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# NEW SCREENING METHODS FOR EVALUATION OF *FUSARIUM* SPORULATION INHIBITION BY *BACILLUS* BIOCONTROL STRAINS

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***Abstract***

*In vitro antagonistic activity of microorganisms is the first approach in the selection of biocontrol microbial strains. The method usually used in this direction is the double culture technique. This method has been improved, in time, in order to be more relevant for the inhibition of some pathogenic growth. However, pathogenic growth reduction is not sufficient in agricultural systems. The vegetative growth inhibition must be complemented by suppression of resistant and/or spreading forms of the pathogens so that the probability of pathogen dissemination will be reduced. Due to these requirements we propose new screening methods to determine the sporulation inhibition, using Fusarium species as a model. Within this study we demonstrated the functionality of these new methods using Bacillus biocontrol strains. The methods are based on the quantification of spores formation, spores dissemination and germination when growing the fungus together with the bacterial biocontrol strains.*

***Key words:*** *sporulation inhibition, biocontrol*

## INTRODUCTION

In the agricultural field diseases suppression is an important step in crop production. Chemical pesticides were found to have the largest inhibition effect against phyto-pathogenic microorganisms [4]. There are many chemical fungicides available to control the important crop diseases caused by different phyto-pathogenic fungi [1]. However, chemical pesticides are not ideal for long term application due to the concerns of exposure risks, residual persistence and tolerance of pathogenic fungi [8]. Biological treatments that reduce fungal and spore formation could be an alternative to chemical pesticides, with benefits for the environment.

For the biological control of phytopathogenic fungi, microbial biocontrol agents and spices are cited for their antifungal and antitoxigenic activities [2, 3].

For the selection of biocontrol alternatives there are numerous analytical and cultural methods described for the detection and quantification of fungal inhibition [5, 6]. Fungal inhibition methods must relay on mycelial growth suppression, sporulation inhibition and mycotoxins reduction.

Since pathogenic growth reduction is not sufficient for plant diseases suppression in agricultural systems, the vegetative growth inhibition must be complemented by suppression of resistant and/or spreading forms of the pathogens so that the probability of pathogen dissemination will be reduced. Due to these requirements we propose new screening methods to determine the sporulation inhibition, using *Fusarium* species as a model. Within this study we demonstrated the efficacy of these new methods using *Bacillus* biocontrol strains. These methods are based on the quantification of spores formation, spores dissemination and germination when growing the fungus together with the bacterial biocontrol strains.

## MATERIALS AND METHODS

### *Fungal material*

The phytopathogenic fungi concerned in *Fusarium graminearum* DSM 4527 strain and *Fusarium oxysporum* f.sp.*radicis lycopersici* ZUM 2407 strain. Fungi were routinely grown on Potato-Dextrose-Agar (PDA) for maintenance.

To obtain fungal spore suspension the fungi were multiplied in Czapek-Dox broth medium at 25°C and 150rpm shaking for 5 days.

The fungal broth culture was then filtered through four cheesecloth layers in order to harvest only the spore suspension, which was adjusted to a concentration of $10^6$ spores /ml, revealed by counting on Bürker-Türk chamber.

### *Bacterial inoculums*

Three bacterial strains were previously selected for their antagonist activity against several phytopathogenic fungi [7, 9].

Bacterial strains used in this study were Romanian isolates of *Bacillus pumilus* OS15 strain, *Bacillus amyloliquefaciens* OS17 and BW strains. The origin of this strain is presented in table 1.

Bacteria were routinely grown on Luria Bertani medium at 28°C.

To obtain the bacterial cell suspension, 48h old broth cultures were centrifuged at 3750rpm, for 20minutes, at 10ºC and pelet was resuspended in saline phosphate buffer up to the desired concentration.

The microbial load of the bacterial suspension was spectrophoto-metrically evaluated at $10^8$cfu/ml when the optical density reached the absorbance of 1 at 600nm wavelength.

Table 1. Biocontrol bacterial strains used in the experiments

| Strain | Source | Provenience |
|---|---|---|
| *Bacillus pumilus* OS15 | Isolated from onion rhizosphere | RDIPP - Bucharest collection |
| *Bacillus amyloliquefacien* OS17 | Isolated from onion rhizosphere | RDIPP - Bucharest collection |
| *Bacillus amyloliquefacien* BW | Isolated from soil | Faculty of Biotechnology Bucharest Collection |

Bacterial inoculum, consisting of filtered supernatant, was obtained from bacterial fresh cultures, where broth cultures were centrifuged and the resulted supernatant was filtered through a 0.22µm membrane (Millex®GP) so that no bacterial cell would be in the final liquid.

### *In situ* evaluation of *Fusarium graminearum* spores disemination inhibition by biocontrol bacterial strains

Spores inhibition test was evaluated *in situ* on wheat straw leftovers which is one of the natural substrates for *F. graminearum* development in the agricultural environments. The experiment was made in situ Roux plate (20cm/16cm/5cm). The growth media concerned in grinded wheat straw moistened with saline phosphate buffer at 3:10 w/v rate.

Plates were inoculated with 4 ml of fungal spore suspension ($1\times10^6$ spores /ml concentration), linearly distributed at 4 cm from the end of the plate. The bacterial treatment consisted in 8ml inoculum of $10^8$cfu/ml, linearly distributed and parallel to the fungal inoculum at 8cm distance from it. Plates were incubated at room temperature in daylight conditions, for one week, when spore traps were installed inside the Roux pates. The spore traps consisted in sterile glass slides, loaded with100µl PDA, and placed obliquely, as in figure 1. After spore traps installation, the plates were incubated for other 3 days on previously mentioned conditions.

At the same time a negative control was prepared only with *Fusarium graminearum* inoculum and chemical control with thiophanate methyl to compare the bacterial treatments with a standardized fungicide, Topsin 500SC, in 0.25% concentration and the same dose as the biological treatments.

To compare the bacterial treatments with a standardized fungicide we prepared a chemical control with Topsin 500SC (thiophanate methyl) in 0.25% concentration and with the same dose as the biological treatments.

At the same time a negative control was prepared only with *Fusarium graminearum* inoculum.

Treatment ability to inhibit spore formation and propagation was evaluated by microscopic analysis of the spore traps.

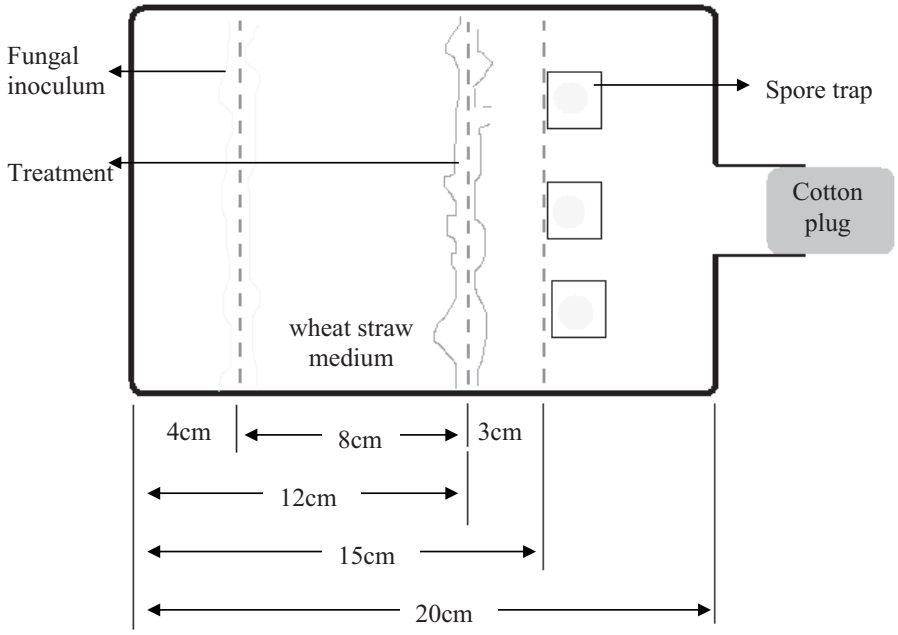


Figure 1. Schematic presentation of the Roux plates prepared for *in situ* evaluation of *Fusarium graminearum* inhibition into spores formation and propagation.

***In vitro* evaluation of *Fusarium oxysporum* f.sp. *radicis lycopersici* sporulation inhibition by biocontrol bacterial strains**

Test was performed on LB broth medium, 10ml/sample. Fungal inoculum consisted in two micelial plugs, 6mm in diameter, colected from fungal growth with the same age. Bacterial treatment concerned in filtred supernatant from bacterial fresh cultures obtained in LB broth medium. Chemical control consisted of thiophanate methyl treatment with Topsin 500SC at 0,14% final concentration. As a negative control we used untreated samples incubated in the same condition with the other experimental variants. Samples were incubated at 28ºC with 150rpm shaking for 5 days. Spores counting was made after three and five days of incubation. Spores concentration was evaluated on Bürker-Türk chamber. The test was performed three times, each sample having three replicates.

***In vitro* evaluation of the ability to delay spore germination of *Fusarium oxysporum* f.sp. *radicis lycopersici* spores using biocontrol bacterial strains**

The experiment was carried out on sterile glass slides loaded with 100µl of PDA medium. Slides were inoculated with 10µl spores suspension of *Fusarium oxysporum* f.sp. *radicis lycopersici* in $2\times10^6$spors/ml concentration. This led to a load of $2\times10^4$ spores/slide. Biological treatments consisted in application of 20µl filtered supernatant from bacterial fresh cultures. Slides were preserved in sterile Petri plates and incubated for 3 hours at room temperature.

Samples visualization was performed with an optical microscope using the 40X objective. The growth of the fungal filaments generated by the spore germination was precisely determined by processing the images captured from the microscope with APS Assess 2.0 soft. In this way the hyphal growth from the spore germination has been measured and compared with those from the untreated control. This appreciation is possible because of the identical conditions of images collection: same microscope (MC1, IOR), distances from the sample, magnification, camera (Model GN-B100/SA/W5, Gen Security) and attributes of the captured images.

## RESULTS AND DISCUSSION

### *In situ* evaluation of *Fusarium graminearum* spores dissemination inhibition by biocontrol bacterial strains

Treatment evaluation of spore inhibition formation and propagation was evaluated by microscopic analysis of the spore traps (photo1).

Spore formation and propagation detected in the negative control were estimated to be above all treated samples. In this experimental variant spores were evaluated as having significantly higher size compared with treated variants. In the chemical control the disseminated spore number was considerably smaller than the negative control (three times smaller). Biological treatments with BW and OS15 registered spore number/ traps was smaller than in chemical control and compared with the negative control were 10X and 4X respectively smaller than in non-treated control variant. OS17 treatment reduced spore dissemination by 2X comparing with the non-treated control variant. Results of the spore dissemination are presented in table 2.

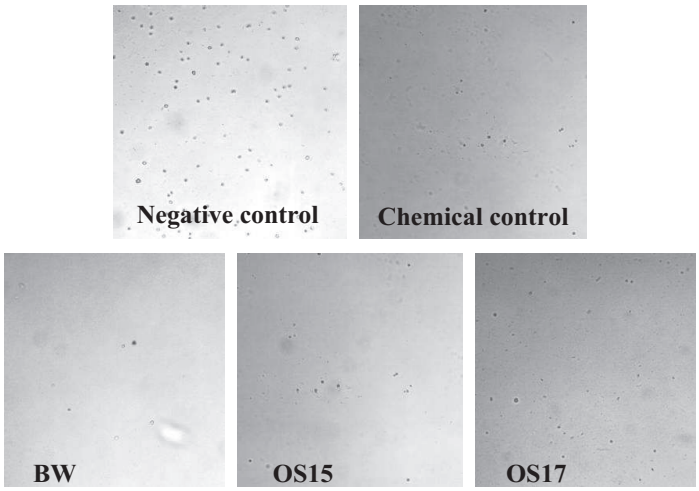


Photo 1. Image captures from the spore traps

Table 2. The inhibition of *Fusarium graminearum* DSM4527 spore dissemination

| Experimental variants | Spore load / trap with 100µl PDA medium |
|---|---|
| Negative control | $2.4 \times 10^6$ spores |
| Chemical control | $7.8 \times 10^5$ spores |
| *Bacillus amyloliquefacien* BW treatment | $2.5 \times 10^5$ spores |
| *Bacillus pumilus* OS15 treatment | $6.3 \times 10^5$ spores |
| *Bacillus amyloliquefacien* OS17 treatment | $1.2 \times 10^6$ spores |

General view of the Roux plates is revealed in photo 2. The visual appreciation of the biological treatments showed a reduced mycelial density than in controls.

### *In vitro* evaluation of *Fusarium oxysporum* f.sp. *radicis lycopersici* sporulation inhibition by biocontrol bacterial strains

According to the representation in table 3, the most efficient treatment in sporulation inhibition was found in the chemical control.

The inhibitory effect of the bacterial filtered supernatant on fungal sporulation revealed that biological treatment with BW strain had similar results with the thiophanate methyl treatment regarding spores inhibition. All treatments reduced sporulation compared to untreated control where an average sporulation of $3.2 \times 10^7$ was found after 5 days of incubation.

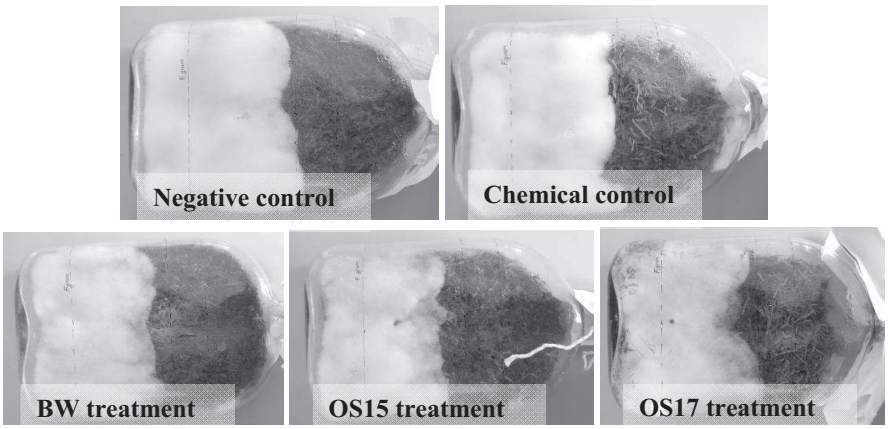


Photo 2. General view from the *in situ* evaluation of *Fusarium graminearum* spores inhibition

Table 3. The inhibition of *Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM 2407 spores formation

| Experimental variant | *Fusarium* spore concentration (spores/ml) | |
|---|---|---|
| | Three day incubation | Five days incubation |
| *Bacillus amyloliquefaciens* BW | $1{,}6 \times 10^5$ | $1{,}7 \times 10^5$ |
| *Bacillus pumilus* OS15 | $5{,}2 \times 10^5$ | $1{,}2 \times 10^6$ |
| *Bacillus amyloliquefaciens* OS17 | $1{,}0 \times 10^5$ | $2{,}3 \times 10^5$ |
| Chemical treatment | $1{,}1 \times 10^5$ | $1{,}3 \times 10^5$ |
| Negative control (Forl) | $5{,}9 \times 10^6$ | $3{,}2 \times 10^7$ |

The inhibitory effect on sporulation increases the efficacy of the treatments, since the sporulation reduction has an additional impact on the fungus capacity of propagation.

***In vitro* evaluation of the ability to delay spore germination of *Fusarium oxysporum* f.sp. *radicis lycopersici* spores using biocontrol bacterial strains**

Compared with the control all treatments reduced spores germination (see photo 3). The most efficient was *B. pumilus* OS15 treatment that reduced spores germination by 90.9% compared with the untreated control, followed by the *B. amyloliquefaciens* OS17 and BW treatments which reduced spores germination by 82.8% and 81.8% respectively.

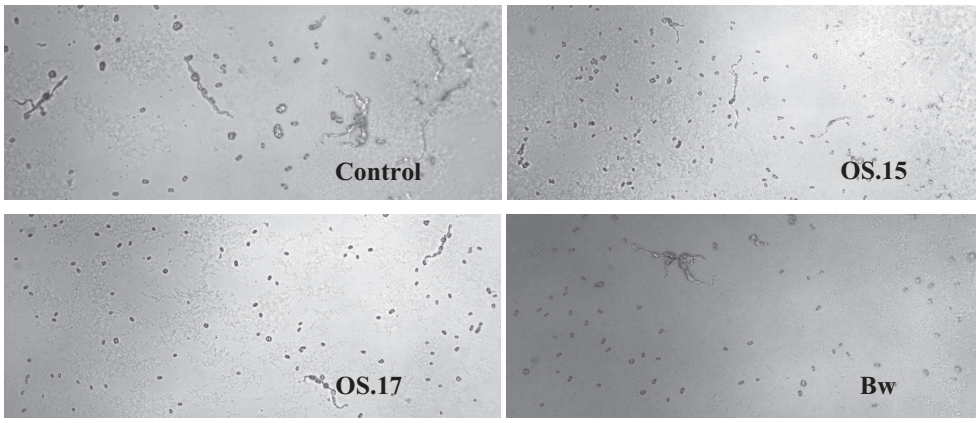


Photo 3. Delayed germination of *Fusarium oxysporum* f.sp. *radicis lycopersici* ZUM 2407 spores induced by the treatments with bacterial filtered supernatant

## CONCLUSIONS

Spores dissemination inhibition assay is an innovative method for the evaluation of *Fusarium* propagation.

The bacterial treatment efficacy into inhibiting spores spreading and reducing spores germination revealed that tested *Bacillus* strains are capable to diminish *Fusarium* disease dissemination.

The tested bacterial strains were able to reduce *Fusarium* sporulation, the results obtained in the biological treatment with BW strain being comparable to those from the chemical control.

Considering the antifungal properties of the tested *Bacillus* strains (OS15 of *B. pumilus*, OS17 and BW of *B. amyloliquefaciens*) from others studies and the sporulation inhibition capacity we can say that these strains are efficient biocontrol agents that can suppress phytopathogenic fungi disease.

The antifungic activity and plant growth promotion proved by these strains in other studies correlated with the results revealed through this paper offers great prospects in plant protection technologies for these biocontrol strains.

Research performed on these bacterial strains proved that they are competitive rhizobacteria which can be successfully used to improve alternative agricultural systems by reducing the incidence of the phytopathogenic fungi attack.

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