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Selection of phytotoxin producing rhizobacteria

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ABSTRACT

In order to select phytotoxin producing rhizobacteria to control weed plants, twenty five bacterial strains previously isolated from the rhizospheres of various plants were grown in a liquid medium and, after cell removal by centrifugation, the liquid phases were freeze-dried and the products were extracted with ethyl acetate/methanol. The extracts were concentrated to dryness under vacuum and dissolved in water and sucrose solution to be submitted to *in vitro* assays of lettuce (*Lactuca sativa* L.) seed germination and wheat (*Triticum aestivum* L.) coleoptile growth. Although most samples affected coleoptile growth, only those from four strains reduced lettuce seed germination. Two strains of *Bacillus cereus*, one strain of *B. pumilus* and one of *Stenotrophomonas maltophilia* were the most promising microorganisms for producing phytotoxin and, consequently, for the development of new weed control products.

Key words: *Bacillus*, *Stenotrophomonas*, weeds, lettuce, wheat.

INTRODUCTION

Although herbicides are essential to obtain the necessary food and fiber, their use increases production costs and causes contamination of humans and the environment with harmful substances (Paoletti and Pimentel 2000, Tu et al. 2006). Thus, less expensive and less toxic methodologies to control weed must be developed. To circumvent such a problem, the use of rhizobacteria appears as a promising alternative, since some of these microorganisms are able to suppress the growth of specific plant species (Kremer and Souissi 2001, Hoagland 2001). They can be used directly in soil as bioherbicides (Mazzola et al. 1995) or to produce active metabolites against weeds. An example is *Serratia plymuthica* (strain A153), which is able to produce haterumalide A. This substance presents potential to control weeds, including the annual herbaceous such as *Stellaria media*, *Thlaspi arvense* and *Chenopodium album* (Gerhardson et al. 2001).

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Since phytotoxin production is one of the mechanisms by which rhizobacteria act against plants (Alstrom and Burns 1989, Loper and Schroth 1986), in a preliminary work crude metabolites from some rhizobacteria were submitted to an assay with lettuce seeds (*Lactuca sativa* L.) to select those potentially useful for the development of new products to control weeds (Carvalho et al. 2007). In order to continue this research, this paper presents the effect of crude metabolites produced by some rhizobacterial strains different from those used in the previous work on wheat coleoptiles (*Triticum aestivum* L.) and lettuce seeds, which are considered excellent phytotoxin detectors (Stonard and Miller-Wideman 1994).

MATERIALS AND METHODS

Production of Rhizobacterial Metabolites

Rhizobacteria used in this study are housed in the Department of Plant Pathology – Federal University of Lavras, state of Minas Gerais, Brazil. They were pre-

viously isolated from plant roots by J.L. Coimbra, unpublished data, who identified the microorganisms by fatty acid methyl ester analyses, which were carried out as described elsewhere (Kloepper et al. 1992, Chavarria-Carvajal et al. 2001). They were grown on tryptic soy agar (TSA – Merck KgaA) for two days, at 28°C, and transferred to 250 mL of tryptic soy broth (TSB – Isofar Indústria e Comércio de Produtos Químicos) by the use of a sterilized needle that was introduced into the colonies grown on TSA. After ten days at 28°C, under constant stirring (100 rpm), bacterial cells were removed by centrifugation (10,000 g) and 70 mL of each supernatant liquid were freeze-dried. To each resulting residue were added 14 mL of a methanol/ethyl acetate (MeOH/AcOEt; 1:1) solution, and the mixture obtained was filtered through a piece of cotton. The filtrates were concentrated to dryness in a rotary evaporator and dissolved in distilled water (70 mL) prior to their submission to the lettuce seed germination assay.

To carry out the wheat coleoptile growth assay, each supernatant liquid (3 mL) was freeze-dried and washed with 12 mL of the MeOH/AcOEt solution. The resulting liquids were concentrated to dryness in a rotary evaporator and dissolved in 6 mL of an aqueous 2% (g/mL) sucrose solution buffered at pH 5.0 with K_2HPO_4 (1.794 g/mL) and monohydrated citric acid (1.019 g/mL) (Nitsch and Nitsch 1996).

Lettuce Seed Assay

Fifty lettuce seeds (cv. Salad Bowl) were disposed in a transparent plastic box (GERBOX-11.4 × 11.4 cm) containing two sheets of germination paper (10.5 × 10.5 cm) embedding an amount of sample to be evaluated that was equivalent to 2.5 times the paper weight. After incubation at 20°C under a 12 h photoperiod per day, for seven days, the following parameters were used to evaluate the experiment: non-germinated seeds and healthy seedlings (Brasil 2009). Distilled water and TSB extract, obtained as described for rhizobacterial supernatant liquids, were employed as negative controls. An aqueous solution of glyphosate at 17.14 mg/L, which was prepared from Agrisato 480 CS, produced by Alkagro do Brasil Ltda, was used as positive control. The experiment was carried out with four replicates, in a randomized design.

Wheat Coleoptile Assay

Wheat seeds (var. BRS-49) were disposed on sterilized humid sand, without light incidence, for three days. The first 2 mm (apical part) of five etiolated seedlings were discarded and coleoptiles (the following 4 mm) were placed into 2 mL solutions containing samples to be evaluated. TSB extract, obtained as described for rhizobacterial supernatant liquids, was dissolved in an aqueous 2% (g/mL) sucrose solution buffered at pH 5.0 to be used as negative control. Aqueous solutions of 2,4-dichlorophenylacetic acid (2,4-D; Sigma Chemical, Saint Louis, MO, USA) at 1.5 mg/L and glyphosate at 7.968 g/L (prepared from Agrisato 480 CS, produced by Alkagro do Brasil Ltda) were used as positive controls. After 20 h at 25 ± 2°C, in darkness, the coleoptiles were disposed on a flat black surface and photographed with a digital camera. Images were amplified (3.28 times) and a ruler was employed to measure coleoptile length in the printed figure. Then, coleoptiles were freeze-dried and weighed. The experiment was carried out with three replicates, each one comprising five coleoptiles.

Statistical Analysis

Values of non-germinated seeds and healthy seedlings, proceeding from the lettuce assay, were directly submitted to analysis of variance, followed by comparison of means by Scott and Knott (1974) calculations ($P \leq 0.05$). Regarding the wheat assay, length (fifteen coleoptiles) and dry mass of three replicates (each one comprising five coleoptiles) were, separately, transformed into percentages before calculations. Statistical analyses were done using SISVAR software (Ferreira 2008).

RESULTS

Only metabolites produced by the two strains of *Bacillus cereus*, *Bacillus pumilus* (strain 83-20) and *Stenotrophomonas maltophilia* (strain 56-21) presented phytotoxic effects in the lettuce seed germination assay (Table I). Except for the strains 83-01, 83-20, 54-11, 58-10 and 62-17, the length or mass of wheat coleoptiles was statistically smaller than the observed for TSB (after freeze-drying and extraction). Nevertheless, just

TABLE I
Effect of rhizobacterial metabolites on the germination of lettuce seeds and growth of wheat coleoptiles.

Rhizobacteria and controls	Plant source	Lettuce[a]		Wheat coleoptile[a]	
		Healthy seedlings[b]	Nongerminated seeds	Length (%)	Mass (%)
Water		45.2[b]	4.0[a]	–	–
Sucrose at 2%		–	–	91[d]	102[d]
Glyphosate – 01[c]		0.0[a]	2.0[a]	–	–
Glyphosate – 02[d]		–	–	56[a]	53[a]
TSB[e]		45.0[b]	2.0[a]	100[e]	100[d]
2,4-D[f]		–	–	109[f]	130[e]
Bacillus cereus Frankland and Frankland (strain 83-01)	*Zea mays* L.	0.0[a]	2.2[a]	110[f]	96[d]
B. cereus (strain 83-02)	*Zea mays* L.	0.0[a]	6.7[a]	92[d]	92[c]
Bacillus megaterium de Bary (strain 54-12)	*Coffea arabica* L.	43.5[b]	2.5[a]	77[b]	61[a]
Bacillus pumilus Meyer and Gottheil (strain 55-08)	*Solanum lycopersicum* L.	43.7[b]	3.2[a]	97[d]	99[d]
B. pumilus (strain 55-26)	*Trapaeolum majus* L.	47.7[c]	0.7[a]	77[b]	86[c]
B. pumilus (strain 55-28)	*Coffea arabica* L.	48.5[c]	1.0[a]	90[d]	97[d]
B. pumilus (strain 56-28)	*Solanum lycopersicum* L.	42.0[b]	2.0[a]	88[c]	97[d]
B. pumilus (strain 83-12)	*Zea mays* L.	48.0[c]	0.7[a]	71[b]	52[a]
B. pumilus (strain 83-13)	*Zea mays* L.	47.0[c]	1.7[a]	85[c]	76[b]
B. pumilus (strain 83-14)	*Solanum lycopersicum* L.	46.2[c]	1.7[a]	76[b]	87[c]
B. pumilus (strain 83-15)	*Zea mays* L.	46.5[c]	2.2[a]	87[c]	91[c]
B. pumilus (strain 83-20)	*Solanum lycopersicum* L.	0[a]	49.2[c]	104[e]	96[d]
B. pumilus (strain 84-02)	*Zea mays* L.	46.2[c]	1.2[a]	93[d]	71[b]
B. pumilus (strain 84-03)	*Crotalaria* sp.	48.5[c]	1.0[a]	91[d]	108[d]
B. pumilus (strain 84-07)	*Crotalaria* sp.	47.7[c]	2.0[a]	91[d]	87[c]
B. pumilus (strain 85-10)	*Solanum lycopersicum* L.	48.0[c]	1.7[a]	95[d]	95[d]
Klebsiella pneumoniae (Schroeter) Trevisan (strain 58-15)	*Solanum lycopersicum* L.	48.0[c]	0.7[a]	92[d]	78[b]
Stenotrophomonas maltophilia Palleroni and Bradbury (strain 54-11)	*Trapaeolum majus* L.	46.7[c]	1.5[a]	102[e]	101[d]
S. maltophilia (strain 56-14)	*Ricinus communis* L.	46.2[c]	2.2[a]	95[d]	84[c]
S. maltophilia (strain 56-03)	*Coffea arabica* L.	48.2[c]	1.0[a]	86[c]	88[c]
S. maltophilia (strain 57-09)	*Ricinus communis* L.	47.2[c]	1.7[a]	91[d]	73[b]
S. maltophilia (strain 58-10)	*Solanum melongena* L.	43.2[b]	6.2[a]	107[f]	64[a]
S. maltophilia (strain 58-25)	*Solanum lycopersicum* L.	47.0[c]	2.2[a]	85[c]	73[b]
S. maltophilia (strain 62-17)	*Solanum lycopersicum* L.	48.5[c]	0.7[a]	105[f]	105[d]
S. maltophilia (strain 56-21)	*Phaseolus vulgaris* L.	2.0[a]	18.2[b]	88[c]	63[a]
Coefficient of variability		6.4%	21.9%	8.8%	8.8%

[a]Values with the same letter in each column do not differ statistically according to Scott and Knott (1974) calculations ($P \leq 0.05$); [b]seedlings with developed shoots and no necrosis on primary and seminal roots (Brasil 2009); [c]17.14 mg/L; [d]7.97 g/L; [e]after freeze-drying and extraction; [f]1.5 mg/L; (–) not evaluated.

as verified for the auxin (2,4-D), one strain of *Bacillus cereus* (strain 83-01) and two of *S. maltophilia* (strains 62-17 and 58-10) caused increases in the wheat coleoptile length.

DISCUSSION

The toxic effects of *B. cereus* (strain 83-02) metabolites on lettuce seed germination and wheat coleoptile growth seemed reasonable (Table I), since in a previous work with another strain of this bacterium, isolated from *Ricinus communis* L., the corresponding metabolites affected lettuce seed germination and signalgrass (*Brachiaria decumbens* Stapf) seed germination to a great extent (Carvalho et al. 2007). According to the authors, metabolites from *B. cereus* (strain 57-02) caused necrosis on 82.6% of lettuce roots and afforded 48% of abnormal signalgrass seedlings (seeds exposed to water presented 20% of abnormal seedlings). Nevertheless, metabolites produced by strain 83-01 of *B. cereus* enhanced coleoptile length. Although these results may seem antagonistic to each other at a first glance, they are in accordance with the ability of *B. cereus* to produce indole-3-acetic acid (IAA), a plant growth promoter (Egamberdieva et al. 2008) that is toxic to plants when used at inhibitory concentrations, which should be above 10^{-4} M to deleteriously affect oat coleoptile sections (Taiz and Zeiger 2006). Like IAA, the effects of other substances on plants are dose-dependent. An example is 2,4-dichlorophenylacetic acid (2,4-D), which is commercially employed as a herbicide, but can be used as an auxin to promote plant growth in tissue cultures (Pasqual 2001). Particularly in this work, 2,4-D caused increases in both length and mass of coleoptiles (Table I).

Among the thirteen *B. pumilus* strains studied, only the one denominated 83-20 affected lettuce, preventing seed germination. A correlation with the plant from which the bacteria were isolated seemed improbable, since other strains isolated from the same plant species presented no effect on lettuce seed germination (Table I). Except for the strain 83-20, all the others reduced wheat coleoptile growth. Perhaps the apparent lack of relationship between these two assays can account for different concentrations of active substances in crude metabolites. Anyway, the activities detected seem in accordance with the reported use of *B. pumilus* as a bioherbicide (Japan Tobacco Inc. 1998).

S. maltophilia (strain 56-21) reduced the lettuce seed germination and wheat coleoptile growth (Table I), which is in accordance with the activity of such bacterium against the weed *Bromus tectorum* L. reported by Mazzola et al. (1995). Except for the strain 54-11, all the others (strains 56-14, 56-03, 57-09, 58-10, 58-25, 62-17, and 56-21) affected wheat coleoptile growth, which in most cases was lower than the observed for those coleoptiles exposed to the control (TSB) (Table I). Probably this is a consequence of *S. matophilia* ability to produce IAA, the inhibiting or promoting effect of which, on plants, may be dose-dependent (Pasqual 2001).

Although *Bacillus megaterium* can present deleterious effect on weed growth (Kim and Kremer 2005) and on lettuce when combined with the fungus *Glomus constrictum* (Marulanda-Aguirre et al. 2008), lettuce seed germination was not affected by metabolites produced by such bacterium (Table I). However, the reduction of both length and mass occurred for wheat coleoptiles in contact with substances produced by *B. megaterium*. Similarly, *K. pneumoniae* metabolites presented no effect on lettuce seed germination, but reduced the wheat coleoptile length and mass. This result seems to be in accordance with the previously reported absence of phytotoxic effects on lettuce seeds by the metabolites of such bacterium (Carvalho et al. 2007).

Regardless of bacterial species used, the lack of relationship between the lettuce seed and the wheat coleoptile assays was easily observed, which may be partially due to the dose-dependent effects of some substances on plants (Pasqual 2001) and to the ability of some strains to produce more than one active substance (Karadeniz et al. 2006). An example is the production of the plant hormones IAA, giberelic acid, zeatin and abscisic acid by *B. cereus* grown in brain heart broth (Karadeniz et al. 2006). It is also possible that the absorption of active substances by plants have influenced the results, since a similar behavior can be observed for some commercial herbicides that are preferentially absorbed by leaves (Deuber 1992), which are more similar to coleoptiles than to seeds. Still worth mentioning is the difference among plants species used to carry out the assays that can result in various susceptibilities

to the bacterial metabolites. Like the commercial herbicides sulfosate and glyphosate, perhaps the active substances produced by rhizobacteria presented increased toxicity to monocotiledons (Deuber 1992, Maciel et al. 2002). Future studies will be carried out under field and greenhouse conditions to evaluate the toxic effects of *Bacillus cereus* (strains 83-01 and 83-02), *B. pumilus* (strain 83-20) and *Stenotrophomonas maltophilia* (strain 56-21) on greenery crops and weeds.

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RESUMO

Com o objetivo de selecionar rizobactérias produtoras de fitotoxinas para uso no controle de plantas daninhas, vinte e cinco isolados bacterianos previamente obtidos das rizosferas de diferentes plantas foram cultivados em meio líquido e, após remoção das células por centrifugação, as fases líquidas foram liofilizadas e os resíduos obtidos foram submetidos à extração com acetato de etila/metanol. Os extratos foram concentrados sob vácuo até secura e dissolvidos em água e solução de sacarose para serem submetidos a testes *in vitro* de germinação de sementes de alface (*Lactuca sativa* L.) e de crescimento de coleóptilos de trigo (*Triticum aestivum* L.). Embora a maior parte das amostras tenha desfavorecido o crescimento dos coleóptilos de trigo, somente as provenientes de quatro isolados reduziram a germinação das sementes de alface. Dois isolados de *Bacillus cereus*, um isolado de *B. pumilus* e um de *Stenotrophomonas maltophilia* foram os microrganismos mais promissores para a produção de fitotoxinas, com possibilidade de uso no desenvolvimento de novos produtos para o controle de plantas daninhas.

Palavras-chave: *Bacillus*, *Stenotrophomonas*, plantas daninhas, alface, trigo.

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