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SHORT COMMUNICATION

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Alleviation of noxious effects of cattle ranch composts on wheat seed germination by inoculation with *Azospirillum* spp.

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Abstract Two commonly-used composts from dairy cow manure that are used to improve poor structure and fertility of desert soils have inhibitory effects on wheat seed germination, probably as a result of their high levels of humic acids. Inoculation of wheat seeds with two species of the plant growth-promoting bacteria *Azospirillum brasilense* Cd and *A. lipoferum* JA4 (separately) prior to sowing in these amended soils improved germination, similar to the natural level of germination of seeds in desert soil without compost amendment. Both compost amendments increased height of wheat seedlings in the range of 20–25%, increased shoot dry weight by 15–19%, but severely decreased (51–54% less) root dry weight. Inoculation of wheat seeds with *A. brasilense* Cd, but not with *A. lipoferum* JA4, significantly increased plant growth parameters (height, shoot and root dry weight) over control plants grown in soil-compost mixtures. This bacterial species could survive for a period of 20 days in compost humic acid solution, could increase its population when the humic acids served as the sole carbon source, and may change the composition of humic acids in which it grows. We suggest that inoculation with *A. brasilense* may alleviate noxious effects on germinating seeds caused by compost application by possibly transforming the composition of humic acids in the compost.

Keywords *Azospirillum* · Bacterial inoculants · Compost · Humic acids · Plant growth-promoting rhizobacteria

Introduction

Many barren desert soils in the southern Sonoran Desert of Mexico are low in organic matter and available nutrients, and therefore, prohibit normal growth of most plants, even when irrigated (Bashan et al. 2000; Carrillo-Garcia et al. 2000). These soils are increasingly used for agriculture, especially modern crop production. The use of composts instead of chemical fertilizers is increasingly popular (Warman and Harvard 1998; Stamatiadis et al. 1999). Although composts significantly increase organic matter content of most desert soils and improve soil structure and fertility (El Nadi et al. 1995; Bernal et al. 1998), some compost preparations might have noxious effects on crops (De Brito Alvarez et al. 1995). This probably happens because of the high content of polyphenolic compounds and humic acids produced in the composting processes (Chen 1992; Valdrighi et al. 1995), especially of cow manure (Inbar et al. 1990). Sometimes improper composting is the cause of the high levels of these compounds (H. Antoun, personal communication). Nevertheless, humic acids in proper concentrations can enhance plant and root growth (Hartwigsen and Evans 2000; Singaravel and Govindasamy 1998). Negative effects on plant growth might also occur from non-composted organic matter added to soils (Staman et al. 2001). Two dairy cow composts suggested for soil improvement in intensive agriculture in the Vizcaino Desert in northern Baja California Sur partially inhibit germination of wheat seeds (unpublished data).

Composts are also known to protect plants against soil borne pathogens (Pascual et al. 2002), and stimulate the biocontrol-plant growth-promoting bacteria (biocontrol-PGPB) population in the rhizosphere (De Brito Alvarez et al. 1995). Thus, the objective of this study was to determine whether inoculation with two species of common PGPB *Azospirillum* (Bashan and Holguin 1997) alleviates stress and enhances growth of germinating wheat seeds under growth chamber conditions when applied with two dairy cow composts used in desert soil agriculture.

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Materials and methods

Organisms and growth conditions

Azospirillum brasilense Cd (DSM 1843, Braunschweig, Germany) and *A. lipoferum* JA4 (donated by V.L.D Baldani, CNPBS, Rio de Janeiro, Brazil) served as model bacteria, and were cultivated and prepared for inoculation according to Bashan et al. (1993). Wheat (*Triticum aestivum* cv. Rayon) susceptible to inhibition of germination by dairy cow compost served as a model plant. Seeds were disinfected and prepared for inoculation as described previously (Bashan 1986). Then they were inoculated with bacteria suspended in liquid 0.06 M, pH 7.0 phosphate-buffer supplemented with 0.15 M NaCl (PBS) at a final concentration of 10^6 cfu ml^{-1} by the standard vacuum infiltration method (Puente and Bashan 1993).

Substrates for testing seed germination

Three growth substrates were used: (1) a desert soil with the following physical and chemical characteristics has been described earlier: (in mg/kg) total N, 20; total C, 400; total inorganic C, 400; P, 12; K, 619; Fe, 7.5; Mn, 4.4; Zn, 0.3; Cu, 0.8; (in cmole (P^+)/kg) Na, 0.4; Ca, 7.8; Mg, 1.7; CEC, 8.6; (in S/m) electrical conductivity, 0.1; (in %) clay, 8; silt, 8; sand, 82; water holding capacity, 12; pH 7.59 (Bashan et al. 2000; Carrillo-Garcia et al. 2000); (2) the same desert soil mixed with compost produced from cultivated cruciferous waste (cauliflower and broccoli) and dairy cow manure (CW + DCM) at a ratio of 1:2 (soil:compost, v/v, resembling the recommended level of compost application in desert soil agriculture in Baja California Sur, [M. Bacilio, unpublished data]); and (3) the same desert soil mixed with compost produced from dairy cow manure (DCM) and cultivated corn straw waste (4:1, v/v) at the same application ratio as substrate CW + DCM. The physico-chemical composition and characteristics of the composts were: for CW + DCM (in %) total N, 1.9; NO_3^-, 1.32; P, 0.8; K, 0.63; Na, 0.27; Fe, 0.31; Mn, 0.04; Zn, 0.01; Cl, 0.08; SO_4^-, 0.13; organic matter, 10.9; (in mS/cm) electrical conductivity, 1.04; pH 9.2; for DCM (in %) total N, 1.9; NO_3^-, 0.31; P, 0.9; K, 0.86; Na, 0.34; Fe, 0.31; Mn, 0.05; Zn, 0.02; Cl, 0.12; SO_4^-, 0.11; organic matter, 11.5; (in mS/cm) electrical conductivity, 1.13; pH 9.5. The pH of the soil-compost mixtures was 7.92 for soil-CW + DCM and 7.85 for soil-DCM. Substrates were saturated with distilled water before sowing inoculated and non-inoculated seeds.

Germination tests

Mixed compost-soil (60 g) or control desert soil (80 g) of identical volumes was placed in polystyrene petri dishes (85 mm diameter). Uniform seeds (inoculated and non-inoculated serving as controls) were separately placed on the surface of the substrate, carefully touching the substrate. The petri dishes were kept in the dark at 24±1°C in a growth chamber (Conviron TC 16, Controlled Environments, Winnipeg, Canada) for 48 h. Each seed was separated (1 cm) from its neighbor. All petri dishes were covered with lids, but not sealed. Germination of seeds was considered positive when seeds started to show visible germination by rupturing of seed coat and emergence of a radicle.

Plant growth conditions

After the germination tests, 3-day-old seedlings of uniform size were grown in 120-ml plastic pots containing 85 g desert soil or 75 g soil-compost mixture (same volume), previously saturated with distilled water to water-holding capacity. Each pot contained eight seedlings incubated in a growth chamber (Conviron, Winnipeg, Canada) for 15 days at 26±1°C, 200 μmole m^{-2} s^{-1} light intensity, and 70% relative humidity. Plants were irrigated every 2 days with 5–10 ml distilled water, depending on growth size, until saturation of the substrate, but not to excess.

Extraction of plants and dry weight determination

After 15 days, the height of each plant shoot was measured with a ruler. The plants were carefully removed from the substrate. The roots in each pot were excised and gently washed with tap water to remove soil and compost particles. Plants from each pot were first dried with a paper towel to remove excessive water. Then roots and shoots were dried separately in a forced-air oven at 70°C for 2 days. Plants were then placed in a hermetically sealed desiccator to avoid absorption of humidity from the air. Each sample group was then weighed with an analytical balance. Each weight measurement represented the weight of the plants in one pot.

Humic acids extraction from composts and identification

Humic acids from both kinds of compost and soil amended with these composts were extracted using the method of Tomati et al. (2001) as follows: samples (2 g) of dry compost were incubated with shaking for 24 h in 200 ml 0.1 N NaOH under N_2 atmosphere. The solution was centrifuged at 6,000 *g* for 20 min and the pellet was discarded. The supernatant was acidified to pH 1.0 with 6 N HCl and further incubated at room temperature for 24 h. The solution was then centrifuged for 20 min at 6,000 *g* and the supernatant was discarded. Pellets containing humic acids were dissolved with 20 ml of 0.5 N NaOH until they reached pH 12 and then the suspension containing humic acids was filtered through Wathman no. 40 filter paper, and dialyzed against distilled water until neutral pH. The resulting fractions of humic acids were analyzed. Thin layer chromatographic analyses were performed using an analyzer (IATROSKAN MK-5 TLC/FID, Iatron Laboratories, Tokyo) equipped with SIII chromarods (rods of quartz; particle size 5 µm, covered with silica). Samples (2 μl) were applied and dried by hot air for 3 min. The separation by chromatography was run in a methanol, formic acid, and water mixture (80:10:10, v/v) for 30 min with a scan rate of 30 s. The chromarods were then dried at 70°C for 10 min. Identification of compounds was performed as described by Ackman et al. (1990), using the standard humic acid Enersol SC (Hartwigsen and Evans 2000; American Colloid Co., Arlington Heights, Ill.). A Hewlett Packard Vectra Pentium III workstation and integrated software (Peak Simple, version 2.66) were used in the analyses.

Bacterial growth in humic acids

Erlenmeyer flasks (125-ml) containing 50 ml of either saline solution (0.85% NaCl) or saline solution amended with a suspension of 0.02 mg/l standard humic acids (Enersol SC), were inoculated with 3×10^4 cfu ml^{-1} *A. brasilense* Cd and incubated at 30±1°C for 20 days at 120 rpm in a rotary shaker. The number of cells was then counted by the plate count method on nutrient agar medium.

Analysis of possible modifications of humic acids during bacterial growth

This was evaluated by gas chromatography (HP 5890 Series II, Hewlett Packard, USA), using the capillary column Nukol, (2-5326, 15 m; 0.53 mm inner diameter and film thickness of 0.50 µm) under a N_2 flow rate of 30 ml min^{-1} and injector and detector temperatures of 250°C on injected 0.1 µl samples. A workstation (Hewlett Packard Vectra Xm Series 3) with Integrate software (Quem Station HP 3365 Series II version A.03.34) was used to analyze the data.

Experimental design and statistical analyses

All experiments were repeated 3 times. Each germination repetition treatment consisted of 25 seeds. Petri dishes were placed in the

growth chamber randomly. The results were analyzed by Student's *t*-test at $P \leq 0.05$. For plant growth experiments, we used five pots, each containing eight plants per treatment. Height was determined for each plant, and dry weight was determined for the eight plants in each pot. These measurements were analyzed by one-way ANOVA at $P \leq 0.05$. Analysis of soils and of soil-compost mixtures was done in duplicate, using 2-g samples per replicate. Thin layer chromatography (TLC) runs were repeated twice for every determination. Gas chromatography determinations of humic acids were replicated 5 times. Results, in percentage, were transformed to arcsin values before analysis. All statistical analyses were done by Statistica software (Statsoft, Tulsa, Okla.). Graphic data are accompanied by standard error bars.

Results

Humic acid concentrations detected by TLC analyses were 5.1% for CW + DCM; 13.7% for DCM; 4.3% for soil + CW + DCM; and 7.2% for soil + DCM.

Inoculation of seeds with *A. brasilense* Cd improved germination (Fig. 1). *A. lipoferum* did not affect seed germination (data not shown). Both types of composts significantly inhibited germination of the seeds by the order of 7–19% (Fig. 1). Inoculation of seeds with both *Azospirillum* species, but especially with *A. brasilense* Cd, alleviated the inhibitory effect of both types of noxious soil amendments. The percentage of seeds that germinated after inoculation with *A. brasilense* Cd while growing in these mixtures was similar to the percentage of germination in soil without amendments (Fig. 1). *A. lipoferum* JA4 showed a similar, but lower level of stress alleviation (data not shown) than *A. brasilense* Cd.

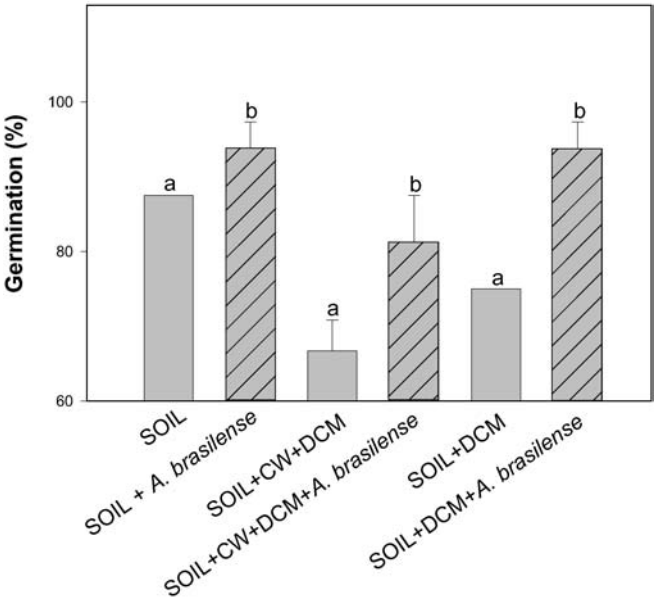

Fig. 1 Germination of wheat seeds in soil and soil amended with compost produced from crucifer waste plus dairy cow manure (CW+DCM) and dairy cow manure (DCM) and inoculated with *A. brasilense* Cd. Pairs of columns denoted by a different letter differ significantly at $P \leq 0.05$ by Students's *t*-test. *Bars* represent standard errors. Absence of bar indicates minimal SE

When the seedlings from the germination experiments were allowed to continue growing, compost amendment increased the height of the seedlings by 20–25%. However, inoculation with *A. brasilense* Cd, but not with *A. lipoferum,* further enhanced plant height (Fig. 2A). Dry weight of foliage was enhanced by the two types of compost amendments (Fig. 2B). Inoculation with *A. brasilense* Cd significantly enhanced foliar biomass of seedlings grown in soil amended with both composts

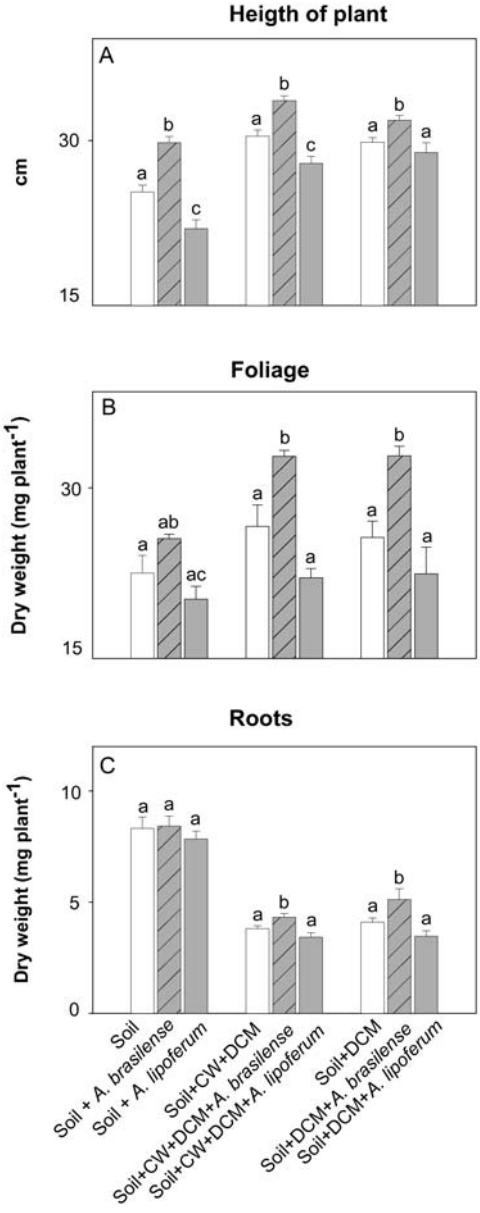

Fig. 2 Effect on growth of wheat (**A** height, **B** dry weight of foliage and **C** roots) inoculated at seed stage with *Azospirillum brasilense* Cd and *A. lipoferum* JA4, when grown in desert soil with composts derived from crucifer waste plus dairy cow manure (*CW + DCM*) or dairy cow manure (*DCM*). Controls were desert soil and desert soil supplemented with either of the composts. *Groups of three columns denoted by different letters for each soil and soil mixture* differ significantly at $P \leq 0.05$ by one-way ANOVA. *Bars* represent standard errors

Table 1 Gas chromatography analyses of changes occurring in two humic acids (retention times 7.5±0.0286 s and 10.47±0.0739 s) in saline solution with *Azospirillum brasilense* Cd grown for 10 and 20 days

	Incubation time (Days)	Humic acid 1		Humic acid 2	
		Retention time (s)	Concentration (μg/ml)	Retention time (s)	Concentration (μg/ml)
Control: standard humic acids	0	7.44	10.15	10.38	75.67
Humic acids + *A. brasilense*	0	7.62	13.62	10.68	21.50
Control: humic acids	10	7.50	35.83	10.50	25.61
Humic acids + *A. brasilense*	10	7.44	25.13	10.32	58.32
Control: humic acids	20	7.50	23.75	10.26	31.17
Humic acids + *A. brasilense*	20	7.56	24.46	10.68	53.00

compared with seedlings without *A. brasilense* Cd (Fig. 2B). *A. lipoferum* did not enhance plant development either in soil or in soil amended with compost (Fig. 2).

Wheat root biomass significantly decreased in soils amended with composts by 51–54%, compared with roots cultivated in poor soil (Fig. 2C). Inoculation with *A. brasilense* Cd increased root biomass in the presence of either type of compost, while inoculation with *A. lipoferum* JA4 had no alleviating effect (Fig. 2C).

Incubation of *A. brasilense* Cd in saline solution with humic acids resulted in a significant increase in the bacterial population after 20 days. Higher concentrations of humic acids supported larger population. In saline solution, the number of bacteria increased from $3.07\times10^4\pm4.05\times10^3$ cfu ml^{-1} at inoculation time to $8.23\times10^4\pm2.9\times10^4$ cfu ml^{-1}. Addition of 0.1 mg ml^{-1} humic acids increased the population to $2\times10^5\pm5.8\times10^4$ cfu ml^{-1}, while addition of 0.2 mg ml^{-1} humic acids increased the population further to $7.75\times10^5\pm2.2\times10^5$ cfu ml^{-1}.

Changes in humic acids composition yielded two major peaks, at 7.5±0.03 s and 10.47±0.07 s retention time. These humic acids were probably altered significantly because of bacterial growth (Table 1). Minor peak distribution (other possible humic acids) detected by chromatographic analyses were randomly changed, and therefore ignored.

Discussion

The combination of inoculation of seeds with plant growth-promoting bacteria (PGPB) with compost amendments is an uncommon practice, albeit these practices, separately, are commonly used in modern agriculture (Hoitink and Fahy 1986; Van Elsas and Heijnen 1990; Bashan 1998). Promoting plant growth with PGPB *Azospirillum* spp. is often observed under less favorable growing conditions, such as soils of low fertility, especially those with low N content (Bashan and Levanony 1990), and water-stressed plants (Sarig et al. 1984, 1990; Alvarez et al. 1996; Creus et al. 1996). In this study, we measured stress alleviation in emerging wheat seedlings by inoculating them with *Azospirillum* spp. and placing the developing seedlings in potted soils with composts with high humic acid content. Some composts have high levels of polyphenolic compounds and humic acids, which can inhibit seed germination (Chen 1992). Although most composts are applied at high dilution rates in soils (Canarutto et al. 1996; Valdrighi et al. 1995), the high level used in this study resembled the suggested application level of compost intended to increase the level of organic matter in soils in this arid agricultural zone.

Inoculation of seeds with *A. brasilense* Cd improved germination and alleviated inhibition of germination resulting from compost application. Therefore, this bacterial species can be considered a stimulator of germination of wheat seeds, as occurs in other plant species (Puente and Bashan 1993; Bhadauria et al. 2000), and to mitigate stress (Creus et al. 1996). Additionally, inoculation with *A. brasilense* Cd significantly enhanced foliar biomass of seedlings grown in the two composts over seedlings grown without inoculation with *A. brasilense*. This makes compost plus bacterial inoculation a synergistic treatment for plants.

It is well established that inoculation of wheat plants with *A. brasilense* Cd, but without compost, is known to enhance growth (Kapulnik et al. 1985). In this study, the compost treatment reduced root mass, but apparently had no negative effect on foliage. It supported more foliage than plants grown in poor soil. This reduction in root biomass might be a disadvantage in cases of low water supply. German et al. (2000) reported a thinner, but larger, root system in common beans inoculated with *A. brasilense* under water stress. The level of *Azospirillum* colonization in germinating seeds and seedlings was not measured in this study, since continuous evaluation of root colonization with these bacterial species in our laboratory from 1994 to 1999 showed that *Azospirillum* spp. always colonized the rhizosphere of wheat, tomato, and cardon cactus growing in this soil (Bashan et al. 1995, 1999).

Indigenous soil microorganisms are known to use humic acids to degrade, transform, mineralize, and in general over long periods of time, significantly reduce their level in soil (Tranvik and Hofle 1987; Haider and Martin 1988; Blondeau 1989; Kontchou and Blondeau 1992; Filip et al. 1999; Filip and Alberts 1994; Filip and Kubat 2001). Growth occurs in *A. brasilense* Cd incu-

bated in a saline solution supplemented with humic acids, and perhaps this bacterial species also metabolized the humic acids, as evidenced by the significant changes in gas chromatography peaks detected after bacterial incubation. This bacterium is also known to survive well for prolonged periods in organic matter (Bashan and Levanony 1988), and has laccase activity allowing metabolism of phenolic compounds (Alexandre and Bally 1999; Diamantidis et al. 2000). All this suggests that *A. brasilense* Cd has metabolic or nutritional relations with compost. Perhaps inoculation affects plant growth and tolerance to inhibitory effects of humic acids by metabolically transforming the chemical composition. However, some reservation about these observations and hypotheses should be considered. The methods used in this study (TLC and GC) to evaluate the outcomes of survival and growth of *Azospirillum* in humic acids did not rule out the possibility that the bacteria grew on minute impurities within the standard humic acid used as substrate. Only ^{1}H and ^{13}C-NMR spectroscopies and Fourier-transform IR (FTIR) spectroscopy can characterize the principal classes of chemical groups, their elemental composition, functional groups, and molecular weight (Inbar et al. 1990; Tomati et al. 2001) to indicate what happened to the humic acids, and to validate whether *Azospirillum* spp. can metabolize humic acids (H. Antoun, personal communication). Nevertheless, as support for this hypothesis, it is known that *Azospirillum* produces IAA and other organic acids (like glucuronic acid; H. Rodriguez, personal communication) that may affect the composition of humic acids and allow bacteria to grow on them.

Another possible explanation for reduced stress in wheat plants induced by compost is through pH reduction of the rhizosphere. High soil pH, as detected in this study, may reduce wheat growth because of reduced availability of phosphates and iron (Jensen and Salisbury 1984). *A. brasilense* Cd inoculation is known to significantly reduce pH of the rhizosphere by proton extrusion and organic acid production in wheat and cardon cactus plants (Amooaghaie et al. 2002; Bashan 1990; Carrillo et al. 2002).

In brief, the main finding of this study is that inoculation of wheat seeds with *A. brasilense* Cd alleviates inhibition of seed germination by humic acid constituents of two commonly used composts.

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