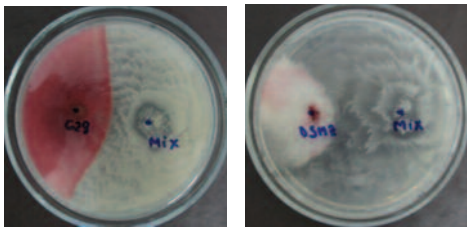

Figure 3. Inhibition of fungal growth (G28 and F183 – DSMZ) by bacterial mixture (B1, B5, BW and BIR)

These findings suggest that the selected bacterial strains could be efficient for the

inhibition of some fusaria. Moreover, previous studies with *B.amyloliquefaciens BW* proved that this strain is able to produce various enzymes associated to plant growth promotion and antifungal activity and could be considered as a competitive rhizobacteria which can be successfully used to bio-activate the vegetal mulch and to control *F.oxysporum* f.sp. *radicis lycopersici* FoRL (Sicuia et al., 2012).
Experiments with bacteria able to suppress various *Fusarium* species development have been reported over time (Sadfi et al. 2001, Johansson et al., 2003, Omar et al., 2006); their results proved the efficiency of natural rhizobacteria as biocontrol agents. The results obtained in this study confirm these findings and the possibility of isolation of new highly antagonistic bacterial strains from natural sources.

Trichothenece production in selected fusaria and influence of bacterial treatment

The genetic ability of *F.graminearum F183, F.culmorum Fc46 and F.culmorum G28* to synthesize DON was previously described, all of them contain *tri 5* gene and *tri7* gene and are able to produce DON at increased level, and to induce severe effects on wheat seeds (no germination was obtained after inoculation) (Cornea et al., 2013). It is well documented that trichothecenes contaminating food and feed are harmful for human and animal health and the prevention of mycotoxins accumulation or their biodegradation is of great interest for scientist. As it was presented above, biological control using bacteria has been explored as an additional or alternative possibility of managing fungal infection and/or mycotoxin accumulation.
In our study, the reduction of DON accumulation in media inoculated with mycotoxigenic fusaria by interaction with natural bacterial isolates was examined. In this respect, antagonistic bacterial strains selected for their significant *in vitro* inhibitory activity against *Fusarium* isolates were co-cultivated with their target on solid PSA medium. After seven days of incubation, the DON was extracted in chloroform and the extracts were examined by TLC methods (figure 4).

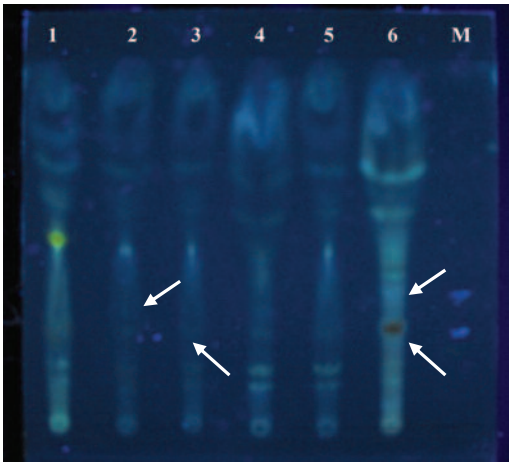

Figure 4. Thin layer chromatography of *F.culmorun* Fc46 extracts: 1 – Fc46 + bacterial mixture; 2 – Fc46 + B1; 3 - Fc46 + B5; 4 – Fc46 + BW; 5 – Fc46 + BIR; 6 – Fc46 control (no bacteria); M – DON standard. The arrows indicate the position of DON

The results proved that in the presence of B1 and B5 bacterial strains, at least in the case of Fc46, no spots corresponding to DON were observed on the TLC plates, most probably due to the biosynthesis inhibition rather than bacterial degradation of mycotoxins, but this conclusion need to be confirmed.
This is, to our knowledge, the first report where inhibition of *Fusarium* species by bacterial biocontrol agents was coupled with chromatographic methods to determine the mycotoxin levels in the fungi-bacteria contact areas. The inhibition areas were observed between the fungi and bacteria but the specific mechanism involved in fungal growth inhibition will be clarified in further experiments.

CONCLUSIONS

The results of this study proved that properly selected bacterial isolates have the potential to significantly suppress the growth of *F.graminearum* and *F.culmorum*.
The efficiency of the inhibition could be influenced by the specific interactions between bacterial strains (competition, antagonism) and by the particularities of target fungal pathogens belonging to the same species. For this reason, the inhibitory efficiency of antagonistic bacteria could be improved by mixing them in specific ration.

The TLC method revealed the effect of biocontrol bacteria on DON biosynthesis/accumulation in tested fusaria, and proved that at least two bacterial strains (B1 and B5, isolated from compost) were able to reduce the level of DON in the extracts of *F.culmorum* Fc46.

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