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Bacillus amyloliquefaciens STRAINS WITH BIOCONTROL POTENTIAL AGAINST *Fusarium* spp. WHEAT PATHOGENS

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Abstract

Wheat contamination with fungi leads to quantitative losses and qualitative depreciation due to mycotoxins accumulation. Currently there are no Fusarium-resistant wheat cultivars. There are only medium tolerant varieties to such infections. In the present study, several Fusarium spp. pathogens were isolated from wheat debris and kernels harvested in 2017 from different lines of Triticum aestivum obtained in the experimental field of NARDI Fundulea. Fungal cultures were identified at genus level based on their microscopic and cultural characteristics on different nutrient media. Fungal growth inhibition of these pathogens was studied in vitro, using strains of Bacillus amyloliquefaciens (BIR, BPA, OS17 and BW) with biological control potential. The biocontrol efficacy, established by biometric measurements, was in the range of 61.9 to 84.4%. Further studies are required to determine the in vivo effects of these biocontrol bacteria on wheat plants.

Key words*: biocontrol, Bacillus amyloliquefaciens, Fusarium sp., wheat.*

INTRODUCTION

Wheat contamination with *Fusarium* species leads to both quantitative loses and qualitative depreciation due to mycotoxin contamination. In Romania, the most common species of *Fusarium* found in wheat and other cereal grains are *F. graminearum*, *F. culmorum*, *F. roseum*, *F. avenaceum*, *F. poae, F. moniliforme* (sin *F. verticillioides*), *F. langsethiae* and *F. nivale* (BASF, 2012; 2019; Şopterean et al., 2012).

In wheat, fusariosis is more virulent on kernels and mature plants, but pathogens can also infect young plants or seeded kernels before emergence.

Currently, there are only tolerant or medium resistant wheat cultivars to fusariosis. Intensive growth of cereal crops, with poor variability in crop rotation, increase *Fusarium* infection pressure.

In special cases, such as plant breading fields and organic farming, where chemical pesticides are forbidden, the infection pressure is very high. Therefore, the only way to reduce pathogen attack is by applying some agrotechnical measures and natural plant protection products (PPPs), as an alternative to chemicals. The list of PPPs, approved by the European Commission, include several microorganisms with antifungal activity as active substances (http://ec.europa.eu).

Among these, *Bacillus amyloliquefaciens* can be used for biological control, either as single strain or as consortia.

Considering these, we focused our study on the characterization of *Fusarium* contaminants isolated from several wheat lines.

The biological control of these fungal pathogens was also analyzed, using four selected strains of *Bacillus*.

MATERIALS AND METHODS

Vegetal material

In order to analyze *Fusarium* contaminants of wheat we used severely infected kernels and spikelet's selected from a breeding field, placed in Călărași County, Romania. *Fusarium* infected samples are presented in Table 1. Samples were collected in 2017 and analysis during 2017 and 2018.

Table 1. *Fusarium* infected samples

Sample code	Vegetal material
E18A 2-4/ Sp4 / 2017 A	Kernels
E18A 2-4/ Sp4 / 2017 B	spikelets constituents
E18A 5-7/ Sp5 / 2017	Kernels
E25A F1-10/ Sp2 /2017 A	Kernels
E25A F1-10/ Sp2 /2017 B	spikelets constituents
E25A F1-10/ Sp3	Kernels
E28A F2-1/ Sp7+Sp8 P2 A	Kernels
E28A F2-1/ Sp7+Sp8 P2 B	spikelets constituents
E24A+F132 = R7S6	Kernels

Biocontrol bacteria

The biocontrol bacteria used in this study were previously isolated and identified as *Bacillus amyloliquefaciens* through Biolog GEN III system and 16S rDNA sequencing (Sicuia et al., 2016). These strains (BIR, BPA, OS17 and BW) were selected due to their antagonistic activity against different fungal pathogens, including *Fusarium* species (Sicuia, 2013; Grosu et al., 2014, 2015).

The Biolog Gen II technique for filamentous fungi was used to establish the biochemical fingerprint of the *Fusarium* isolates.

Fungal isolation

In order to isolate *Fusarium* spp. fungi from naturally infected wheat we use kernels and spikelet constituents presenting clear symptoms of fusariosis. The kernels used were shrivelled, wrinkled, matte, whitish or rosy shades, sometime covered with sporulated mycelia (Figure 1).

Figure 1. *Fusarium* infected wheat samples
a) kernels and
b) spikelet (arrow reveals the infection site)

From the whole wheat spikes were collected only spikelets with sporulated mycelia growing over its constituents (rachilla, glumes, palea and lemma).

The infected kernels and spikelet parts were inoculated on two semi-selective media for *Fusarium* isolation: Nash Snyder medium (also known as Peptone PCNB agar or PPA) and Malachite Green Agar (MGA) supplemented with antibiotics (50 ppm streptomycin and 100 ppm chloramphenicol) (Leslie & Summerell, 2006).

Typical growth of *Fusarium* was subcultured for several times on Potato-Dextrose-Agar (PDA) with antibiotics in order to assure pure cultures.

Fungal characterization

Fusarium cultures were macroscopically characterized when grown on PDA, Maltz extract agar (MA) and Potato-Sucrose-Agar (PSA). Some microscopic aspects were also studied on PDA cultures.

The Biolog Gen II technique for filamentous fungi was used to establish the biochemical fingerprint of some *Fusarium* isolates.

Mycotoxins analysis

The micotoxigenic potential of some fungal isolates was evaluated using TLC analysis (Ursan et al., 2018). Extraction was made from three weeks old *Fusarium* cultures grown on PSA medium.

Biocontrol activity

The biocontrol activity was analyzed by double culture technique. Bacterial strains were placed in spots at 2.5 cm distance from the fungal plugs inoculated in the center of the PDA plates. Incubation was carried out at 28°C. The antifungal efficacy was evaluated compared to the fungal growth obtained in the control plates (Islam et al., 2009).

RESULTS AND DISCUSSIONS

Nine *Fusarium* isolates were obtained from the naturally infected wheat, one from each sample subjected into the analysis.

During isolation better results were obtained on MGA semi-selective agar (Figure 2).

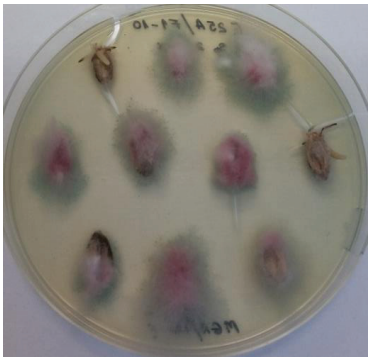

Figure 2. Wheat kernels covered in *Fusarium* growth after incubation on MGA semi-selective medium

MGA medium allowed typical growth of *Fusarium* spp., with less contaminants than PPA. Several other authors also recommend MGA as a better solution that PPA (Alborch, 2010; Bozac et al., 2014). Moreover, due to the fact that PCNB (pentachloronitrobenzene) is carcinogenic, it is less available.

Comparative analysis of the purified stains of *Fusarium* sp. grown on PDA, PSA and MA showed differences in pigment production depending on the growth substrate. Carbohydrate concentration and simple sugars positively affected pigment production in all strains. Red pigments were produced by all Fusarium isolates. Darker red color was obtained on PSA and PDA media, and light red pigmentation was obtained on MA medium (Figure 3).

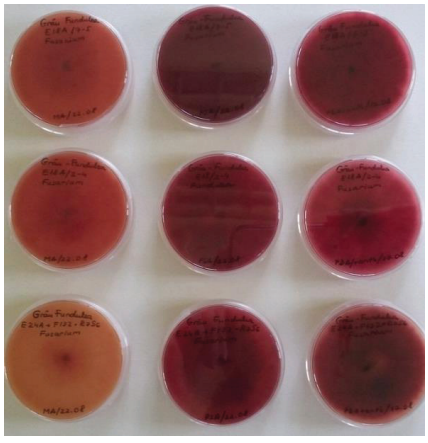

Figure 3. Red pigmentation of three *Fusarium* isolates grown on MA (left), PSA (middle) and PDA (right)

In comparison, in the two weeks old cultures, all *Fusarium* isolates presented the darkest red color on PSA medium, on PDA the pigments were faster developed compared to PSA medium (Figure 4).

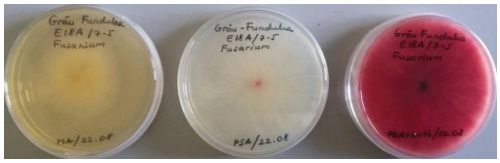

Figure 4. *Fusarium* growth on MA (left), PSA (middle) and PDA (right) after two days of incubation

The highest mycelia growth rate was recorded on PDA medium, followed by PSA. On MA substrate, all fungal isolates had a slower growth rate, with less pigmentation (Figure 4).

The surface color of the cultures varied with strain, and depending on the growth substrate. Pinkish color (mostly on MA) to reddish mycelia (especially on PDA) were noticed, sometime with yellow to orange aerial hyphae on top of the basic red pattern (on PSA and PDA media) (Figure 5).

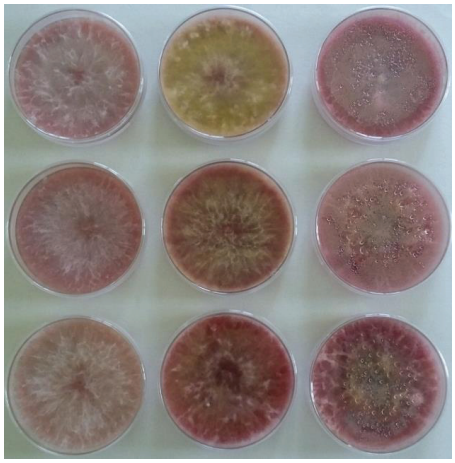

Figure 5. *Fusarium* cultures of E18A/7-5 (top line), E18A/2-4 (center) and E24A+F132=R7S6 (bottom line) grown on MA (left), PSA (middle) and PDA (right)

Studies regarding *Fusarium* pigments mention aurofusarin, neurosporaxanthin, and possibly rubrofusarin, as the most common pigments responsible for surface color in *F. graminearum* (Cambaza, 2018), *F. culmorum, F. avenaceum*, *F. poae* and others, like: *F. acuminatum*, *F. crookwellens*, *F. pseudograminearum*, *F. sambucinum*, *F. sporotrichioides* and *F. tricinctum* (Frandsen et al., 2006).

Microscopic evaluation of the selected fungal isolates was performed only for PDA cultures. Analyzed under light microscopy (40×) all *Fusarium* isolates presented septate filaments, branched in acute angles, hyaline or pale pink colored. Older filaments presented rough surface cell walls (Figure 6a), and the young branches were colorless and smooth. Generally, the abundance of macroconidia was weak. However, in some cultures more than three weeks old, orange sporodochia were formed (Figure 6b), in which abundant macroconidia were present (Figure 6c). Microconidia were detected only in some of the strains. Chlamydospores were produced in both mycelia and macroconidia (Figure 6 d, e). In the mycelia, chlamydospores were found single or in chains.

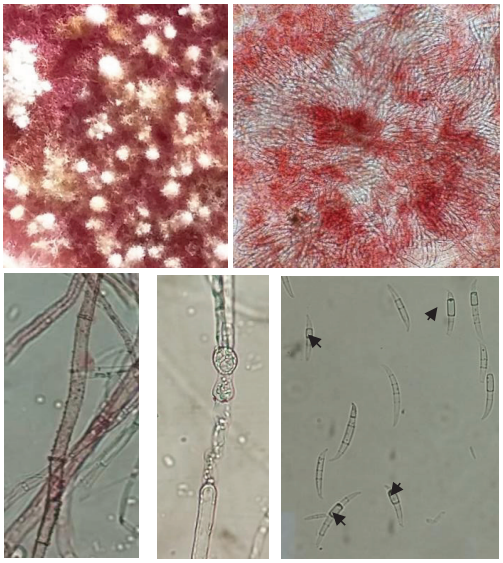

Figure 6. *Fusarium* culture characteristics: sporodochia macroscopic view (a) and microscopic aspects (b), rough filaments (c), chlamydospores within mycelia (d) and macroconidia (e) with chlamydospores (arrow)

Although the isolated fusaria were microscopically analyzed, the identification at specie level was not concluded. However, some of the strains are similar with *Fusarium graminearum*, *F. crookwellense*, *F. chlamydosporium* and *F. tricinctum.* Similar results were also revealed with the Biolog Gen II phenotypic assay for filamentous fungi identification (Figure 7).

Figure 7. Biochemical fingerprint of *Fusarium* sp. E18A/2-4 in Biolog Gen II microplates for filamentous fungi identification

Fusarium infected cereal grains are mostly contaminated with fumonisins and zearalenone (Kim & Vujanovic, 2016). Analyzing the mycotoxigenic potential of E18A/2-4, E18A/5-7 and E24A+F132 = R7S6, it was revealed that the isolated fungi are zearalenone producers (Figure 8).

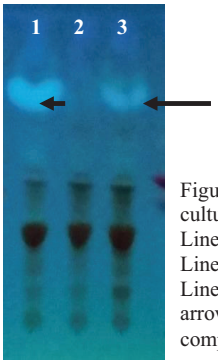

Figure 8. Mycotoxins in *Fusarium* cultures obtained on PSA medium: Line 1- E24A+F132 = R7S6, Line 2 - E18A/2-4 and Line 3 - E18A/5-7 extract, arrow indicates zearalenone compound

The most promising way to reduce mycotoxin contamination is to prevent plants from fungal infection, currently with plant protection products. Microbial based biopesticides such as *Bacillus amyloliquefaciens* can be used in biological control. In the European Union all active ingredients of chemical and biological pesticides must be approved by the European Commission and the commercial product must be authorized for release on the market (http://ec.europa.eu). Currently, in the EU there are four strains of *B. amyloliquefaciens* approved as active substance in pesticide formulations, QST 713, MBI 600, FZB24 and D747. Among these, *B. amyloliquefaciens* (former *B. subtilis*) QST 713 strain is the most common, being approved in 22 countries (BE, CY, CZ, DE, DK, EE, EL, ES, FI, FR, IE, IT, LT, LU, LV, NL, PL, PT, SE, SI, SK, UK).

Considering the biocontrol potential of *B. amyloliquefaciens* strains, we tested four Romanian native strains (BPA, BIR, OS17 and BW) for their antagonistic potential against the isolated *Fusarium* spp (Table 2).

Table 2. *B. amyloliquefaciens* efficacy in reducing *Fusarium* spp. growth

Fungal strain	Biocontrol strains			
	BPA	BIR	OS17	BW
E18A 2-4/ Sp4 / 2017 A	70.5%	70.5%	72.7%	68.2%
E18A 2-4/ Sp4 / 2017 B	74.5%	70.2%	72.5%	70.2%
E18A 5-7/ Sp5 / 2017	75%	71.6%	72.7%	72.7%
E25A F1-10/ Sp2 /2017 A	84.4%	77.8%	77.8%	75.6%
E25A F1-10/ Sp2 /2017 B	66.7%	61.9%	61.9%	61.9%
E25A F1-10/ Sp3	72.5%	67.5%	75%	70%
E28A F2-1/ Sp7+Sp8 P2 A	80.5%	75.6%	73.2%	75.6%
E28A F2-1/ Sp7+Sp8 P2 B	83.3%	83.3%	83.3%	83.3%

Among the tested strains BPA revealed the highest potential in reducing *Fusarium* spp. growth. During *in vitro* trials a synergic effect was noticed between BPA and OS17 strains in reducing fungal growth (Figure 9).

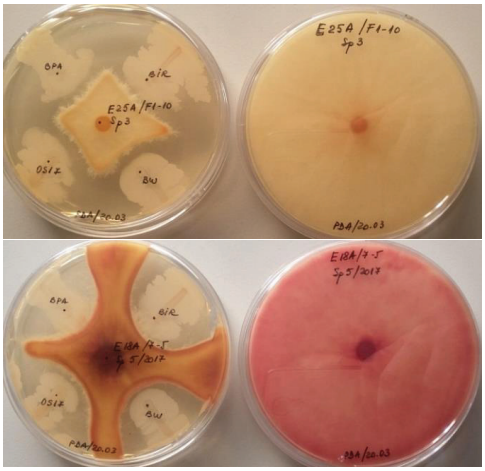

Figure 9. Antifungal activity of *B. amyloliquefaciens* against *Fusarium* spp.

These bacterial strains presented a wide spectrum of antifungal activity. Previous studies mentioned these strains as promising biocontrol agents against mycotoxigenic *Fusarium graminearum* and *F. culmorum* (Grosu et al., 2014), and several other fungal pathogens, like: *Alternaria* spp., *Botrytis cinerea*, *Fusarium oxysporum*, *F. solani*, *Macrophomina phaseolina*, *Penicillium* spp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum* (Dinu et al., 2012; Sicuia et al., 2011; 2012a, b; Grosu et al., 2014; Sicuia et al., 2013).

CONCLUSIONS

Wheat samples (plant debris and grains) severely contaminated with *Fusarium* were purchased from a breeding field, in Călărași County, Romania.

New strains of phytopatogenic fungi belonging to *Fusarium* spp. were detected and characterized. Among the tested *Fusarium* semi-selective media, MGA was found to be more appropriate than PPA. The isolated *Fusarium* spp. were highly virulent, and some proved to be mycotoxin producers.

Biological control methods can be used in order to reduce the fungal growth of such pathogens. Four Romanian native strains of *Bacillus amyloliquefaciens* (BPA, BIR, OS17 and BW) prove to have good inhibitory activity against the newly isolated fusaria, with an efficacy of 61.9 to 84.4%.

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