

Responses of Bean to Root Colonization With *Pseudomonas putida* in a Hydroponic System

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ABSTRACT

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Colonization of bean roots by *Pseudomonas putida* was maintained at $0.6-1.8 \times 10^5$ colony-forming units per gram of root tissue during growth for 18 days under hydroponic culture involving complete as well as iron- and boron-deficient media. Leaves from 18-day-old transplants colonized by *P. putida* had reduced iron contents compared with uninoculated seedlings, and roots had 17-93% higher lignin contents than did uninoculated seedlings. Plants with roots colonized by *P. putida* gained more weight after inoculation with *Fusarium solani* f. sp. *phaseoli*

compared with plants grown without *P. putida*. In plants inoculated with both *P. putida* and *Fusarium*, foliar wilting and onset of lesion formation were delayed by 2-3 days. Colonization by *P. putida* decreased the amount of lignin normally generated at the lesioned root-shoot interface in plants infected by *Fusarium*. These data suggest that *P. putida* may afford some protection against *F. solani* in the early stage of disease development. Protection may involve alteration of the plant's defense potential through an increase in lignin in the root tissues.

Additional key words: nutrition, suppressive soil.

The colonization of plant roots by certain isolates of the group characterized by *Pseudomonas putida* and *P. fluorescens* may have a beneficial effect on plant performance (7,9,10,17,18). Increased growth and crop yields are reported for seedlings treated with *P. putida*. Suppression of *Fusarium* wilts and disease caused by *Gaeumannomyces graminis* var. *tritici* has been observed in pseudomonad-colonized plants (8,15,16).

The growth promotion and disease suppression attributed to pseudomonad colonization are affected by iron nutrition. Growth promotion was not observed for plants grown with adequate iron (7). Suppression of *Fusarium* wilt and take-all was negated by inclusion of iron chelates into the soils (7,16). The importance of iron nutrition may be related to bacterial production of siderophores to chelate Fe^{3+} ions under iron-deficient conditions. These siderophores have higher chelation efficiencies and stabilities than siderophores produced by other soil microorganisms including *Fusarium* species (16). These observations suggest that the pseudomonad siderophores may inhibit growth of deleterious microorganisms in the rhizosphere by limiting their iron availability (18).

We have demonstrated agglutination of cells of certain strains of *P. putida* by a glycoprotein present on bean root surfaces (1). Cells agglutinated by this plant component include protective isolates used by Scher and Baker (15) and Kloepper, et al (7). Because agglutinability may enhance colonization of plant roots, we initiated studies of the interaction between bean seedlings and the isolate of *P. putida* routinely used for our agglutination investigations. Studies reported in this paper examined the ability of this isolate of *P. putida* to colonize bean roots under adequate or nutritionally stressed hydroponic conditions. Additionally, the effect of inoculation with *P. putida* on the severity of root rot caused in bean by *Fusarium solani* f. sp. *phaseoli* was examined. Our previous studies had demonstrated that plants grown under limited iron and boron nutrition displayed enhanced symptom

formation (4). Consequently, effects of the pseudomonad and *F. solani* were studied under adequate nutrient supply as well as under iron- and boron-limited conditions. Hydroponic culture was used to obtain precision in providing defined nutrient conditions.

MATERIALS AND METHODS

Culture of *Pseudomonas* and *Fusarium*. *Pseudomonas putida* (Trevisan) Migula isolate Corvallis (1) was labeled by selecting for resistance to nalidixic acid and rifampicin (Sigma Chemical Company, P.O. Box 14508, St. Louis, MO) by using King's B medium containing the antibiotics at 50 μ g/ml. The nalidixic acid- and rifampicin-resistant isolate, *P. putida* (nal^R rif^R), was maintained on plates of King's B medium containing the antibiotics and was transferred at 3-wk intervals.

Inoculum of *P. putida* nal^R rif^R was obtained by growth in liquid culture in King's B medium lacking antibiotics. Mid-log phase cells were obtained by centrifugation at 10,000 g, washed twice with sterile distilled water, and suspended in 10^{-3} M MgCl₂ before being used to inoculate bean seedlings.

Fusarium solani (Mort.) Sacc. f. sp. *phaseoli* (Burk.) Snyder and Hans. was cultured as described (3,4).

Assessment of bacterial colonization. A qualitative replica-plating procedure was used to assess the degree of colonization of bean seedling roots by colonies of *P. putida* nal^R rif^R. The seedling root was transferred to a sterile petri dish and pressed with a sterile velvet pad which was subsequently replica-plated onto plates of King's B medium containing antibiotics. The plates were incubated at 30 C for 3-5 days and the colony locations were noted.

A quantitative assay was used that involved dividing the root into an upper 5-cm portion adjoining the stem and a lower portion from 5 cm above the root tips. The material was weighed, and where possible 1 g of each portion was transferred to a tube containing 5 ml of sterile water. The root segments were vortexed for 2 min and the tube contents allowed to settle. Aliquots (0.1 ml) were serially diluted, 0.1-ml samples were plated on King's B medium containing the antibiotics, incubated at 30 C for 3-5 days, and the colonies were counted.

Growth of bean seedlings. Seedlings of *Phaseolus vulgaris* L. 'Dark Red Kidney' were grown as described (4). Inoculum of $1.5 \times$

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10^{12} colony-forming units (cfu) of *P. putida* was applied per square meter to designated trays when the roots had just emerged from the seeds. Plants were grown to the first true leaf stage before transfer to hydroponic culture. Conditions for hydroponic culture were as described (4,6). The nutrient combinations were 50 μ M Fe³⁺ plus 25 μ M borate, 5 μ M Fe³⁺ plus 25 μ M borate, 50 μ M Fe³⁺ plus zero borate, and 5 μ M Fe³⁺ plus zero borate. Designated containers were supplemented at time of transplanting with 10^3 conidia of *F. solani*.

Samples of nutrient solution were removed at selected times for examination of pH, *o*-dihydroxyphenol content (13,14), and microbial populations. Bean seedlings, harvested 18 days after transplanting, were examined for the presence of lesions at the root-stem interface. The weight of the whole plant was recorded and fresh weight gain calculated. Samples of plant tissue were either used directly for determination of the degree of bacterial colonization or air-dried at 105 C for 1.5 hr prior to measurement of iron and lignin content using procedures previously described (4,12).

RESULTS

Colonization of bean roots by *P. putida*. Inoculation of bean seedlings with *P. putida* nal^R rif^R resulted in roots which, from imprints of the tissue, were colonized over all of the surfaces. The colonies of *P. putida* nal^R rif^R were present at 0.6 to 1.8 $\times 10^5$ cfu per gram fresh weight of root. Colonization was similar for the lower and upper root sections. This range of colonization was observed for plants removed from the flats 2 days after inoculation and for seedlings examined throughout the 18-day hydroponic growth period. The iron and boron status of the hydroponic solutions, or the presence of *Fusarium* inoculum, did not consistently influence bacterial numbers from the stated range. The weight gains of plants inoculated and uninoculated with *P. putida* was not statistically different under any of the growth conditions.

A comparison of bacterial growth on King's B and King's B plus antibiotics for samples from the pseudomonad-inoculated plants showed that *P. putida* nal^R rif^R accounted for 60–90% of the colonies obtained from the roots. Roots from plants not inoculated with *P. putida* possessed no nal^R rif^R colonies whether assayed by

velvet screening or by plating of root washings. However, plating these samples onto King's B without antibiotics revealed assorted microflora, including some fluorescent pseudomonads, to be present.

Effect of root colonization by *P. putida* on subsequent root infection by *Fusarium*. Seedlings infected with *F. solani* reached greater weights when preinoculated with *P. putida* than did uninoculated plants (Table 1). Each seedling infected with *F. solani* developed a red-brown lesion at the root-stem interface. Lesions were obtained even when the seedlings were preinoculated with *P. putida* although the reddish streaks first formed 2–3 days later than in the pseudomonad-nontreated plants. The presence of *P. putida* also delayed for 2–3 days the foliar wilting observed in all plants inoculated with *F. solani*. Preinoculation with *P. putida* of plants inoculated with *F. solani* had no significant effect on the lengths of the lesions obtained after 18 days at the stem-root interface. Lesion size was significantly greater for plants grown in 5 μ M FeCl₃ without added borate (3.0 \pm 0.4 cm) than for the other nutrient regimes (1.9 \pm 0.3 cm).

Iron contents of bean leaves. The iron contents of bean leaves after 18 days in hydroponic culture were related to the nutrient media and the microbial inocula (Table 2). With 50 μ M FeCl₃, infection by *Fusarium* increased the iron content of terminal leaves compared to those of uninoculated plants. This effect was correlated with the noticeably greener leaves of the infected plants. With 5 μ M FeCl₃, leaf chlorosis appeared in uninoculated seedlings after 16 days and in infected seedlings after 18 days. Iron contents of leaves from these plants were similar. However, with 5 μ M FeCl₃, the pseudomonad-inoculated plants developed chlorosis after 7 days with *P. putida* alone and after 12 days with the pseudomonad plus *Fusarium* treatment. Leaf iron contents were lower in the pseudomonad-inoculated plants than in uninoculated plants. Boron deficiency did not consistently alter the iron levels detected in the leaves.

Plants growing under iron-limited conditions are reported to have lower rhizosphere pH (2). Hydroponic media with 5 μ M FeCl₃ and containing uninoculated plants had a rapid pH reduction from

TABLE 1. The effect of *Pseudomonas putida* on weight of bean seedlings infected with *Fusarium solani* and grown with different levels of iron and borate^a

Microorganism treatment	Fresh weight gain (g) and nutrient levels			
	50 μ M FeCl ₃		5 μ M FeCl ₃	
	25 μ M borate	Zero borate	25 μ M borate	Zero borate
<i>P. putida</i> plus <i>F. solani</i>	3.1 a	2.0 a	2.7 a	2.4 a
<i>F. solani</i>	0.3 b	0.5 b	0.6 b	0.3 b

^aPlants were preinoculated with *P. putida* grown in hydroponic culture at the different nutrient levels, and inoculated with *F. solani* as described in Materials and Methods. Means with a common letter within columns were not significantly different according to Duncan's new multiple range test, $P = 0.05$.

TABLE 3. The effect of *Pseudomonas putida* and *Fusarium solani* and levels of iron and borate on the lignin contents at the root-shoot interface of bean seedlings

Microorganism treatment ^a	Lignin (mg/g dry wt.) and nutrient levels ^b					
	50 μ M FeEDDHA		50 μ M FeCl ₃		5 μ M FeCl ₃	
	25 μ M borate		25 μ M borate	Zero borate	25 μ M borate	Zero borate
Control	177 a		160 a	160 a	168 a	157 a
<i>P. putida</i>	192 a		162 a	185 a	172 a	152 a
<i>P. putida</i> plus <i>Fusarium</i>	218 b		188 a	220 b	153 a	186 b
<i>Fusarium</i>	259 c		230 b	246 b	185 a	189 b

^aPlants were preinoculated with *P. putida* and challenged with *F. solani* as described in Materials and Methods.

^bLignin was extracted from dried tissue at the root-shoot interface of seedlings grown hydroponically for 18 days under the described nutrient conditions. Means with a common letter within a column were not significantly different according to Duncan's new multiple range test, $P = 0.05$.

TABLE 2. The effects of *Pseudomonas putida* and *Fusarium solani* and mineral nutrition on iron content of terminal leaves of bean seedlings

Microorganism treatment ^a	Leaf iron content (μ g/g) and nutrient levels ^b			
	50 μ M FeCl ₃		5 μ M FeCl ₃	
	25 μ M borate	Zero borate	25 μ M borate	Zero borate
Control	370 a	450 a	379 a	323 a
<i>P. putida</i>	351 a	360 b	297 b	200 b
<i>P. putida</i> plus <i>F. solani</i>	730 b	878 c	242 c	262 c
<i>F. solani</i>	645 c	720 d	320 d	310 a

^aPlants were preinoculated with *P. putida* and challenged with *F. solani* as described in Materials and Methods.

^bThe iron content of leaves from plants grown under described iron treatment conditions was determined after 18 days of hydroponic growth. Means with a common letter within columns were not significantly different according to Duncan's new multiple range test, $P = 0.05$.

6.5 to 3.8 between days 5 and 8 after transplanting. In contrast with 5 μM FeCl_3 , the pH of media containing plants inoculated with either the pseudomonad or *Fusarium* alone, or both together, remained between 6.2 and 6.8. With 50 μM FeCl_3 , the pH of the nutrient solutions ranged between 6.3 for the uninoculated plants and 7.2 for the plants inoculated with the pseudomonad plus *Fusarium*. Plants inoculated with the pseudomonad or with *Fusarium* alone did not alter the pH of the nutrient solutions from the initial value of 6.7.

The concentrations of *o*-dihydroxyphenols in the nutrient solutions were monitored simultaneously with pH because of a proposed role of these compounds in promoting iron reduction (14). Whether 5 or 50 μM FeCl_3 was used as the iron source, nutrient media containing uninoculated plants and plants inoculated with *P. putida* showed an increase in *o*-dihydroxyphenol concentration from zero at day 2 to 4.4 (\pm 0.1) μM at day 8. In contrast, 2 days after inoculation with *Fusarium*, with or without *P. putida*, the concentration of *o*-dihydroxyphenols was 6.1 (\pm 1.0) μM for 5 μM FeCl_3 and 8.6 (\pm 1.0) μM for 50 μM FeCl_3 . These *o*-dihydroxyphenol concentrations declined daily to 5.0 (\pm 0.2) μM for 5 μM FeCl_3 and 7.0 (\pm 0.5) μM for 50 μM FeCl_3 media by day 8.

Lignin formation. Lignin contents of the root-shoot interface were not altered consistently by inoculation with the pseudomonad alone compared with control plants (Table 3). Lesion development caused by *F. solani* increased the amount of lignin extracted from the root-shoot interface. Plants grown in 50 μM Fe^{3+} and inoculated with the pseudomonad plus *Fusarium* showed lignin contents that were reduced 18–22% compared to those of plants with *Fusarium* alone. With 5 μM FeCl_3 , lignin contents of plants inoculated with *Fusarium* plus the pseudomonad were reduced 21% with borate but only 2% without borate when compared to plants inoculated with *Fusarium* alone.

The presence of both microorganisms also affected the lignin contents of the lower roots (Table 4). Inoculation with *Fusarium* stimulated lignin formation above that in uninoculated plants. The presence of *P. putida* reduced the amount of lignin formed in plants inoculated with *Fusarium*, although the difference was not always statistically significant. Inoculation with *P. putida* alone also enhanced lignification by 26–93% over that observed in uninoculated plants. To confirm this effect of colonization by *P. putida*, the lignin contents of lower roots from plants harvested at the time of transplanting into hydroponic culture were determined (Table 5). In three separate trials, the lignin contents of roots of plants inoculated with *P. putida* were higher by 9–50% than for the uninoculated plants.

DISCUSSION

The aggressive colonization of bean root surfaces by the *P. putida* isolate Corvallis agrees with reports of effective colonization

of roots of other plant species by strains of the group characterized by *P. putida* and *P. fluorescens* (17, 18). Our data demonstrate that colonization occurred on lateral roots as well as the main root, and that it was maintained in the presence of *F. solani* f. sp. *phaseoli* and under growth conditions varying in iron and boron availability. Under low iron availability, pseudomonad colonization of bean caused chlorosis and reduced foliar iron contents. The production of siderophores by the *P. putida* Corvallis isolate (F. Fekete and A. J. Anderson, unpublished) may be involved in this chlorotic response. The siderophores may chelate the iron in a form which is unavailable to the plant.

Pseudomonas putida as well as the challenging pathogen *F. solani* f. sp. *phaseoli*, affected two other phenomena related to iron nutrition. Many plants respond to low iron availability with acidification of the rhizosphere (2). Although acidification of the nutrient solution was observed with low-iron nutrition for uninoculated plants, root colonization by *P. putida* and/or *Fusarium* alleviated this pH drop. The secretion by plant roots of dihydroxyphenolic compounds has been proposed as a mechanism to promote reduction of rhizosphere Fe^{3+} ions to Fe^{2+} ions (14). In our studies, production of dihydroxyphenolic compounds was affected more by the microbial populations than by the iron status. The enhanced phenolic accumulations from plants inoculated with *P. putida* and/or *F. solani* may have resulted from changes in root wall permeability and in microorganism-mediated changes in phenol metabolism.

Altered phenolic metabolism was demonstrated by increased lignin formation in roots colonized by *P. putida* or *F. solani*. Formation of lignin, whether stimulated by inocula of the pseudomonad or *Fusarium*, responded similarly to iron nutrition. Reduction in lignin content associated with low available iron may relate to an essential role of peroxidases containing heme iron in the synthesis of the polymer. The slight increase in lignin observed in boron-deficient plants when inoculated with pseudomonads or *Fusarium* is contrary to the hypothesis of Lewis (11). Lewis predicted that boron deficiency may impair polymerization of the monomeric precursors to lignin. Our observations would be consistent if the factors that control phenol metabolism differ in plants that are responding to a microbial challenge rather than being at equilibrium with the biotic environment.

Enhanced production of oxidized phenols has been documented for many plant-pathogen interactions including that of *F. solani* and bean. However, root colonization by the saprophytic *P. putida* alone caused increased production of ligninlike polymers. This observation suggests that *P. putida* may contribute to plant resistance to pathogens by increasing lignin levels. The subtle effects of colonization by *P. putida* on infection by *F. solani* support this suggestion. The time of onset of lesion formation was lengthened and the amount of lignin was reduced in plants inoculated with *P. putida* versus uninoculated plants challenged with *F. solani*. The failure of *P. putida* to prevent infection by *F. solani* agrees with the lack of suppression of this pathogen in natural soils suppressive to *Fusarium* wilts (R. Baker, personal communication). Results of greenhouse and field studies of suppression of take-all and of *Fusarium* wilts by *P. putida* demonstrated that a high level of protection occurred only when available iron was low. Scher and Baker (16) and Kloepper et al (8) suggest that the siderophores produced by the protectant bacteria under low-iron conditions limited iron availability to the pathogen

TABLE 4. Effect of microorganisms and mineral nutrition on lignin content of the lower roots of bean seedlings harvested after 18 days in hydroponic culture

Treatment ^y	Lignin (mg/g dry weight) ^z			
	50 μM FeCl_3 25 μM borate	50 μM FeCl_3 Zero borate	5 μM FeCl_3 25 μM borate	5 μM FeCl_3 Zero borate
Uninoculated	112 a	98 a	109 a	90 a
<i>Pseudomonas putida</i>	160 b	190 b	138 b	150 b
<i>P. putida</i> plus <i>Fusarium</i>	164 b	204 b	150 b	210 b
<i>Fusarium</i>	190 b	297 c	160 b	225 b

^yPlants were preinoculated with *P. putida* and challenged with *F. solani* as described in Materials and Methods.

^zLignin was extracted from dried tissue of the lowest 5 cm of roots of seedlings grown hydroponically for 18 days under the described nutrient conditions. Means with a common letter within columns were not significantly different according to Duncan's new multiple range test, $P = 0.05$.

TABLE 5. The effect of microorganisms on the lignin content of the lower roots of greenhouse-grown bean seedlings

Treatment ^a	Lignin (mg/g dry weight) trial number ^b		
	1	2	3
Uninoculated	86	128	76
Pseudomonad-inoculated	100	140	114

^aPlants were preinoculated with *P. putida* as described in Materials and Methods.

^bLignin was extracted from dried tissue of the lowest 5 cm of roots of seedlings grown in vermiculite to the first trifoliate leaf stage.

and consequently hindered fungal growth. In the system involving *F. solani* and *P. putida*, some protective effects are apparent with iron levels that should not stimulate the production of the bacterial siderophores. Rather, results of our examination of lignin contents of root tissue suggest that the interaction between *P. putida* and the plant that enhances lignification also could be crucial in the protection phenomenon. If lignin functions as a defensive barrier in the plant (5), then the enhanced level associated with colonization by *P. putida* may contribute to an impaired root-pathogen interaction. Consequently, we suggest that in addition to direct effects upon pathogens, beneficial isolates of *P. putida* also may strengthen the defensive potential of the plant.

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