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Isolation and Characterization of New *Bacillus Spp. S*trains – Useful as Biocontrol Agents of Plant Pathogens

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Abstract

Bacillus spp. strains have been identified as producers of a different kind of inhibitory compounds for filamentous fungi. Many inhibitory bacilli produce small peptides with a long fatty moiety, the so-called lipopeptide antibiotics.

Different Bacillus spp. strains are isolated to be used as biocontrol agents and to be improved biosynthetically. Mutants were isolated after NTG treatment and analysed for inhibitory activity. The inhibitory compounds from the culture medium were extracted according to the method applied for iturin. When different strains of Bacillus spp. were interacted with filamentous fungi, the best results were obtained with the strain BE2 and with the mutant designated as B209. No plasmids were isolated from the selected strains ant it was concluded that the genes responsible for the biosynthesis of inhibitory compounds are chromosomal rather than plasmidial.

It was also observed that the simultaneous cultivation of the inhibitory bacterial strain and the sensitive fungi allow the increase of the diameter of inhibitory zone produced by methanolic extract. Moreover, in some interactions, between bacilli and sensitive fungi (Sclerotinia sclerotiorum) a precipitation line was observed that is similar to the line when lectins interact with glycoproteins.

Keywords: antifungal compounds, *Bacillus spp.*, phythopathogenic fungi, mutagenesis

Introduction

Spp Bacilli are Gram-positive bacteria that are present in substantial numbers in nearly all agricultural soils and in other environments. Among their other activities (biosynthesis of different enzymes), various strains of *Bacillus spp.* exhibit antifungal abilities. Recently, it was shown that new strains of bacilli, isolated in Romania, inhibited different species of filamentous fungi: *Fusarium sp., Alternaria sp., Sclerotimia sp.*

The lipopeptide nature of inhibitory compounds was established for some antifungal strains of *Bacillus spp.* On the basis of structural relationships, the lipopeptides identified in *Bacillus subtilis* are classified into three different groups: the surfactin group, the plipastatin-fengycin group and the iturin group [1].

It was also shown that, in some bacterial strains, lytic enzymes are responsible for inhibitory activities; instead, in other strains, lipopeptidic compounds determine the absence of growth of fungal phytopathogens. Among fungi, *Sclerotinia sclerotiorum* and *Alternaria tenuis* are two of the most important plant pathogens in our country, which produced serious damages to the crops.

The improvement of the level of inhibitory substances produced by bacilli strains is a goal of various studies performed in specific laboratories. "Classical" methods of improvement, such as mutagenesis as well as "modern" techniques of recombinant DNA were usually used in bacilli [2,1].

The aim of our researches is the isolation and characterization of new *Bacillus spp.* strains, able to inhibit the development of *Sclerotinia sclerotiorum* strains, in order to use them as biocontrol agents. We were also interested in the improvement of biosynthetic properties of these strains, both by mutagenesis and by recombinant DNA methods.

Material and Methods

Microbial strains: Five different *Bacillus spp.* strains were isolated from soil samples obtained from agricultural fields cultivated with sunflower or tomato. The new isolates were subjected for genus identification according to Bergey's Manual (1975) and they were designated as B1, B14, BA, BE1 and B2 and they were compared for their inhibitory activity with the *B.subtilis* ATCC 6633 reported in literature as producer of inhibitory compounds [2]. In the experiments were used five species of fungi: *Sclerotinia sclerotiorum, Fusarium oxysporum, Fusarium solani, Alternaria tenuis, Rhizopus nigricans, Monilia spp., Trichoderma viride.* These strains were isolated from different diseased plants and were obtained in pure cultures on specific media using the current mycological methods [3].

Culture conditions. Bacteria were maintained on LB broth or agar medium and the fungi were cultivated on different media: Sabouraud, YPG (yeast extract-peptone-glucose), PGA (potato-glucose-agar).

Detection of antagonistic activities. The test of the antagonistic effect has been done by Manka method [4]. The fungi - bacilli interactions were performed in inoculated in juxtaposition, 2 cm from each other, on Petri dishes containing solid medium. The inhibitory effect of *Bacillus spp.* strains and their methanol extracts were determined by measuring the fungi colonies diameter at five and ten days after the seeding.

Isolation of mutants. In order to obtain mutants with improved abilities of biosynthesis, cultures of B1 and B2 were treated with N-methyl-nitrosoguanidine and dilutions were plated on LB agar medium [5]. Mutants were selected in two steps: firstly we s detected the ability to degrade proteins and/or starch and, secondly three of the mutants, designated as: B107 (prt^- amy^-), B108 (prt^+amy^-) – derived from B1 and B209 (prt^+amy^-), derived from B2 were tested for inhibitory activities against fungi.

DNA techniques. Plasmid isolation was purified by emphazing the alkaline lysis method as described by Bron (1990) and the detection of DNA was carried out electrophoretically, in agarose gel [6].

Extraction of inhibitory compounds from culture medium was performed according to the indications of Phae et al. for iturin [7]: after three days' cultivation on solid

medium, the inhibitor was extracted for 1h with 45 ml of methanol for each 15g of medium. When the bacteria were cultivated in liquid medium, culture broth was centrifuged at 8000 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. The extracts obtained were tested for inhibitory action and were subjected to separation by thin layer chromatography (using chloroform/ methanol/ethanol/distilled water: 70/30/35/15, v/v/v/v, and revealed by staining with 1% ninhydrin reagent in acetone).

The inhibitory compunds produced by *Bacillus spp.*B2 and B209 were extracted in different concentrations of ethanol (95%, 70%) and tested against some fungi.

Results and Discussions

Selection of antifungal bacterial strains

Development of biocontrol systems has begun when effective inhibitory strains were identified. Most results in this area were obtained with *Trichoderma* species which presented antifungal abilities both "in vitro" and "in vivo" experiments. In contrast, fewer results were reported with antifungal bacilli but in that case strong activities against a large number of fungi species was obtained. The inhibitory activities in bacilli seems to be rather due to antibiotic type compounds than lytic enzymes.

In our experiments we isolated from soil samples, five bacterial strains belonging to *Bacillus* genus, and according to their morphological and biochemical properties, to *B.subtilis* species.

These strains were tested for antagonistic activities against different species of phytopathogenic fungi **(Table 1)**.

Among the bacteria tested, the best results were obtained with the strain B2, which presented a good inhibitory activity against majority of fungi strains **(Figure 1)**.

This strain was used for further studies: characterization of the inhibitory compound; detection of the presence of plasmid DNA; modification of biosynthetic properties by chemical mutagenesis.

Table 1. Interaction between different *Bacillus spp.* strains and filamentous fungi

Fungal strain	Bacillus sp.strains			
	B1	B2	B17	B209
Rhizopus nigricans N2	-	-	ND	+
Sclerotinia sclerotiorum 2	+	+	ND	++
Trichoderma viride UV	-	-	-	-
Monilia sp.	+	++	ND	++
Alternaria tenuis VP	++	+	ND	++
Alternaria tenuis a1	++	++	+	+++
Alternaria tenuis a2	+	+	+	++
Fusarium solani	+	+	ND	-
Fusarium oxysporum	+	++	+	++
Trichoderma viride VP	+	++	+	++

B1 and B2 are new isolates; B17 = *Bacillus subtilis* ATCC 6633; B209 = mutant derived from B9 after NTG treatment, ND = undetermined

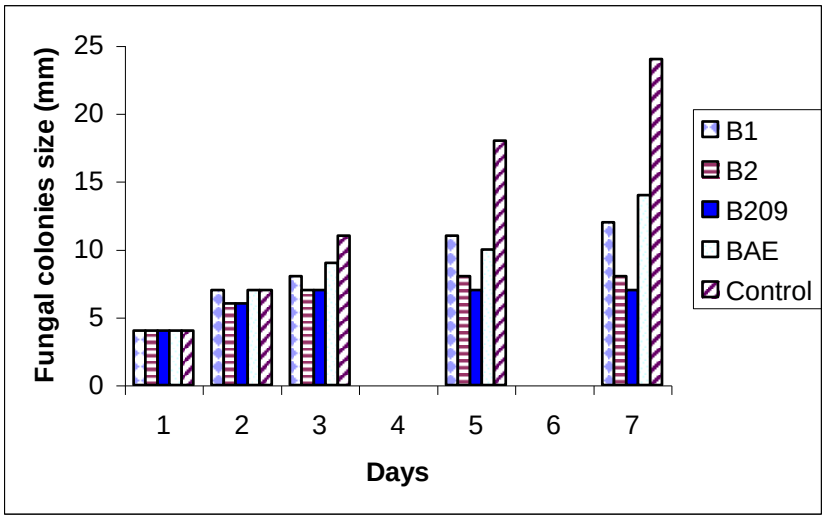

Figure 1. Inhibitory effect of some bacterial strains against *Alternaria alternata*

As it could be observed from figure1, the best results were obtained with *B.subtilis spp.B2* (wild strain) and B209 (mutant strain).

Characterization of selected bacteria

The proteic nature of inhibitory compounds produced by selected bacilli was tested by ammonium sulphate precipitation. The precipitation was performed with 70% ammonium sulphate and the precipitate dissolved in water was dialysed for 24 h against water. Small amounts (100 µl) of solution were applied in wells in Sabouraud plates seeded with fungi. The absence of fungal growth was checked. It was shown that the solution obtained by this treatment had no activity against sensitive fungi.

However, the application of the method described by Ohno [8] for the isolation of inhibitory compounds similar to iturin (lipopeptide family) allowed to obtain a precipitate which inhibited only one of the sensitive strains (*Scletorinia sclerotiorum* and *Alternaria tenuis)* **(Figure 2)**.

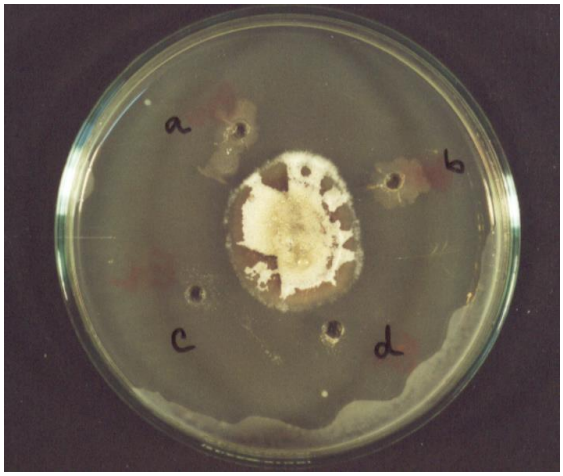

Figure 2. Inhibitory action of bacterial cultures of *Bacillus sp. B1* (a), *Bacillus sp. B2* (b) and the corresponding methanolic extracts (c, respectively d) against *Alternaria tenuis* a1

The methanolic extracts obtained were tested for inhibitory action and were subjected to separation by thin layer chromatography (using chloroform/methanol/ ethanol/distilled water: 70/30/35/15, v/v/v/v, and revealed by staining with 1% ninhydrin reagent in acetone) **(Figure 3)**.

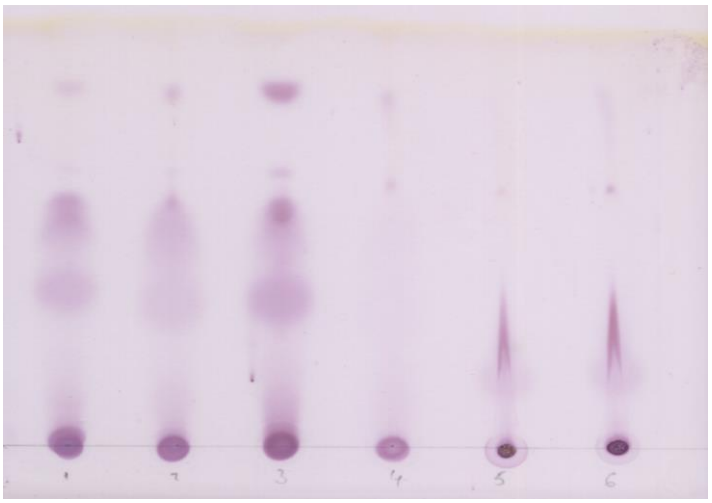

Figure 3. Chromatography on silicagel plates (TLC) of the methanolic extracts from different *Bacillus spp.* strains: B17 (1); B2 (2); B209 (3), reported to the precipitates resulted after extraction

In parallel, the inhibitory compunds produced by *Bacillus spp.*B2 and B209 were extracted in different concentrations of ethanol and tested against some fungi, the results obtained being presented in **(Figure 4 and 5)**.

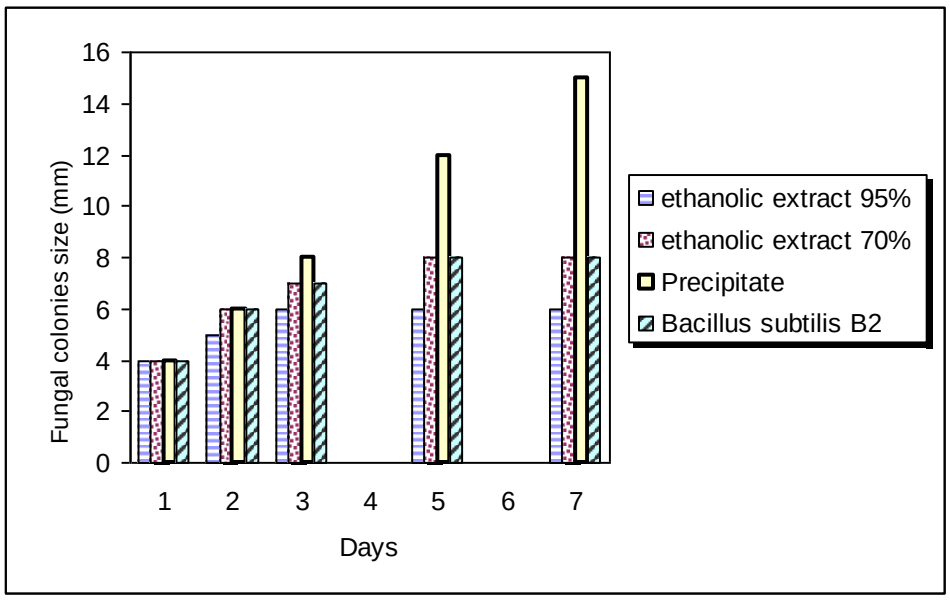

Figure 4. Inhibitory effect of ethanolic extracts obtained from B2 against *Alternaria tenuis*

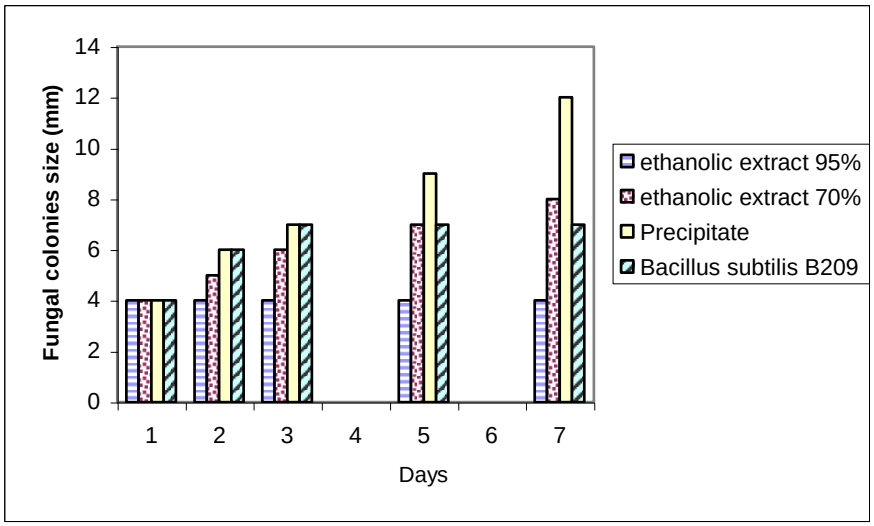

Figure 5. Inhibitory effect of ethanolic extracts obtained from B209 against *Alternaria tenuis*

In order to characterize the bacilli used in our experiments, they were subjected to different plasmid DNA isolation protocols but no plasmids were identified **(Figure 6)**. These data suggested that the genes involved in the biosynthesis of inhibitory compounds are located rather on chromosom than on extrachromosomal elements. The chromosomal DNA isolated from bacili strains is pure and in good quantity to be used in other experiments like RAPD and RFLP, for taxonomical identification of the strains.

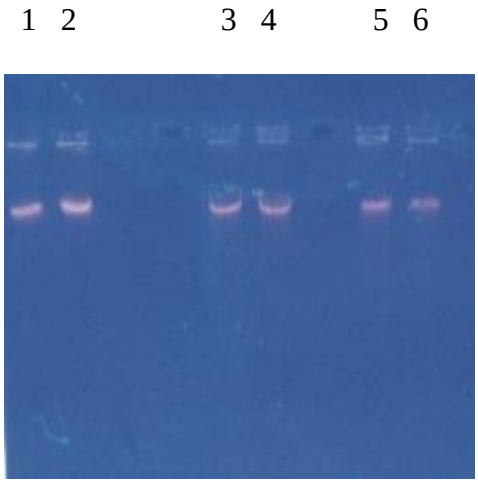

Figure 6. 0,8% Agarose gel electrophoresis of DNA isolated from different *Bacillus spp* strains: B1 (lanes 1,2), BE2 (lanes 3,4) and B17 (lanes 5,6)

Characterization of genetically modified bacterial strains

A special attention was concerned to the improvement of selected inhibitory strains, by chemical mutagenesis. In this respect, the mutagenesis with nitrosoguanidine was applied. The best results were obtained when a 50µl/ml final concentration of NTG was used **(Table 2)**.

Table 2. The action of NTG on *Bacillus sp.*B2

NTG concentration (μg/ml)	CFU/ml	Viability (%)
0	8×10^7	100
25	$6{,}9 \times 10^7$	86,25
50	$5{,}2 \times 10^7$	65
100	$0{,}4 \times 10^7$	5

As it was mentioned before (material and methods), the selection of mutants was performed both for the identification of auxothrophs and for improvement of inhibitory action. Among the colonies grown on after NTG treatment, one the mutants designated as B209 (prt^+amy^-), derived from B2, exhibited a larger inhibition area when they were tested against sensitive fungi **(Figure 7)**.

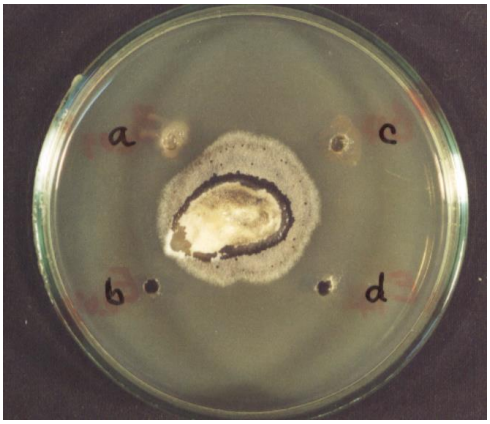

Figure 7. The inhibitory action of mutant strain B209 (b) and of the methanolic extract (d) against *A.tenuis* comparing with *B.subtilis* ATCC 6633 (a and c).

It was observed that the simultaneous cultivation of the inhibitory strain and the sensitive fungi allow the increase of the diameter of inhibitory zone produced by methanolic extract, comparing with the action of the extract obtained from solid medium cultivated with inhibitory strain alone. Moreover, in some interactions between bacilli and sensitive fungi (i.e. *Sclerotinia sclerotiorum*) there was observed a precipitation line similar to that produced when lectins interact with glycoproteins **(Figure 8)** [9].

Figure 8. Interaction between *Bacillus spp.* B209 and *Sclerotinia sclerotiorum.* A precipitation line (arrow) could be observed near the bacilli.

It is possible that the diffusible compounds produced, most probably, by fungi strains are associated with the recognition and inhibition produced by *Bacillus sp.* strains and stimulate the inhibitory effect of bacilli. Similar aspects were observed by Barak and Chet [10] analysing *Sclerotium rolfsii-Trichoderma spp.*interaction: they isolated a lectin produced by *S.rolfsii* which adsorb only to conidia of *T.hamatum* T-244, the antagonist of *S.rolfsii* and not to those of non-antagonistic strains of *Trichoderma.* These authors suggested that the fungal lectin determines the specificity of the parasitic interaction. However, there are necessary supplementary studies to be able to conclude the role of a specific lectin in antagonistic bacteria-fungi interactions.

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