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Effect of Bacteria and Amoebae on Rhizosphere Phosphatase Activity

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The contributions of various components of soil microflora and microfauna to rhizosphere phosphatase activity were determined with hydroponic cultures. Three treatments were employed: (i) plants alone (*Bouteloua gracilis* (H.B.K.) Lag. ex Steud.) (ii) plants plus bacteria (*Pseudomonas* sp.), and (iii) plants plus bacteria plus amoebae (*Acanthamoeba* sp.). No alkaline phosphatase was detected, but an appreciable amount of acid phosphatase activity (120 to 500 nmol of *p*-nitrophenylphosphate hydrolyzed per h per plant) was found in the root culture solutions. The presence of bacteria or bacteria and amoebae increased the amount of acid phosphatase in solution, and properties of additional activity were identical to properties of plant acid phosphatase. The presence of bacteria or bacteria and amoebae increased both solution and root phosphatase activities at most initial phosphate concentrations.

Organic phosphorus comprises a large percentage of the phosphorus in grassland soils (2), and the labile organic phosphorus fraction has been shown to be very important in the phosphorus cycle of native grassland systems (8). Labile organic phosphorus compounds are mineralized very rapidly in soils (1) by enzymes that catalyze the hydrolysis of esters and anhydrides of phosphoric acid (5). Phosphomonoesterases are the most widely studied soil phosphatases and are classified into acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphatases (5). Acid and alkaline phosphatases are produced by bacteria, fungi, and yeasts (4, 11, 16). Acid phosphatase is also produced by protozoa (14), mycorrhizal fungi (12), and plant roots (20). However, most soil acid phosphatase is primarily of plant origin (6, 20). Because plant roots do not produce alkaline phosphatase, soil alkaline phosphatase activity is primarily of microbial origin (6). Ridge and Rovira (17) measured the acid phosphatase activity of young wheat roots in the presence and absence of microorganisms. They found that inoculation with a known phosphatase-producing microorganism had no effect on the phosphatase activity of the root surface and that adding a soil inoculum decreased root phosphatase activity. However, Estermann and McLaren (6) found that soil microorganisms increased surface phosphatase activity of barley roots. The objectives of this study were to determine the effect of bacteria and amoebae on the rhizosphere phosphatase activity of blue grama.

MATERIALS AND METHODS

The phosphatase substrate *p*-nitrophenylphosphate (disodium salt, trihydrate) (PNP) was obtained from Calbiochem, La Jolla, Calif. The organisms used were blue grama grass (*Bouteloua gracilis* (H.B.K.) Lag. ex Steud.), a bacterium (*Pseudomonas cepacea*), and an amoeba (*Acanthamoeba polyphaga*). The bacterium and the amoeba were isolated from the roots of blue grama (9).

Solution alkaline phosphatase activity was measured by adding 1.0 ml of root culture solution to 1.0 ml of substrate solution containing 10 mM PNP in 1.0 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.2 (18). The reaction was stopped with 4.0 ml of 0.2 M K_2HPO_4. Solution acid phosphatase activity was assayed by adding 1.0 ml of root culture solution to 1.0 ml of substrate solution containing 50 mM PNP in 0.2 M sodium acetate buffer, pH 5.5 (15). The samples were incubated for 2.0 h; the reaction was stopped with 4.0 ml of 0.2 M K_2HPO_4 solution. The samples were filtered, and the *p*-nitrophenol produced by hydrolysis of the substrate was measured spectrophotometrically at 410 nm. The root acid phosphatase activities were assayed by rinsing the roots in deionized water and placing each plant in a test tube containing 4.0 ml of substrate solution (acetate buffer, pH 5.5) diluted twofold. After the test tubes were shaken on a reciprocating shaker for 15 min, the plants were removed, and 8 ml of 0.2 M K_2HPO_4 was added to the test tubes. The samples were filtered, and the *p*-nitrophenol produced was measured spectrophotometrically at 410 nm. All enzyme assays were done at 20°C. All of the enzyme activities are expressed as either nanomoles or micromoles of PNP hydrolyzed per hour per plant.

The bacteria were grown in a defined medium (RSSB) (9). The amoebae were grown in a defined medium (RSS) (9) amended with 1% proteose peptone, 1% glucose, and a phosphate buffer. Log-phase cultures were centrifuged, aseptically washed with sterile 1/7 strength Hoagland solution, and resuspended in sterile 1/7 strength Hoagland solution. One milliliter

of the bacterial suspension was added to the culture solution of each plant in treatments requiring bacteria, and 1.0 ml of the amoeba suspension was added to each plant in treatments requiring amoebae.

Blue grama seeds were sterilized by soaking in 15% Chlorox for 1 h and then rinsed three times with sterile deionized water. Seeds were allowed to germinate in Erlenmeyer flasks containing a thin film of water and stored in the dark at 25°C for 3 days. Germinated seedlings were transferred to nutrient agar (0.2% peptone, 0.3% yeast extract, 0.5% glucose, and 0.5% agar), allowed to grow for 128 days, transferred to test tubes (25 by 200 mm) containing 15 ml of sterile 1/7 strength Hoagland solution (10), and plugged to maintain sterility. Sterility was verified by spreading several drops of root culture solution on nutrient agar. The plants were grown in a controlled-environment chamber for 12 h at 32.2°C, 4,750 lx, and 12 h of 15.6°C night. The three biotic treatments (plants, plants and bacteria, and plants plus bacteria plus amoebae) used 20 plants per treatment. The bacterial inoculum contained 9.6×10^7 bacteria per ml, and the amoeba inoculum contained 1.2×10^5 amoebae per ml. After the initial inocula were counted, all plants were allowed to grow for 28 days. The experiment was then terminated, and alkaline and acid phosphatase activity in solution, final numbers of bacteria and amoebae, and plant dry weights were determined. The acid and alkaline phosphatase activities of the growth media were assayed by the previously described procedures. Plant dry weights were measured after drying at 60°C for 48 h. Bacteria and amoebae were counted using a Petroff-Hauser counting chamber (9). The plant growth media from each treatment were pooled, centrifuged at 37,000 $\times$ *g* for 20 min, and dialyzed against deionized water at 4°C (20 ml of enzyme solution was dialyzed against 100 ml of water) for 3 days. Michaelis constants of the phosphatase were measured by using a range of substrate concentrations varying from 0.1 to 10 mM, and the data were plotted with a Hofstie plot (v versus v/s) (7). Heat stability was measured by preincubating the samples for 2 h at 80°C, cooling the samples, and determining the phosphatase activity. Cultures of both the *Pseudomonas* and *Acanthamoeba* spp. were sonically treated for 15 min at 60 W, and extracts were centrifuged at 37,000 $\times$ *g* for 20 min. The cell-free extracts were dialyzed, and the acid phosphatase activities were characterized in the same manner as was the root culture solution.

An additional experiment was carried out to evaluate the effect of initial phosphate concentration on rhizosphere phosphatase activity. Blue grama seeds were sterilized, germinated, and planted in nutrient agar, and after 77 days the seedlings were transplanted into 1/7 strength Hoagland solution in the same manner as in the previous experiment. Three biotic treatments and three initial phosphorus concentrations with 10 plants per treatment were used. The bacterial inoculum contained 4.4×10^8 bacteria per ml, and the amoeba inoculum contained 2.7×10^5 amoebae per ml. The initial phosphorus concentrations were 4.87, 9.03, and 18.43 μg/ml, and the three biotic combinations noted previously were used. The plants were allowed to grow for 28 days, and the experiment was then terminated. Initial and final numbers of bacteria and amoebae, solution phosphate concentrations, final plant weights, and solution acid phosphatase and root acid phosphatase activities were determined. The ascorbic acid-reduced molybdate method was used to determine solution phosphate concentrations (19). The experiment was analyzed as a randomized block analysis of variance. Tukey's honest significant difference was used for mean separation tests when appropriate.

RESULTS AND DISCUSSION

The numbers of bacteria and amoebae in the culture solutions were determined, but no estimates of the populations on the root surfaces were obtained. Bacteria grew significantly in the treatments with bacteria added, and the final population of amoebae was slightly higher than the initial population. Because Hoagland solution contains only inorganic nutrients, the bacteria presumably obtained organic carbon from root exudates and sloughed root material. Amoebae obtained all their nutrients by feeding on bacteria.

No alkaline phosphatase was detected in the root culture solutions, but acid phosphatase activity was found (Table 1). Plant weight was unaffected, but acid phosphatase activity increased significantly when bacteria or bacteria and amoebae were present (Table 1). The additional acid phosphatase activity in the presence of bacteria and amoebae could be caused by the plant roots, the bacteria, the amoebae, or all three. The proteolytic activity of bacteria probably destroys some phosphatase activity. Thus, the activity observed in the presence of bacteria could be somewhat lower than the total amount of enzyme produced. The acid phosphatase properties in culture solutions were compared with those in cell-free extracts of amoebae, which contained both extracellular and intracel-

TABLE 1. *Plant weights and phosphatase activities in root culture solutions in the presence of bacteria and amoebae (initial phosphate-P concentration, 4.87 μg/ml)*

Treatment	Plant wt (mg)	Total alkaline phosphatase activity (nmol of PNP hydrolyzed per h per plant)	Total acid phosphatase activity (nmol of PNP hydrolyzed per h per plant)
Plants	79.0	0	193[a]
Plants plus bacteria	76.4	0	413
Plants plus bacteria plus amoebae	75.4	0	475

[a] Significantly different at the 0.1% level.

lular phosphatases. Because no appreciable phosphatase activity could be detected in the cell-free extract of bacteria, only the amoeba extract was studied. The properties of acid phosphatase activities in root culture solutions were similar for all treatments, including plants alone and those with the various biotic components (Table 2). The properties of the amoeba acid phosphatases differed from the properties of the phosphatases in any of the treatments (Table 2). Thus, it is likely that plant roots were the primary source of the additional phosphatase activity in the culture solution containing bacteria or bacteria and amoebae. Woolhouse (20) found that adenosine triphosphatase activity of *Agrostis tenuis* roots was depressed by high phosphate concentrations in the culture solution. Low inorganic phosphate concentrations caused by bacterial uptake of phosphorus may derepress phosphatase synthesis. However, microorganisms have been shown to stimulate roots to release organic carbon (13), and the bacteria and amoebae may have stimulated the blue grama roots to produce additional phosphatase.

When the experiment was terminated, the final populations of amoebae were lower than the initial numbers in all treatments, but the bacterial populations were higher.

No significant differences in plant weights among treatments were observed. Solution phosphatase activities of treatments containing bacteria and plants were not significantly greater than those with plants alone (Fig. 1). Solution phosphatase activities were greatest in treatments with plants, bacteria, and amoebae (Fig. 1). The initial phosphorus concentration did not affect the phosphatase activity of any biotic treatment (Fig. 1). At initial phosphorus concentrations of 9.03 and 18.43 μg/ml, all of the solution phosphate was not taken up in any of the treatments (Table 3). Thus, the increased phosphatase activity was not caused by bacteria and amoebae affecting the solution phosphate concentration. The increased phosphatase activity in the presence of bacteria and amoebae could be caused by those organisms stimulating plant roots to produce phosphatase.

Root surface phosphatase activities and solution activities showed similar trends (Fig. 1 and 2). Treatments containing bacteria and amoebae had higher phosphatase activities than those with plants alone at initial phosphate concentrations of 9.03 and 18.47 μg/ml (Fig. 2). Because the properties of root surface activity were not studied, the source of the additional activity could not be determined. Total root surface phosphatase activities were between 4 and 14 times greater than the total solution phosphatase activities.

Bacteria stimulated plant roots to produce acid phosphatase, but the increase in phosphatase activity was greatest when both bacteria and amoebae were present, and the initial phosphate concentration did not affect phosphatase

TABLE 2. *Properties of acid phosphatase activity in root culture solutions of the three treatments and the cell-free extract of the amoebae*

Treatment	K_M (mM)	Heat stability (% activity remaining after 2.0-h preincubation at 80°C)
Plants	0.346	15
Plants plus bacteria	0.304	18
Plants plus bacteria plus amoebae	0.347	16
Amoebae	1.09	32

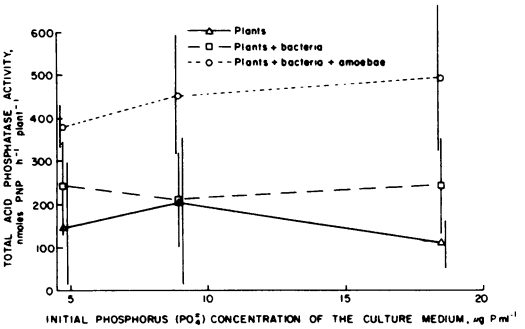

FIG. 1. *Final root culture solution acid phosphatase activity at three initial phosphate concentrations in the presence and absence of the biotic components. Vertical lines correspond to 1 standard deviation.*

TABLE 3. *Final phosphate-P concentrations at various initial phosphate levels*

Treatment	Final phosphate-P concn (μg/ml)
Initial phosphorus concn, 4.87 μg/ml	
Plants	0.86
Plants plus bacteria	1.22
Plants plus bacteria plus amoebae	0.79
Initial phosphorus concn, 9.03 μg/ml	
Plants	4.34
Plants plus bacteria	5.26
Plants plus bacteria plus amoebae	4.57
Initial phosphorus concn, 18.43 μg/ml	
Plants	13.74
Plants plus bacteria	15.09
Plants plus bacteria plus amoebae	14.32

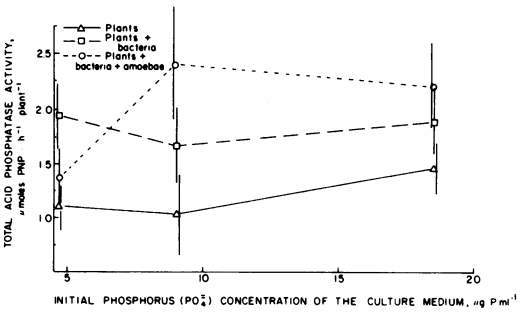

FIG. 2. *Final root surface acid phosphatase activity at three initial phosphate concentrations in the presence and absence of the biotic components. Vertical lines correspond to 1 standard deviation.*

activity. Rhizosphere microorganisms have been shown to produce hormones such as indolyl-3-acetic acid and gibberellins that stimulate plant growth (3). However, no effect on plant growth was observed in the presence of bacteria and amoebae, but similar compounds produced by these organisms may affect the production of plant root phosphatases.

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