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Phytohormones and antibiotics produced by *Bacillus subtilis* and their effects on seed pathogenic fungi and on soybean root development

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Summary

Bacteria belonging to the genus *Bacillus* were isolated from soil samples of Paraná State, Brazil, with the aim of evaluating their potential biological control of soybean seed pathogens. Strain PRBS-1 was selected, showing similar effectiveness to that of the strain AP-3, used as a reference due to its known antibiotic potential. The sequencing of the ribosomal 16S rRNA gene confirmed that both strains belong to the species *B. subtilis*, although showing high genetic diversity in relation to this species. Both strains inhibited five soybean seed pathogenic fungi *in vitro*, *Rhizoctonia solani, Colletotrichum truncatum*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina* and *Phomopsis* sp. Furthermore, the metabolites of AP-3 increased production of root hairs, while the metabolites of PRBS-1 stimulated outgrowth of lateral roots in soybean. The antibiotic effect of both strains seemed to be related to compounds of the iturin group, while the root growth promotion by PRBS-1 was at least partially related to the production of indoleacetic acid. The results have shown the potential of using selected strains of *B. subtilis* in the biological control of seed pathogens, as well as in promoting soybean growth.

Introduction

The inhibitory effect of *Bacillus subtilis* on plant pathogenic fungi has been frequently reported in laboratory, greenhouse, and field studies (Merriman *et al.* 1974; Pusey & Wilson 1984; Cubeta *et al.* 1985; Bettiol & Kimati 1990; Bettiol *et al.* 1994; Araújo & Hungria 1995; Araújo *et al.* 1995; Melo 1998). *B. subtilis* is able to synthesize more than 60 different types of antibiotics, mainly polypeptides, many of which possessing anti-fungal effects and belonging to the iturin family (Phae & Shoda 1991). Besides the anti-fungal effects some compounds produced by *B. subtilis* may act as well as plant growth promoters (Turner & Backman, 1991).

The continuous use of chemical inputs in agriculture is one of the main causes of unbalances in the soil microbial community, which leads to the outbreak of many diseases in crop plants. Cultivated soybean is affected by several species of fungi that cause severe yield losses and the majority of these pathogenic agents are seed-transmitted, thus demanding chemical seed treatment. As one example, Brazil, which is the world's second largest producer of soybean has more than 90% of the farmers treating seeds with fungicides (Hungria *et al.* 2005). However, besides the negative effects on the environment and human health, one main problem reported in the country is that fungicides often drastically reduce the viability of *Bradyrhizobium* cells, decreasing nodulation and nitrogen fixation rates (Hungria *et al.* 2005). In this context, the use of microorganisms as biological control agents may represent an alternative method to control pathogenic fungi (Cubeta *et al.* 1985; Bernal *et al.* 2002).

Preliminary studies performed by our group have indicated the feasibility of using *B. subtilis* against five soybean seed-pathogenic fungi (Araújo *et al.* 1995). Besides, the co-inoculation of *B. subtilis* (or its metabolites) and *Bradyrhizobium* has increased nodulation and soybean yield under greenhouse and field conditions (Araújo & Hungria 1995, 1999), confirming previous results described for both alfalfa and soybean (Li & Alexander 1988, 1990). In the U.S.A. there are also reports on the benefits to nodulation of soybean through the inoculation of *Bacillus cereus* (Halverson & Handelsman 1991) and *Bacillus megaterium* (Liu & Sinclair 1990), related to production of phytohormones, pectinases, or molecular signals, such that *Bacillus*-based products have been successfully commercialized (Raupach & Kloepper 1998).

The identification of compounds resulting from *B. subtilis* secondary metabolism is important for the elucidation of the beneficial effects observed either by the inoculation of the bacterium or by the use of its metabolites. It is also possible that several mechanisms

are involved, a reason by which the beneficial effects of *B. subtilis* inoculation are frequently reported for different crops and under different environmental conditions (Kilian *et al*. 2000). The estimates are that about 60% of Brazilian farmers use inoculants containing *Bradyrhizobium*, such that 25 million doses were commercialized in the 2003/2004 crop season (Hungria *et al*. 2005). However, in the Brazilian market there are no products based on *Bacillus* or other microorganisms aiming at either the biological control of seed pathogens or plant growth-promoting effects. Within this context, the objective of this study was to evaluate the potential of using selected *B. subtilis* isolates as anti-fungal agents and soybean growth promoters.

Material and methods

Bacillus strains

Bacillus sp. strain AP-3, isolated from rice rhizosphere (Bettiol & Kimati 1990) was used as a reference strain. In this study, bacteria from the genus *Bacillus* were isolated from soil samples collected in six different locations under soybean cropping, in three different counties of the State of Paraná (Londrina, Ponta Grossa and Campo Mourão). For the bacteria isolation, 10 g of soil were drawn from each sample and were then diluted in 100 ml of sterile distilled H_2O. These soil slurries were then subjected to thermal shock (70 °C for 10 min.) in order to select microorganisms which are resistant to this treatment, as the genus *Bacillus* (Buchanan & Gibbons 1975). Afterwards, 1-ml aliquots of each soil slurry were transferred to Petri dishes containing nutrient agar medium (10 g dextrose; 10 g bacto-peptone; 1 g acid casein and 15 g agar per litre of water, pH 7.0). After incubation at 28 °C for 48 h, colonies were isolated and characterized as belonging to the genus *Bacillus* according to the characteristics described by Collins & Lyne (1984) and Li & Alexander (1988). Through these procedures, twenty strains were isolated.

Inhibition of pathogenic fungi in vitro

The pairing method on standard potato-dextrose (PDA) medium (250 g potato; 10 g dextrose; 15 g agar per liter of water) was used for testing the antagonistic activity against the following species of plant pathogenic fungi, *Rhizoctonia solani*, *Colletotrichum truncatum*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina* and *Phomopsis* sp. Pure cultures of these fungi were initially grown in Petri dishes containing standard PDA medium and incubated at 28 °C for 48 h. After this period, 5-cm disks were cut from the edge of the active growth of colonies of each fungus with the aid of a cork borer. Four disks were then transferred to equidistant sites of new 9-cm Petri dishes containing PDA medium, 24 h prior to the inoculation of each *Bacillus* spp. isolate in the centre of the plate. Each treatment had four replicates and the Petri dishes were then incubated at 28 °C for 7 days. After this incubation period, the inhibition halos were measured for each strain in relation to the plant pathogenic fungi. The *Bacillus* strain showing the highest inhibitory activity against the five plant pathogenic fungi was selected and designated as PRBS-1 (=A3-5).

Taxonomic position of Bacillus strains

The DNA from the strains AP-3 and PRBS-1 were extracted and amplified for the 16S rRNA ribosomal region by PCR with Y1 and Y3 primers, as described by Chen *et al*. (2000). The PCR fragments were amplified again using 3.2 pmol reaction^{-1} of primers and the kit 'Big Dye' (PE Applied Biosystems), as described by Chen *et al*. (2000) and run on Perkin Elmer ABI 377 (PE Applied Biosystem) analyzer. The sequences generated were confirmed in both strands and were submitted to the GenBank database using BLAST search (NCBI, 2004), to seek for significant 16S rRNA alignments.

Extraction of Bacillus metabolites

Strains AP-3 and PRBS-1 were grown in 500-ml Erlenmeyer flasks containing 250 ml of liquid potato-dextrose medium, under constant agitation (100 rev/min) at 28/23 °C (night/day), for 7 days. After this period, the broth was centrifuged at 15,000 × g for 10 min in a refrigerated 'Sorvall' centrifuge RC-5B in order to separate the cells from the supernatant. The supernatants containing the metabolites were used for detecting the plant growth-promoting and antibiotic compounds.

Detection of índole-3-acetic acid (IAA) and abscisic acid (ABA) in the Bacillus metabolites

Strains AP-3 and PRBS-1 were grown in potato-dextrose medium, in triplicate, for 7 days, in the dark, to avoid IAA oxidation by light. The broth was then centrifuged at 5000 × *g* for 20 min and the supernatant was collected. A 2.0 ml sample was transferred to a 50-ml Becker flask and the pH of the solution was adjusted to 2.8 using diluted H_3PO_4. This sample was then transferred to a funnel and the extraction proceeded by the use of 15 ml of ethyl acetate, repeated three times. The ethyl acetate solution was dried in a rotary evaporator at 40 °C and the final residue was suspended in 2 ml of methanol. The material was filtered and 20 μl were injected into HPLC apparatus (Millipore, Waters model 570) with photodiode detector, under the following conditions, column (reverse phase) C-18 of 15 cm, 10.7 mm diameter, mobile phase, 60:40 of methanol: 1% phosphoric acid in H_2O, 1.0 ml min^{-1} flow (isochromatic) for 30 min, at 30 °C. Peaks were monitored at 278 nm wavelength. Results were compared to analytical standards of IAA and ABA (Sigma), prepared in deionized water and injected under the same conditions.

Effects of cells and metabolites of strains AP-3, PRBS-1 and pure IAA on soybean root phenotype

An experiment was carried out under axenic conditions in order to study the phenotype of soybean roots in response to addition of *Bacillus* supernatant, containing the bacterial metabolites. Soybean seeds (cv. BR-16) were surface-sterilized with ethanol and 10% sodium hypochlorite as described before (Vincent 1970). Seeds were treated with cells and the supernatants of strains AP-3 and PRBS-1 in a proportion of 10^7 cells seed^{-1} and of 1 ml of metabolites 10 g^{-1} seeds, respectively. The experiment was performed with five replicates per treatment and the seeds were incubated in a germinator at 25 °C for 72 h. After incubation, seed germination, length and thickness of the main root and the presence of root hairs were evaluated according to the methodologies described by Hadas & Okon (1987) and Hungria *et al.* (1996). Grades ranging from 0 to 5 were attributed from the lowest to the highest concentration of root hairs, respectively.

Another experiment was conducted in order to study the effects of IAA and of the *Bacillus* supernatants on the root phenotype of soybean. The treatments consisted of 10 ml of the supernatant (metabolites) of each strain and of 0.30 mg of IAA ml^{-1} of deionized H_2O to 10 g of soybean seeds (cv. BR-37), with five replicates. After being treated, the seeds were incubated in a germinator at 25 °C for 72 h. Fifty seedlings per treatment were then evaluated in relation to seed germination, root length, percentage of seedlings bearing secondary roots and the presence of root hairs, as described by Hadas & Okon (1987) and Hungria *et al.* (1996).

Detection of antibiotics produced by the Bacillus strains

Analyses were performed aiming at preliminarily characterization of the metabolites of AP-3 and PRBS-1 for antibiotic activity. For the extraction of the metabolites, after centrifugation ($5000 \times g$/20 min) each supernatant was acidified to pH 2.0 adding concentrated HCl and the precipitate formed was separated by centrifugation ($20{,}000 \times g$/15 min.) using a refrigerated 'Sorvall' centrifuge. The supernatant was then discarded, the precipitate containing the antibiotics was solubilized in methanol and the alcoholic solution was centrifuged again ($20{,}000 \times g$/10 min). The supernatant containing the solubilized antibiotics was subsequently collected as methanol extract. The technique described by Matsuno *et al.* (1992) was used for the identification of antibiotic compounds. From the methanol extract, a 20-μl aliquot was drawn and injected into the HPLC (Millipore, Waters model 570), with photodiode detector, equipped with a C-18 reverse phase column (10.7 mm diameter × 250 mm), and the mobile phase consisted of acetonitrile,10 mM of ammonium acetate, (2:3, v:v) at a 1 ml min^{-1} flow. Reading was monitored at 280 nm wavelength and peaks were collected.

For the evaluation of the antibiotic activity, samples from each peak were evaporated to dryness, resuspended and assayed in triplicate for the antagonistic activity to *Phomopsis* sp. A 200-μl aliquot corresponding to each HPLC peak was poured into a 0.5-cm diameter orifice in the center of a Petri dish containing PDA medium. Subsequently, four 0.5-cm disks containing the fungus mycelia were equidistantly placed in different sites around the orifice as already described in the item concerning inhibition of plant pathogenic fungi *in vitro*.

Results

Analysis of the 16S rRNA gene sequences

Partial sequences of 16S rRNA ribosomal gene were obtained for both *Bacillus* strains. Strain AP-3 showed a high (98%) identity of nucleotide bases (694/703 bp) with *Bacillus* strain TKSP21 (number of access of the GenBank of the NCBI, ABO17591.1) isolated in Korea. Similarly, it presented 98% identity of nucleotide bases (693/703 bp) with *B. subtilis* strain BS-2 isolated in China (number of access, AY172513). Levels of identity of similar magnitude were obtained with other isolates of *Bacillus* and strains of *B. subtilis* (NCBI, 2004). Strain PRBS-1 also presented high identity of bases (97%) with strains TKSP21 and BS-2, respectively 646/664 and 643/664 bp. Therefore, the two strains used in this study may be classified as *B. subtilis*, although the number of different bases found in relation to several other strains of this species, particularly PRBS-1 (more than ten different bases) might indicate a new species. The sequences obtained were deposited in the data bank of the NCBI, receiving the access numbers AY504952 (PRBS-1) and AY504953 (AP-3).

Phytohormones produced by strains PRBS-1 and AB-3

Concerning the effect of *B. subtilis* cells and their metabolites (supernatants) on the root phenotype, the metabolites of strain AP-3 stimulated the outgrowth of root hairs. An increment was also observed with the metabolites of strain PRBS-1, although the difference was not statistically significant (Table 1).

Phytohormones were detected in the metabolites (supernatants) of both strains, and PRBS-1 synthesized more IAA than ABA, while AP-3 produced both phytohormones in similar proportions (Table 2).

In another bioassay, the addition of metabolites of the strain PRBS-1, as well as the addition of purified IAA in the same concentration detected in the metabolites (Table 2), increased the outgrowth of lateral roots (Table 3). On the other hand, the metabolites of AP-3 did not provide higher outgrowth of lateral roots but did confirm a stimulus in the number of root hairs (Table 3) as previously observed (Table 1).

Table 1. Effect of soybean (cv BR-16) seed treatment with cells or the supernatant (metabolites) of *Bacillus subtilis* (strains AP3 and PRBS-1) on the seed germination and on root phenotype *in vitro*[a].

Treatment	Germination (%)	Main root		Root hairs (Grades)
		length (cm)	thickness (mm)	
Metabolites (AP-3)	60.4 a	2.38 a	1.49 a	2.73 a
Metabolites (PRBS-1)	54.2 a	2.90 a	1.55 a	2.15 ab
Cells (AP-3)	53.7 a	2.81 a	1.42 a	2.01 b
Cells (PRBS-1)	52.1 a	2.76 a	1.44 a	1.93 b
Control	50.0 a	2.78 a	1.46 a	1.86 b
C.V.(%)	20.1	18.5	15.5	25.6

[a]Means of five replicates followed by the same letter in each column are not statistically different by the Tukeýs test (5%).

Table 2. Detection of phytohormones [indole-3-acetic acid (IAA) and abscisic acid (ABA)] in the supernatants (metabolites) of *Bacillus subtilis* strains AP-3 and PRBS-1[a].

Strains	IAA (mg ml^{-1})	ABA (mg ml^{-1})
AP-3	0.171	0.170
PRBS-1	0.310	0.015

[a] Means of three replicates.

Inhibition of fungal growth by strains PRBS-1 and AB-3

Table 4 shows the performance of the two *B. subtilis* strains in relation to the inhibitory activity against five fungal species. Strain AP-3, previously shown to have antagonistic activity against pathogenic fungi of rice (Bettiol & Kimati 1990), cucumber and zucchini squash (Bettiol *et al.* 1997) was also effective against soybean seed fungi and a similar performance was achieved with strain PRBS-1.

Using the same conditions established by Phae & Shoda (1991) for the HPLC analysis of the metabolites of *B. subtilis* strain NB-22, four peaks were detected in the metabolites of strain PRBS-1, three of them showing the same retention time as that of the antibiotic compounds belonging to the iturin group (Figure 1). Another peak with a different retention time was detected in the metabolites of PRBS-1, just after peak 1; it might represent a new compound, but was not considered in this study, as it had no similarity with the compounds described by Phae & Shoda (1991). Four peaks were also observed in the metabolites of AP-3, with similar retention time as peaks 1, 2, 3 and 4 of NB-22 (Figure 1).

The comparison of the chromatogram profiles obtained with the metabolites of strains PRBS-1 and AP-3 with that obtained by Phae & Shoda (1991) for strain NB-22 indicates that active fractions showed similar retention time to that of the antibiotic compounds 1, 2, 3 and 4 belonging to the iturin group (Figure 1). The analysis of each fraction in relation to the inhibitory potential against *Phomopsis* sp. revealed that fraction 4 was the most active (Table 5).

Discussion

The antagonistic potential of *B. subtilis* to plant pathogens has been frequently reported, but differences among strains in relation to the inhibitory effect have

Table 3. Effect of soybean (cv BR-37) seed treatment with indole-3-acetic acid (IAA) and with the supernatant of the liquid culture medium where the strains AP-3 and PRBS-1 of *Bacillus subtilis* were grown, on seed germination and on root phenotype[a].

Treatment	Germination (%)	Main root Length (cm)	Lateral roots (% on the seedlings)	Root hairs (grades)
Metabolites (AP-3)	90.0 a	3.84 a	6.0 ab	2.85 a
Metabolites (PRBS-1)	90.0 a	4.12 a	9.5 a	2.21 ab
Indoleacetic Acid [b]	82.0 a	4.67 a	10.5 a	2.40 ab
Control	77.0 a	3.43 a	4.0 b	1.55 b
C.V. (%)	25.1	21.0	27.8	23.0

[a]Means of five replicates followed by the same letter in the columns are not statistically different by the Tukey's Test (5%). [b]0.30 mg of IAA ml^{-1} of deionized H_2O for 10 g of seeds.

Table 4. Growth inhibition against five species of phytopathogenic fungi (*Rhizoctonia solani, Colletotrichum truncatum, Sclerotinia sclerotiorum, Macrophomina phaseolina and Phomopsis* sp.) by *Bacillus subtilis*[a].

Strains	Diameter of the inhibition halo (mm)				
	R. solani	*C. truncatum*	*S. sclerotiorum*	*M. phaseolina*	*Phomopsis* sp.
AP-3	29	49	29	31	38
PRBS-1	32	48	21	30	17

[a] Means of four replicates.

Table 5. Effect of compounds (peaks of the HPLC) present in the metabolites of the strains AP-3 and PRBS-1 of *Bacillus subtilis* on *Phomopsis* sp. mycelial growth inhibition *in vitro*[a].

Metabolites	Inhibition halo (mm)					
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 6	Control[b]
AP-3	25	16	19	31	N.D.[c]	38
PRBS-1	15	N.D.[c]	N.D.[c]	28	19	35

[a] Means of three replications. [b] Two-hundred μl of the supernatant (metabolites) of each strain. [c] N.D., non-detected in the metabolites through HPLC.

also been observed (Dunleavy 1955; Aldrich & Baker 1970; Bettiol & Kimati 1990; Krebs *et al.* 1993; Luz 1994; Bernal *et al.* 2002). One example is strain AP-3, isolated from the rhizosphere of rice and showing antagonistic activity to different fungi associated not only with rice, but also with peach tree, cucumber and zucchini squash (Bettiol & Kimati 1990; Tratch *et al.* 1992; Bettiol *et al.* 1994, 1997). The good performance of AP-3 was confirmed in this paper against five soybean seed pathogenic fungi evaluated *in vitro*. In general the *Bacillus* isolates obtained from soils cropped with soybean showed poor performance against the phytopathogens studied and the most promising one, PRBS-1, was selected. The analysis of the 16S rRNA gene confirmed that both AP-3 and PRBS-1 fit into the *B. subtilis* species, but the high number of different nucleotide bases in relation to other strains belonging to this species, particularly for PRBS-1, might indicate a new species.

The main antibiotics produced by *B. subtilis* belong to the iturin group, being cyclic polypeptides with a structure comprising seven residues of α-amino acids, with a variable aliphatic chain, and β-amino acids comprising, η-C_{14}-β-amino acid, anteiso-C_{15}-β-amino acids, iso-C_{15}-β-amino acids, η-C_{16}-β-amino acids, and iso-C_{16}-β-amino acids. The iturins differentiate *B. subtilis* from other peptidic antibiotic-producing *Bacillus* species (Katz & Demain 1977; Maget-Dana *et al.* 1992). Iturins have been frequently cited for the antagonistic activity to other microorganisms, mainly plant pathogenic fungi (McKeen *et al.* 1986; Besson & Michel 1987; Matsuno *et al.* 1992), but they may also be effective against bacteria (Maget-Dana & Peypoux 1994; Bernal *et al.* 2002). In one study on iturins, Phae & Shoda (1991) demonstrated that five HPLC peaks showed antagonistic activity to eight phytopathogens, and those peaks were related to peaks 1–5 described by Matsuno *et al.* (1992). Furthermore, peak 6 of the study of Phae & Shoda (1991) was identified as comprising the chain η-C_{17}-β-amino acid. In our study, using the same methodology described by Phae & Shoda (1991), four peaks with similar retention time to those of Phae & Shoda (1991) were observed in the supernatants of strains AP-3 and PRBS-1, and all of them inhibited the growth of *Phomopsis* sp. *in vitro*. Nevertheless, in our study peak 6 was only detected in the metabolites of the strain PRBS-1, while peak 4 was well defined in both strains (Figure 1). Despite some variation in relation to the previous work of Phae & Shoda (1991), the chromatographic analyses performed indicated that the compounds detected ought to be included in the group of iturins.

Besson *et al.* (1979) concluded that iturins react mainly with cholesterol and this mechanism may explain the effects on eukaryotic cells (fungi), rich in cholesterol in the external plasmatic membrane. In general the effectiveness of the iturins against phytopathogenic fungi is found in concentrations ranging from 5 to 100 μg ml^{-1}, similar to the action of conventional fungicides (Kilian *et al.* 2000). In this study, the antifungal effect found in the compounds extracted from *B. subtilis* (AP-3 and PRBS-1) supernatants was expressed in lower concentrations. There are few reports on antibiotic production by *B. subtilis* in the rhizosphere as well as on their effect in suppressing root pathogenic fungi. Nevertheless, in a study with *Pseudomonas fluorescens*, Maurhofer *et al.* (1992) detected the presence of antibiotics on the roots of cucumber and the suppression of the development of *Pythium* sp. Therefore the production of iturins by *B. subtilis* strains could subsidize the selection of more efficient isolates towards the inhibition of phytopathogens, being an important parameter to be considered in the selection programmes.

In relation to the influence of *B. subtilis*, or its metabolites, on root phenotype, a stimulus was found on the outgrowth of root hairs induced by the metabolites of the strain AP-3. An increase in the number of root hairs could be related to a higher emission of molecular signals between the host plant and the inoculated bacteria (Hungria *et al.* 1996; Hungria & Stacey 1997), or to the production of growth regulators by the bacteria (Hadas & Okon 1987, Molla *et al.* 2001), although a role of hormones *in situ* in the rhizosphere has not been demonstrated yet.

The hormonal regulation of root development is related to auxins, such as IAA and ABA and the concentration of each hormone in the roots is responsible for the reduction or elongation of the root cells (Jacobs 1979; Evans 1984). These authors reported the existence of an interaction between IAA and ABA, which act by balancing the regulation of root development. In the analyses performed during this study on the metabolites of *B. subtilis*, the presence of these hormones was detected and it was found that strain PRBS-1 produced IAA in higher quantity, while strain AP-3 presented similar concentrations of both hormones. Production of

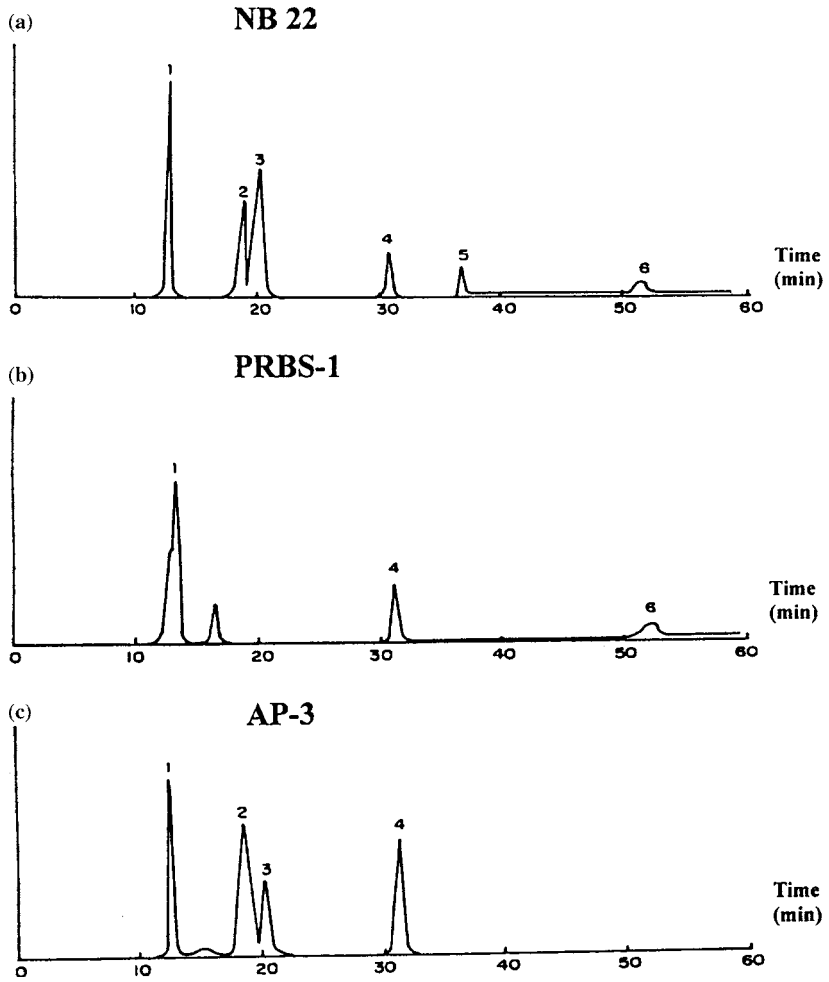

Figure 1. HPLC identification of compounds produced by *Bacillus subtilis*. (a) chromatogram built from the HPLC analysis of strain NB 22, as described by Phae & Shoda (1991); (b) chromatogram of the strain PRBS-1; (c) chromatogram of the strain AP3.

different amounts of IAA has been observed in plant-growth-promoting bacteria (Loper & Schroth 1986). In this experiment, the treatment with IAA, or with PRBS-1 metabolites, increased the emission of soybean lateral roots.

From the results accomplished in this study the potential of utilizing selected strains of *B. subtilis*, such as AP-3 and PRBS-1, in the biological control of fungi pathogenic to soybean seeds becomes evident. Moreover, effects of metabolites of these two strains on the promotion of root growth has also been detected. Together, these benefits may explain the increments observed in the field when those two strains and their metabolites were used in co-inoculation with *Bradyrhizobium* (Araújo & Hungria 1999). Further research is needed to determine the chemical structure of the antibiotic produced by AP-3 and PRBS-1, but the use of those strains, or their metabolites, as bioantagonistics plays a promising role.

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