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# In vivo biocontrol activity of *bacillus* spp. strains on *alternaria tenuis*

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## Abstract

*Alternaria diseases are among the most common diseases of many plants in the world [1]. They affect primarily the leaves, stems, flowers and fruits of annual plants, especially vegetables [2]. The overuse of chemical pesticides has caused soil pollution and harmful effects on human beings. Accordingly, biological control of soil-borne diseases has been attracting attention. Many reports or reviews in this area have already appeared [3, 4, 5, 6]. Biological control agents are potential alternatives for the chemical fungicides presently used in agriculture to fight plant diseases. Bacillus ssp, strains are an example of a promising safe fungal biological control agents. This paper, describes that B.subtilis B2, B. licheniformis B40, B.subtilis mB2.9, B.subtilis tB2.2, B. licheniformis mB40.5, B. licheniformis tB40.2, which showed in vitro antibiotic activities against two strains of Alternaria tenuis [7, 8], were subjected to a pot test to investigate their ability to protect the plant against fungal disease.*

Keywords: biocontrol, antifungal compounds, Bacillus spp., phythopathogenic fungi,

## Introduction

Fungal diseases can have major constraints on crop production and, in conventional agriculture, chemical fungicides are routinely used to provide disease control. However, as these chemicals are often toxic and potentially harmful to both man and the environment, alternative methods of control are needed. Biological control is a potential alternative approach to chemical treatment and there are a number of fungal biocontrol products commercially available in Europe, including some that are registered as biopesticides. These are likely to increase in number in the coming years.

Our researches was focused primarily to select *Bacillus* strains with antifungal potential, to obtain improved strains with increased activity against the plant pathogen *Alternaria tenuis*, to optimize culture conditions for the production of antifungal compounds [7, 8].

The aim of our research presented in this paper was to test *in vivo* biocontrol ability of our *Bacillus* strains on the phytopathogen *Alternaria tenuis. On planta* were tested both culture broth and antifungal compounds extracted in alcohol.

## Material and methods

*Microbial strains*: in the experiments two fungal strains were used: *Alternaria tenuis alt a1*, *A.tenuis alt a2* and seven bacterial strains: *B.subtilis* B2, *B.subtilis* mB2.9, *B.subtilis* tB2.2, *B. licheniformis* B40, *B.licheniformis* mB40.5, *B.licheniformis* tB40.2, *B.subtilis lob1*(from Dr Flori Constantinescu, Institute of Plant Protection, Bucharest).

*Media*: L agar (meat extract 10g; peptone 10g, NaCl 5g; agar 15 – 20g, water 1000ml, pH =7,2); medium for iturin production (polypeptone 1%; glucose 1%; $KH_2PO_4$; $MgSO_4$ x $7H_2O$ 0,05%; pH =7,0); Sabouraud medium (glucose 15g; peptone 5g; agar 10g; distilled water 1000 ml; pH = 5,5 – 5,7). Culture conditions were presented previously [7].

*Extraction of inhibitory compounds from the culture medium* was performed according to the indications of Phae et al. for iturin [9]. After three days of cultivation on solid medium, the inhibitory compounds were extracted for 1h with 45 ml of methanol for each 15g of the medium. When the bacteria were cultivated in liquid medium, culture broth was centrifuged at 7000 rpm for 10 minutes to remove cells and the pH of the supernatant was adjusted to 2.0 with conc. HCl to allow precipitation of peptides. The precipitates resulted were re-collected by centrifugation (10.000 rpm for 10 minutes) and extracted with methanol of 1/10 of the culture volume. In ano*ther experimental variant we used ethanol of different concentrations (95%, 70%) for extraction of antifungal compounds [10]. Finally both extracts (methanolic and ethanolic) were used in further experiments.

*Thin layer chromatography* (TLC) was performed on silicagel plates using chloroform/ methanol/ethanol /distilled water: 70/30/35/15, v/v/v/v, and revealed by staining with 1% ninhydrin reagent in acetone in order to separate antifungal compounds.

*Detection of antagonistic activities*.

*In vitro*, detection of the antifungal action of alcoholic extracts with antifungal activities has been done by Manka method [11].

*In vivo* testing of antifungal potential of bacilli strains has been done by measuring on pepper length and counting number of leaves at five days, respectively by counting germinated cucumber at every two days. The soil used in those studies was autoclaved for 60 min at $121^0$C four times at 12-h intervals.

*Pepper transplants* were chosen to be almost similar as length and number of leaves and were planted in plastic pots with sterilized soil. Cucumber seeds were surface-disinfected in 5% (w/v) sodium hypochloride for 30 min and rinsed with sterile distilled water. and placed on plastic pots. Bacteria were applied to the soil by spraying culture broth*. In a similar way were applied alcoholic extracts, respectively fungal spore suspension. In the uninoculated control, the soil was sprayed with sterile distilled water.

## Results and discussion

*Extraction of inhibitory compounds from culture medium*

The application of the method described by Phae for the isolation of inhibitory compounds similar to iturin (lipopeptide family) allowed us to obtain concentrated antifungal sample compounds.

Both methanolic and ethanolic extracts presented inhibitory action against *Alternaria*. The activity was preserved even after 30 days of fungal strain cultivation (fig.1).

*In a similar way, alcoholic extracts and fungal spore suspension respectively were applied.

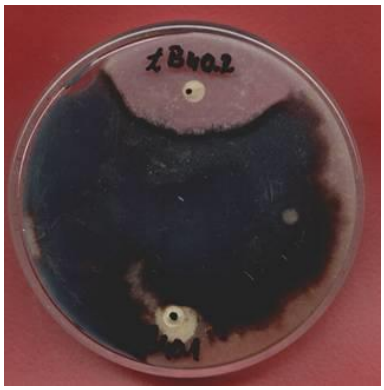



**Fig.1**. Inhibitory action of ethanolic extracts of *Bacillus licheniformis* tB40.1 and tB40.2, after 30 days of cultivation

The extracts obtained were subjected to separation by thin layer chromatography. As can be seen in fig 2 and 3, the alcoholic extracts obtained from mutant strain mB2.9 and transformant strain tB2.2 contain more increased quantities of antifungal compounds than parental strain B2 (the spots were intensly colored).

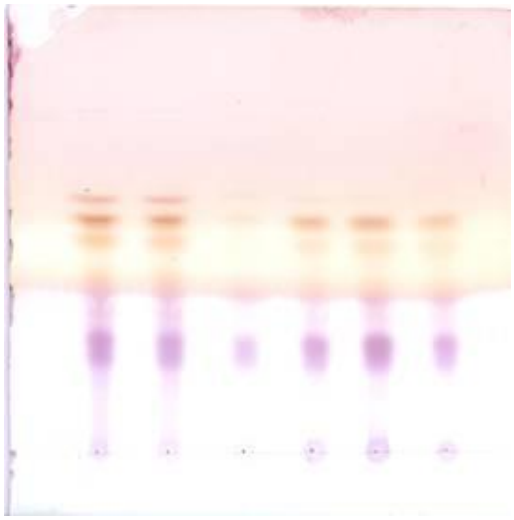


**Fig.2.** Chromatography on silicagel plates (TLC) of the ethanolic extracts from different *Bacillus spp.* strains: B1 (1), B2(2), B17(3), tB1,3(4), mB2.9(5), tB2.2(6)

Even if is preferable to run known and unknown compounds on the same TLC plate, in the absence of iturin control, we ran TLC under the same conditions presented in the literature by different authors [12] and then we compared the $R_f$ of our compounds with the $R_f$ of iturin.

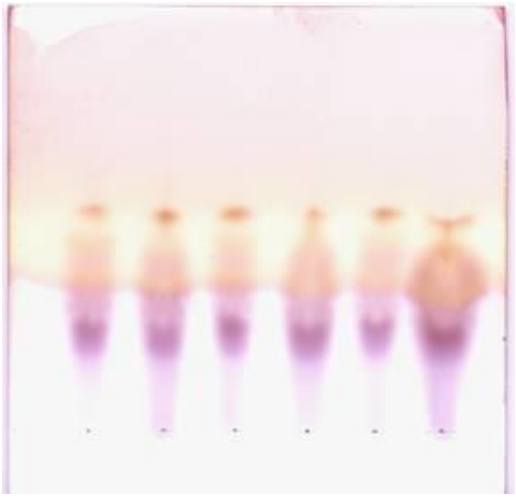


**Fig.3**. Chromatography on silicagel plates (TLC) of the methanolic extracts from different *Bacillus spp.* strains: B1 (1), B2(2), B17(3), tB1,3(4), mB2.9(5), tB2.2(6)

All antifungal compounds were soluble and were extracted in alcohol. The precipitate obtained after extraction didn't exhibit any antifungal abilities as compared to alcoholic extracts and culture broth (fig 4). The best results were obtained with ethanolic extract 95% (colonies of *Alternaria* diameter have maximum 5 mm).

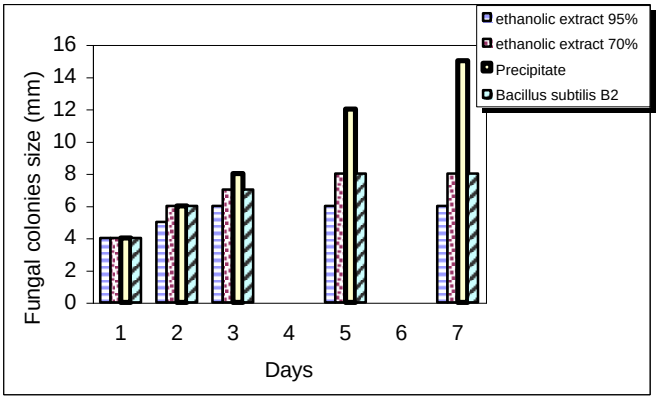



Fig.4. Inhibitory effect of ethanolic extracts obtained from B2 against *Alternaria tenuis*

*In vivo detection of antagonistic activities*

Antagonistic activities were also detected in vivo by using pepper transplants and cucumber seeds. The pepper transplants were put in plastic pots with sterilized soil. Three kinds of experiments were made: (A) plants were treated at the same time with fungi and biocontrol agents (*B.subtilis* B2, *B.subtilis* mB2.9, *B.subtilis* tB2.2, *B.licheniformis* B40, *B.licheniformis* mB40.5, *B.licheniformis* tB40.2, *B.subtilis lob1*), (B) plants were treated first with fungal suspension and after one week with biocontrol agents, (C) plants were treated first with biocontrol agents and after one week with fungal suspension.

Bacteria were applied to the soil by spraying 2 ml culture broth*. In a similar way were applied alcoholic extracts (200µl and 1800µl sterile distilled water), 2 ml fungal spore suspension respectively. In the uninoculated control, the soil was sprayed with 2 ml sterile distilled water. The detection of the antifungal action has been done by measuring the length of plants and counting the number of leaves, every 5 days up to 25 days. Experiments were run in triplicate.

In all three experiments, transformant strains had increased the antifungal potential more than the parental strains (confirming the results obtained *in vitro*) (fig 5). The best results were obtained in experiment (C), when biocontrol agents were applied before fungal infection.

(1) (2)

**Fig.5**. *In vivo* detection of antifungal potential on pepper transplants of B2, mB2.9 (1), B40, mB40.5 (2) against *Alternaria tenuis* a1 as compared to the plant infected with fungal suspension (M II). (exp. C)

Experiments (A) and (B) also exhibit good results and show that even biocontrol agents were applied at the same time or after fungal infection, those can protect plants against *Alternaria* disease.

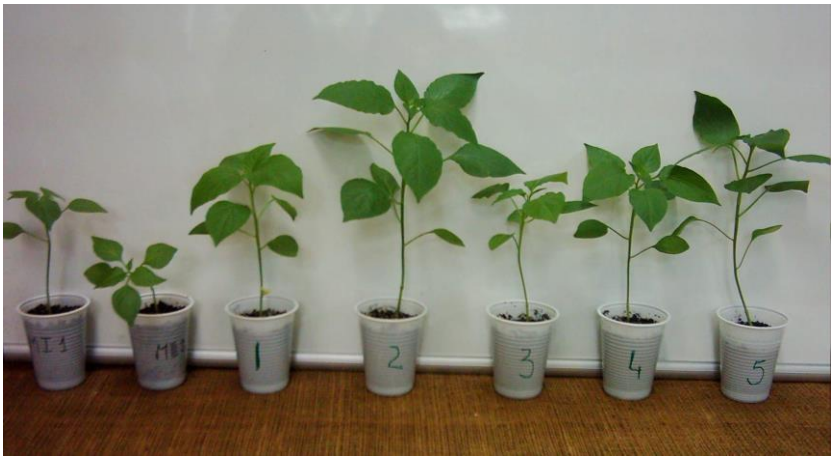



**Fig.6**. *In vivo* detection of antifungal potential on pepper transplants of B2 (1), tB2.2 (2), *B. sub*tilis lob1 (3), B40 (4), tB40.2 (5) against *Alternaria tenuis* a1 comparing with control plant (M I 1) and the plant infected with fungal suspension (M II). (exp. C)

*In a similar way, alcoholic extracts (200 µl and 1800 µl sterile distilled water), and 200 ml fungal pore suspension respectively were applied.

An interesting observation was that both bacteria and alcoholic extracts stimulated the growth of plants designated 1, 2, 3, 4, 5 in fig 7 in all three experiment (A, B, C) (fig 7).

Even trought they were treated with fungal suspension, plants designed 1, 2, 4, 5 in fig 7, grow better than control plants, so *in vivo* analysis of this strain proved that it can be used both for antifungal protection as well as plant growth promoting bacteria.

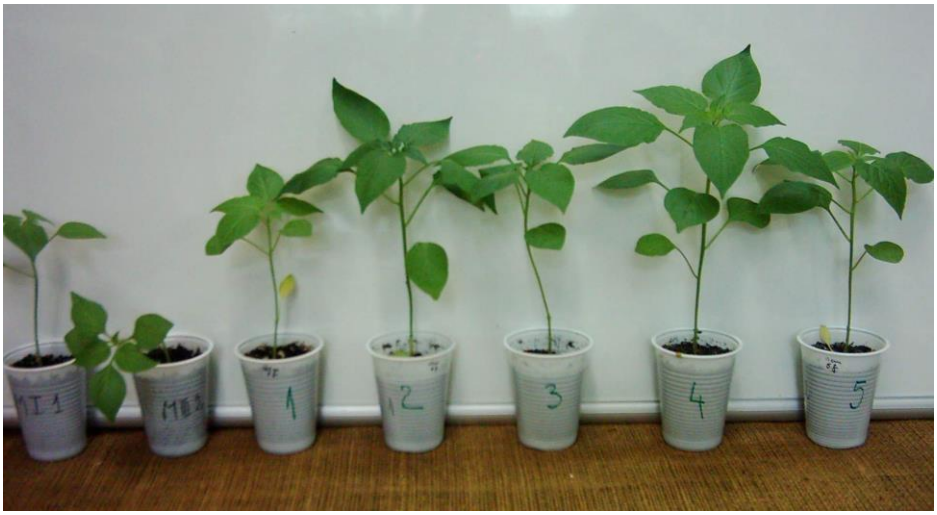
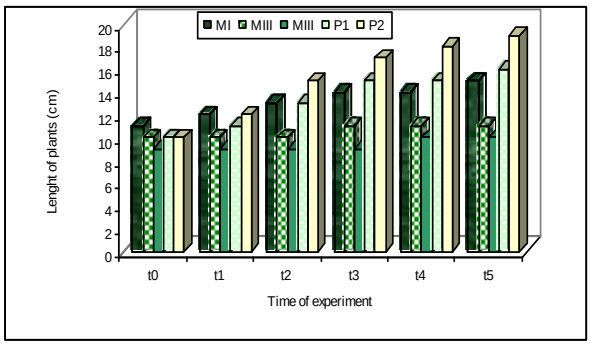
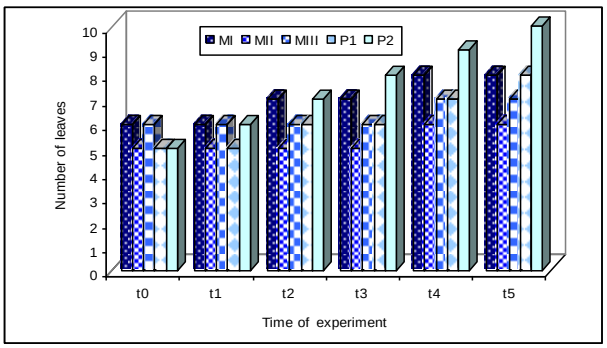



**Fig.7**. In vivo detection of antifungal potential on pepper transplants of *B. sub*tilis lob1 (1), B2 culture broth (2), methanolic extracts from B2 (3), B40 culture broth (4), methanolic extracts from B40 (5) against *Alternaria tenuis* as compared to control plant (M I 1) and the plant infected with fungal suspension (M II). (exp. C)

Comparing the length of pepper plants and the number of leaves growth in control pot with the A, B, C variants we see that the best growth promoting effect was obtained with culture broth (fig 8).




(1)




(2)

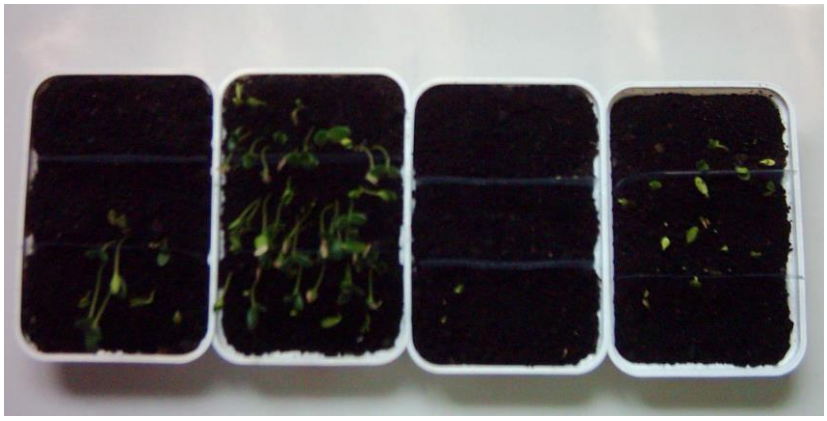
**Fig.8.** *In vivo* detection of antifungal potential on pepper transplants of B40: length of plants (1), number of leaves (2). Methanolic extract from B40 – P1, culture broth from B40 –P2 against *Alternaria tenuis* a1 as compared to control plant -M I, plants infected with fungal suspension -M II and plants treated with methanol – M3; t0-first day of experiment, twenty-fifth day (exp. C)

If *in vitro* determination, alcoholic extracts have increased potential than culture broth because were more concentrated, *in vivo*, culture broth has increase antifungal potential. That means, culture broth of our *Bacillus* strains can be used in order to obtain new products for improvement of plant establishment and plant growth.

The major mechanisms for disease suppression by plant growth promoting bacteria are based on competition for nutrients (competition for iron) and antibiosis [13]. Our understanding at the molecular level of the interaction between bacteria and fungi is poor and in this moment is hard to explain all mechanisms involved.

When cucumber seeds were used in experiments, bacteria were applied to the soil by spraying 15 ml culture broth. In a same way was applied fungal spore suspension (15 ml). In the uninoculated control, soil was sprayed with 15 ml sterile distilled water. The detection of the antifungal action has been done by counting germinated cucumber seeds every two days up to 14 days. Experiments were run in triplicate.

Two kind of experiments were made: (V1) plants were treated first with fungal suspension and after five days with biocontrol agents (*B.subtilis* B2, *B.subtilis* mB2.9, *B.subtilis* tB2.2), (V2) seeds were treated in the same time with fungi and biocontrol agents (*B.subtilis* B2, *B.subtilis* mB2.9, *B.subtilis* tB2.2).



1 2 3 4

**Fig.9.** *In vivo* detection of antifungal potential on cucumber seeds of B2 –experiment V1 (1), B2 –experiment V2 (2), against *Alternaria tenuis* a1 comparing with pot infected with fungal suspension (3) and control pot (4) after 8 days of seeding

After 8 days of seeding, real differences between variants were observed (fig. 9): the best results (both antifungal and germination promoting action) were obtained with V2.

Similar results were exhibit after 14 days of seeding (fig. 10).

1 2 3 4

**Fig.10**. *In vivo* detection of antifungal potential on cucumber seeds of B2 –experiment V1 (1), B2 –experiment V2 (2), against *Alternaria tenuis* a1 comparing with pot infected with fungal suspension (3) and control pot (4) after two weeks of seeding.

According to our results, the mutant mB2.9 and the transformant tB2.2 had increased antifungal potential compared to parental strain B2 (confirming the results obtained *in vitro*). The best results were obtained in experiments V2, when biocontrol agents were applied in the same time with fungal infection (fig.11)

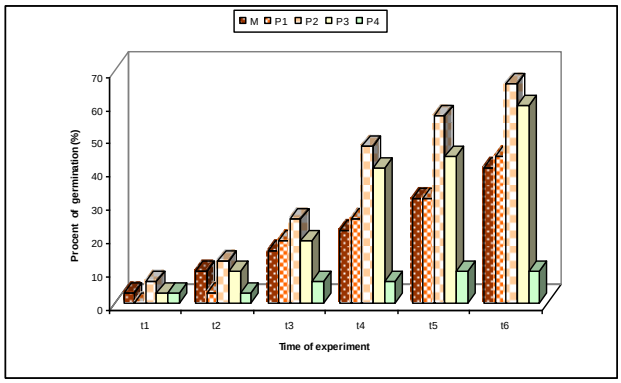



**Fig.11**. *In vivo* detection of antifungal potential on cucumber seeds of B2 (P1), tB2.2 (P2), mB2.9 (P3) against *Alternaria tenuis* a1 comparing with pot infected with fungal suspension (P4) and control pot (M).

In V1 experiments, at the beginning the germination was reduced compared to control pot (fig 11) but at the end of the experiments the percentage of germinated seeds was significantly higher than pots uninoculated control.

**Fig.11**. *In vivo* detection of antifungal potential on cucumber seeds of B2 – experiment V1 comparing with control pot after two weeks of seeding.

These results proved that biocontrol agents applied after fungal infection can protect seeds against *Alternaria* disease and stimulate their germination.

## Conclusions

Although *B. subtilis* is not considered a representative rhizosphere species, like *Pseudomonas* spp., the rhizosphere population density, as well as the persistence of the

bacterium in soil, is an important factor in suppression of damping-off caused by *Alternaria tenuis*.

*B. subtilis* organisms are stable in soil as spores, and this is advantageous for the use of this bacterium as a biocontrol agent mainly because of the spore's stability and ease of handling. This study showed that treatment with the culture broth could be effective as a biological control and plant growth promoting bacteria.

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## References

1. ROCO A, PEREZ L.M. *Electronic Journal of Biotechnology*, Vol.4, no.2, (2001)
2. BASIM H., KATIRCIOGLU Y.Z. *Rev.Plant Pathology,* 1156-1160, (1997)
3. BERNAL G., ILLANES A., CIAMPI L., *Journal of Biotechnology*, Vol.5, no.1, (2002)
4. MATSUNO Y., ANO T., SHODA M., *J. Ferment. Bioeng*., 73, 261-264, (1992) 73, 261-264, (1992)
5. HASE **C**., HOTTINGER M., MOËNNE-LOCCOZ Y., DÉFAGO G.**,** *Biol Fertil Soils* 32:217–221 (2000)
6. DUNNE C, DELANY I, FENTON A, O'GARA F, *Agronomie* 16:721–729(1997)
7. CORNEA, C.P, MATEESCU,R., GREBENIŞAN,I., VAMANU, E., CÂMPEANU, G., Roum.Biotechnol.Lett., vol 8, nr. 1, 1115 – 1122, (2003)
8. MATEESCU,R., CORNEA,C.P, GREBENIŞAN,I., DRAGOŞ, Ş., SIREAN, O., JURCOANE, ŞT., CÂMPEANU, G., *Roum.Biotechnol.Lett*., vol 8, nr. 4, 1367 – 1374, (2003)
9. PHAE, C.G., SHODA M., KOBUTA H., J.Ferment.Bioing. 69, p 1-7, (1990)
10. OHNO A., J.Ferment.Bioing. 70, (1990)
11. MANKA K., MANKA MALGORZATA, Buletin OILB/SORP XV, 1, p 73-75 (1992)
12. CONSTANTINESCU F., *PhD thesis*, Bucureşti, (2000)

**COOK J.R., *Annual Review of Phytopathology,* 31, 53-85, (1993)**