

## Effect of *Pseudomonas putida* on the Stimulation of *Pythium ultimum* by Seed Volatiles of Pea and Soybean

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### ABSTRACT

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*Pseudomonas putida* strain NIR increased the emergence of soybean and pea in soil infested with *Pythium ultimum* when applied to the seeds as a cell suspension. Hyphal growth from soil-produced sporangia of *P. ultimum* was stimulated by volatiles from germinating seeds of pea and soybean, and the stimulation was reduced when the seeds were treated with NIR. Ethanol and acetaldehyde (10  $\mu$ l/L) also stimulated hyphal growth from *P. ultimum* soil inoculum. Treatment of seeds with NIR reduced the concentration of ethanol and acetaldehyde produced by

germinating pea and soybean during the 24 h period after imbibition. NIR used ethanol as a sole carbon source in culture, completely metabolizing 15–500  $\mu$ l/L within 48 h. Ethanol use was partially inhibited by glucose, but was detected even at 1,000  $\mu$ g/ml glucose. NIR may reduce damping-off by competing for and using volatile exudates from germinating pea and soybean seeds. These volatile seed exudates may serve both inductive and nutritional functions in the pathogenesis of *Pythium* damping-off disease.

*Pythium ultimum* Trow is a widely distributed Oomycete fungus that causes preemergence and postemergence damping-off and root rot of agronomic and vegetable crops. Germinating seeds of several crops have been experimentally protected against *Pythium* damping-off by treatment with fluorescent pseudomonads (1,5,16,24). Much of this work has focused on two species, *Pseudomonas putida* and *P. fluorescens*, which have controlled other soilborne diseases such as Fusarium wilt of flax (29), take-all of wheat (39), and Rhizoctonia damping-off of cotton (9).

Three mechanisms have been proposed to explain the antagonism of fluorescent pseudomonads against *Pythium* spp. First, antibiotic production, which was strongly implicated in the biocontrol of take-all of wheat by *P. fluorescens* (37), also may act against *Pythium* damping-off (10,36). Second, most fluorescent pseudomonads produce and excrete siderophores called pyoverdines, which are yellow-green colored secondary metabolites that fluoresce under ultraviolet light. Produced under iron-limiting conditions, these compounds chelate the ferric ion ( $\text{Fe}^{3+}$ ), creating a siderophore- $\text{Fe}^{3+}$  complex that is transported into the microbial cell (19). Some researchers have postulated that siderophores might antagonize plant pathogens in the rhizosphere by competing for iron needed by the pathogen for spore germination, mycelial growth, and pathogenesis (13,32). For example, mutants of *P. fluorescens* deficient in siderophore production demonstrated a reduced ability to control *Pythium* damping-off of cotton (16). Finally, the germination and hyphal growth of *Pythium* propagules are stimulated by root and seed exudates (7), and saprophytic growth before infection might be important in pathogenesis. Competition with microflora for available, labile carbon substrates may limit *Pythium*, and it has been proposed to explain the suppressiveness of composted container media to *Pythium* diseases (2,3,5,17).

*P. putida* NIR, a biocontrol agent of Fusarium wilt of cucumber (4,25), also controlled *Pythium* damping-off (27). Studies were undertaken to determine the mechanism of control against this disease. Although this bacterial strain does not produce antibiotics in vitro, mycelial growth but not germination of *P. ultimum* was inhibited by NIR-produced siderophores in low iron medium, and this inhibition was reversed by the addition of iron. Mutants of NIR deficient in siderophore production, which were obtained

by Tn5 mutagenesis, did not inhibit mycelial growth of *P. ultimum*. However, loss of siderophore production did not affect the biocontrol ability of these mutants against *P. ultimum*, as measured in a bioassay in three different agricultural soils (27,28). Because of this work, the hypothesis that antagonism of *P. putida* against *P. ultimum* was due to iron competition was rejected. An alternative premise was explored that antagonism was the outcome of competition by *P. putida* for seed-generated volatile carbon substrates that stimulate the germination and growth of *P. ultimum*.

The purpose of this study was to investigate stimulation of *P. ultimum* by volatile exudates of pea and soybean, including ethanol and acetaldehyde. This research also investigated the effect of seed treatment with *P. putida* NIR on stimulation of *P. ultimum* and production of ethanol and acetaldehyde in the spermosphere. It is postulated that *P. putida* can rapidly metabolize volatile seed exudates, interfere with the stimulation of *P. ultimum*, and reduce seed rot.

### MATERIALS AND METHODS

**Organisms.** *P. ultimum* isolate N1 (14), which produces only sporangia but no oospores, was maintained on water agar (2% agar, w/v) and transferred monthly. Soil-sporangia inoculum of *P. ultimum* was produced according to the methods of Paulitz and Baker (26). A soil amended with 1% (w/w) ground rolled oats was autoclaved in 1-L mason jars for 45 min on two consecutive days. The sterile amended soil was infested with four plugs of a water agar culture of *P. ultimum*. After 3 wk of incubation, this inoculum contained approximately  $4.0 \times 10^5$  cfu/g, as measured by dilution plating on selective medium (18).

*P. putida* NIR (4), a spontaneous rifampicin-resistant mutant, was stored at  $-80^\circ\text{C}$  in nutrient broth + 10% glycerol. To produce bacterial cells for seed inoculations, the bacterial strain was retrieved from storage and streaked on King's Medium B (KMB) agar (11). After 24 h, bacterial cells were suspended in sterile  $\text{MgSO}_4$  (0.1 M) and pelleted by centrifugation at 2,000 g. The pellet was resuspended in sterile distilled water and adjusted to an optical density of  $A_{640\text{nm}} = 0.5$  (approximately  $10^9$  cells per milliliter).

**Seed treatment.** The bacterial suspension, adjusted to approximately  $10^9$  cells per milliliter, was mixed with a 2% (w/v) solution of pelgel, which served as a sticker. Fifteen pea seeds (*Pisum sativum* L. 'Laxton Progress') or 20 soybean seeds (*Glycine max*

(L.) Merr. 'Maple Arrow') were placed in a 12- × 12- × 2.3-cm plastic weigh dish and covered with 10 ml of the bacterial-pelgel suspension. Seeds were soaked in the bacterial suspension for 2 h before being placed in moist sterile sand. Seeds soaked in distilled water + 2% pelgel were used for control treatments.

**Stimulation of hyphal growth of *P. ultimum* by seed volatiles of pea and soybean.** Forty grams of nonsterile sand was placed in a 85- × 24-mm plastic petri dish and moistened with water to 15% (v/w). Five treated pea seeds or seven treated soybean seeds were buried in the moist sand. A 7-cm filter paper (Whatman No. 1) was moistened with sterile water and placed on the inside of the petri dish lid. Approximately 1 g of soil-sporangia inoculum of *P. ultimum* was spread on the moist filter paper. The lid was placed on the petri dish and sealed with three layers of Parafilm. Three treatments were used: no seeds in dish; seeds treated with pelgel only; and seeds treated with NIR + pelgel. After 24 h of incubation at 26 C, the soil inoculum was removed from the lid of the petri dish, and hyphal length was measured. Approximately 0.1 g of soil inoculum was placed in a 1.5-ml microfuge tube with 1 ml of water. The tube was shaken on a Vortex mixer (Bohemia, NY) for 1 min, and a 30- $\mu$ l sample was examined under the compound microscope. Using an ocular grid, the number of intersections between the lines of the grid and hyphae were counted. Ten counts were taken from each slide, and four slides were taken from each sample. Using the line intersect method of Newman (22), the total hyphal length was calculated and adjusted to the dry weight of the soil, which was determined after drying the samples at 70 C.

To test for the production of volatile antifungal compounds by NIR, a split plate experiment was performed. KMB agar was poured in one side of a 100- × 15-mm plate, and 10 imbibed soybean seeds were placed in the other half with moist sand. The KMB agar was streaked with NIR in one treatment, but left unstreaked in the other. Inoculum of *P. ultimum* was placed on the lid, as previously described, and the plates were sealed with three layers of Parafilm. After 24 h, hyphal length was measured.

The effect of ethanol and acetaldehyde on the stimulation of *P. ultimum* was tested in 100- × 15-mm petri dishes. One milliliter of aqueous solutions containing 0, 100, 300, 1,000, 3,000, 10,000, 30,000, or 100,000  $\mu$ l/ml of ethanol or 0, 11, 22, 111, 222, 1,111, 2,222 or 11,111  $\mu$ l/ml of acetaldehyde was placed on a 7-cm-diameter filter paper (Whatman No. 1) on the bottom of the petri dish. Soil inoculum of *P. ultimum* was placed on the lid of the plate, and the plate was sealed with three layers of Parafilm. The actual concentration in the headspace was calculated by placing aqueous solutions with various concentrations of ethanol or acetaldehyde in 60-ml serum bottles sealed with rubber stoppers. After 24 h, the headspace was analyzed with gas chromatography and the actual concentration in the headspace was calculated. Hyphal length was measured 24 h later, using the same methods as described above. In all experiments, each treatment was replicated four times, and each experiment was repeated once.

**Biological control assay.** The effectiveness of *P. putida* in controlling *Pythium damping-off* on pea and soybean was tested in the following bioassay. Soil-sporangial inoculum of *P. ultimum* was mixed with pasteurized greenhouse soil to achieve a final concentration of 50 cfu/g, determined by dilution plating of the soil. Pea and soybean seeds were treated with strain NIR as previously described. Five seeds were planted in each 100-cm-diameter plastic pot filled with 500 g of soil. Three treatments were used: noninfested control (pasteurized soil); infested, no NIR (soil infested with *P. ultimum*, and seeds treated with 2% pelgel); and infested + NIR (seeds treated with NIR + 2% pelgel and planted in infested soil). Each treatment was replicated six times, and the experiment was repeated twice. After 7 days, percentage of emergence was recorded.

**Effect of NIR on production of ethanol and acetaldehyde by pea and soybean.** Forty-five grams of sand was placed in 60-ml serum bottles and moistened with 7 ml of distilled water. Five seeds of pea or seven seeds of soybean, treated or not treated

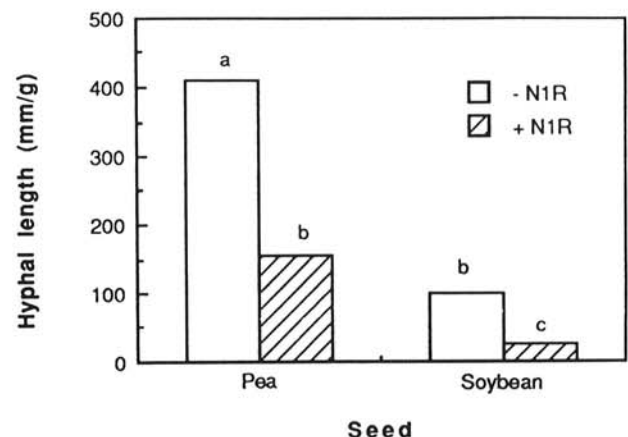
with NIR, were placed in the bottle and covered with 12 g of sand that was moistened with an additional 3 ml of distilled water. Each treatment was replicated three times at each sampling time. The uncovered bottles were enclosed in a 40- × 50-cm plastic bag with moist paper towels on the bottom and incubated at 26 C. Two hours before analysis, the bottles were sealed with a rubber septum. To analyze the samples, 1 ml of gas from the headspace of each bottle was injected into a Varian 3400 gas chromatograph (Palo Alto, CA) equipped with a flame ionization detector and a 183- × 0.4-cm glass column packed with Chromosorb W with a 10% loading of cyanopropyl silicones (CSP-509, Chromatographic Specialties, Brockville, Ontario, Canada). The column was maintained at 50 C, with helium as the carrier gas, at a flow rate of 50 ml/min. Peaks were integrated with a Varian 4290 integrator and compared with the retention time and peak areas of known standards. A standard curve was used to convert peak area to actual concentration.

**Utilization of ethanol by NIR in culture.** A salt medium was prepared by adding 1 g of  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.2 g of KCl, and 0.2 g of  $\text{MgSO}_4$  to 1 L of water. The medium was dispensed into 125-ml Erlenmeyer flasks (50 ml per flask) and autoclaved. After cooling, ethanol (95%) was added to the flasks to give a final concentration of 0, 15, 31, 125, 250, 500, or 1,000  $\mu$ l/L. NIR was transferred to half of the flasks (one loopful of bacteria per flask). Each treatment had four replicates.

In another experiment, the repression of ethanol catabolism by glucose was tested by adding glucose at 0, 31, 62, 125, 250, 500, or 1,000  $\mu$ g/ml to each of the flasks containing 250  $\mu$ l/L of ethanol. A loopful of NIR was transferred to each flask. In both experiments flasks were incubated at 26 C on a rotary shaker (150 rpm) for 48 h. The bacteria were removed from the culture broth by centrifugation, and 1  $\mu$ l of filtrate from each flask was injected in a Varian 3400 gas chromatograph with a 183- × 0.4-cm glass column packed with Chromosorb 102, maintained at 125 C. The data were treated as described above.

## RESULTS

**Stimulation of hyphal growth of *P. ultimum* by seed volatiles of pea and soybean.** When inoculum of *P. ultimum* was exposed to volatiles from germinating seeds of pea or soybean for 24 h, an extensive network of hyphae grew out of the soil. The presence of NIR on the seeds of pea or soybean significantly reduced the hyphal growth of *P. ultimum* exposed to volatiles from germinating seeds (Fig. 1). Volatiles of pea seeds were more stimulatory than volatiles from soybean. When NIR was grown on agar opposite germinating seeds, no significant reduction in



**Fig. 1.** Effect of *Pseudomonas putida* NIR on the hyphal stimulation of *Pythium ultimum* by volatiles from germinating pea and soybean seeds. Seeds treated with NIR were soaked in suspension of 2% pelgel + bacteria ( $10^9$  cells per milliliter) for 2 h. Hyphal length in *P. ultimum*-soil inoculum was measured 24 h after exposure to seed volatiles. Values presented are means of four replicates, and values followed by a common letter do not differ significantly, according to Student's *t* test ( $P < 0.05$ ).

hyphal stimulation was observed. Hyphal growth also was stimulated by 7.6 or 27.2  $\mu\text{l/L}$  of ethanol, or by 9.5  $\mu\text{l/L}$  of acetaldehyde (Fig. 2). The magnitude of stimulation by acetaldehyde was much lower than that observed with ethanol.

**Biological control assay.** Treatment of soybean and pea seeds with NIR significantly improved seedling emergence compared with the unprotected treatment (Fig. 3). Emergence of soybean treated with NIR and planted in infested soil was similar to that of seeds planted in noninfested soil. However, NIR was not as effective on peas, which are more susceptible to damping-off. Only 30% of NIR-treated peas emerged, compared with 93% in the noninfested treatment.

**Effect of NIR on production of ethanol and acetaldehyde by pea and soybean.** When pea seeds were treated with NIR, significantly less ethanol was detected at 18 h compared with the non-treated seed (Fig. 4A). However, in the repeat experiment, significant differences also were detected at 2–8 h. Acetaldehyde concentrations were significantly lower in NIR-treated pea seed at all sampling times and were below the detection limits in many samples (Fig. 4B).

Similar trends were observed with soybean, although overall concentrations of ethanol and acetaldehyde were less than in peas. Ethanol production was significantly reduced in the treated seed from 6 to 19 h after imbibition, and acetaldehyde was diminished at all sampling times except 19 h (Fig. 5).

In all experiments, both treatment and time had significant main effects. In nontreated seeds, both ethanol and acetaldehyde concentrations increased during the first part of the experiment, and declined 10–20 h after imbibition.

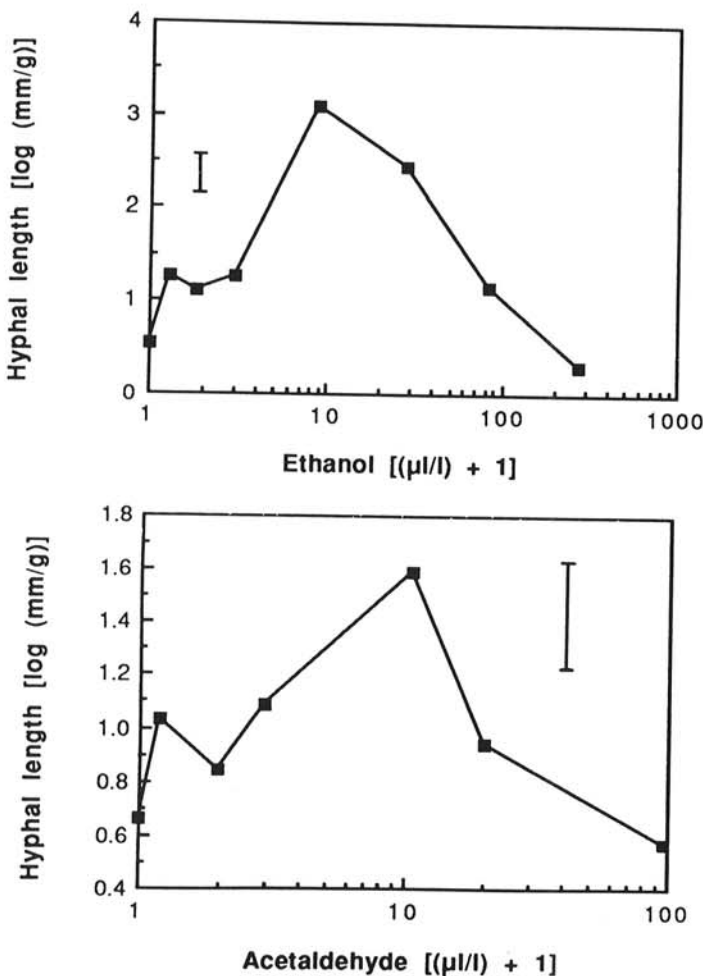


Fig. 2. Effect of ethanol and acetaldehyde on the hyphal stimulation of *Pythium ultimum*. Soil inoculum of *P. ultimum* was exposed to various concentrations for 24 h. Values presented are the means of four replicates. Bars represent least significant difference (LSD), calculated from the error mean square of the experiment.

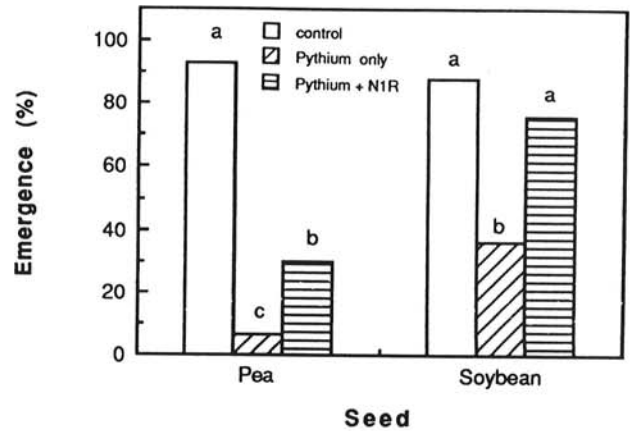


Fig. 3. Biological control of *Pythium* damping-off of pea and soybean by seed treatment with *Pseudomonas putida* NIR. Control: seeds planted in pasteurized greenhouse soil. *Pythium* only: seeds treated with 2% pelgel were planted in pasteurized soil infested with 50 cfu/g of *Pythium ultimum*. *Pythium* + NIR: seeds soaked in a suspension of 2% pelgel + NIR ( $10^9$  cells per milliliter) were planted in infested soil. Emergence was recorded 7 days after planting. Values are means of five replicates, and values followed by a common letter do not differ significantly, according to Duncan's multiple range test ( $P < 0.05$ ).

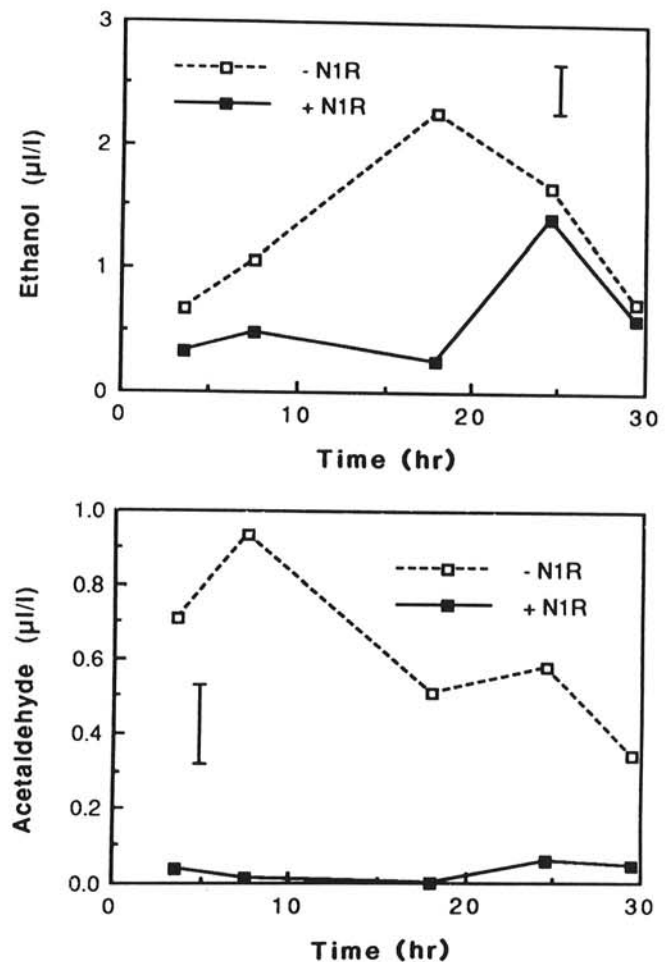


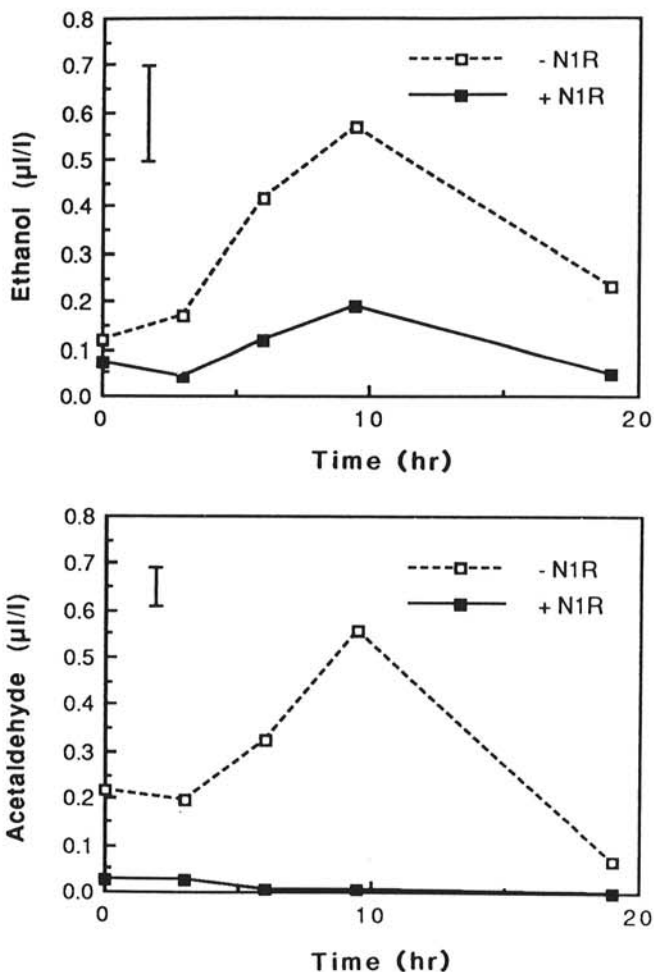
Fig. 4. Effect of seed treatment with *Pseudomonas putida* NIR on the production of ethanol and acetaldehyde by imbibed pea seeds. -NIR: seeds soaked in 2% pelgel solution for 2 h. +NIR: seeds soaked in suspension of 2% pelgel + bacteria ( $10^9$  cells per milliliter). Time represents number of hours after start of imbibition when bottles were sealed with rubber stopper. The headspace was analyzed by gas chromatography 2–3 h later. Each treatment was replicated three times. Least significant difference values for A and B were 0.35 and 0.20  $\mu\text{l/L}$ , respectively.



**Use of ethanol by NIR in culture.** NIR, in culture with ethanol as a sole carbon source, completely metabolized 15–500  $\mu\text{l/L}$  of ethanol after 48 h (Fig. 6A). This catabolism of ethanol was partially inhibited by 62–1,000  $\mu\text{g/ml}$  of glucose, but some ethanol metabolism was still detected at the highest glucose level (Fig. 6B).

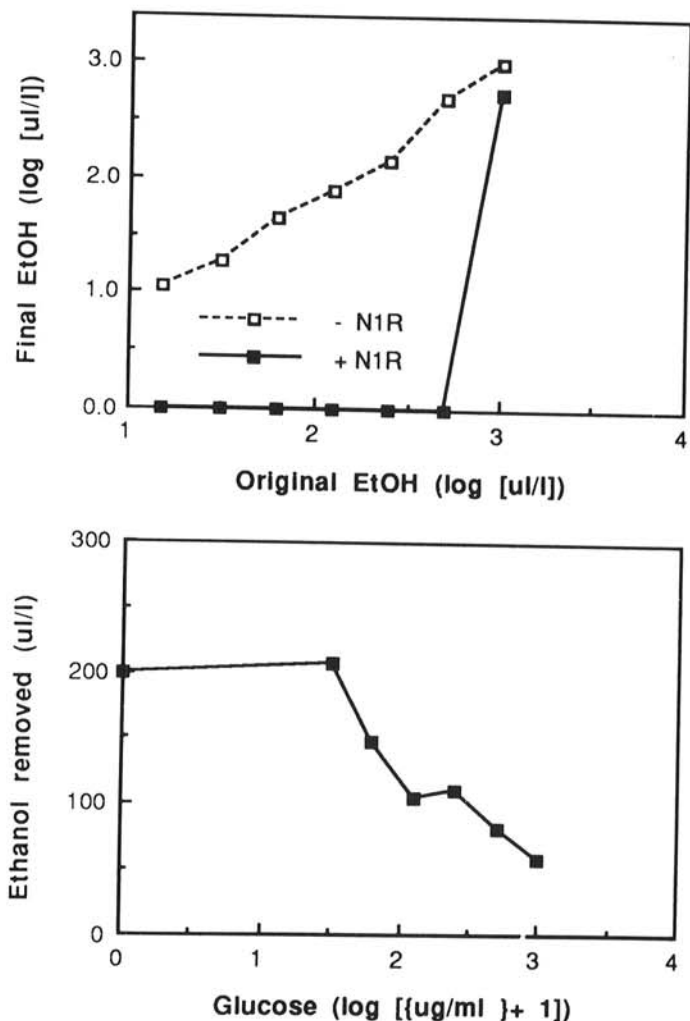
## DISCUSSION

Germination and growth of *P. ultimum* is stimulated rapidly by germinating seeds (7,15,34,35). This stimulation has been attributed to water soluble sugars and amino acids exuded by the seed or seedling (12,30,31). However, volatile compounds such as ethanol and acetaldehyde also are produced rapidly by germinating seeds (6,38,40) and might be more important in the rapid infection by *P. ultimum*. Volatile exudates are produced sooner than water soluble exudates (20) and may diffuse farther and faster, especially in unsaturated soils, making volatile exudates a likely candidate for an early germination signal or nutrient source. For example, the population of *P. ultimum* increased dramatically in an autoclaved soil that was exposed to volatiles from aged pea seeds (23). *P. ultimum* can germinate within 1–3 h after exposure to nutrients and can colonize the seed coat of cucumber (15) or the pericarp of sugar beet (24) within 24 h after planting.



**Fig. 5.** Effect of seed treatment with *Pseudomonas putida* NIR on the production of ethanol and acetaldehyde by imbibed soybean seeds. -NIR: seeds soaked in 2% pelgel solution for 2 h. +NIR: seeds soaked in suspension of 2% pelgel + bacteria ( $10^9$  cells per milliliter). Time represents number of hours after start of imbibition when bottles were sealed with rubber stopper. The headspace was analyzed by gas chromatography 2–3 h later. Each treatment was replicated three times. LSD values for A and B were 0.20 and 0.10  $\mu\text{l/L}$ , respectively.

The results demonstrate that germination and growth of *P. ultimum* from sporangia formed on rolled oats in sterile soil was stimulated by volatiles from pea and soybeans, and this stimulation was mimicked by ethanol and acetaldehyde. Nelson (20) also demonstrated that sporangia of *P. ultimum* were stimulated to germinate by pea and soybean volatiles, but he was unable to reproduce the effect with ethanol or acetaldehyde. In his work, the sporangia were grown on grass extract agar and may respond to nutrients quite differently than sporangia produced on plant tissue. Sporangia grown in agar culture were stimulated by sugars and amino acids, but sporangia formed in plant tissues were not (21). The sporangia used in these experiments were produced on rolled oats in sterile soil, and their response to volatile compounds may be closer to that of sporangia formed in natural soils. The levels of ethanol and acetaldehyde that were stimulatory to *P. ultimum* were higher than the levels sampled in the headspace around germinating seeds. However, ethanol is very soluble in water, and most of the ethanol remains in the aqueous phase rather than volatilizing. It is possible that the ethanol concentration in the film of water surrounding the soil particles in the spermosphere was substantially higher than that measured in the headspace.



**Fig. 6.** Utilization of ethanol by *Pseudomonas putida* NIR in liquid broth. A, NIR added (+NIR) or not added (-NIR) to flasks containing mineral salts and various concentrations of ethanol as the sole carbon source. B, NIR added to flasks containing mineral salts, 250  $\mu\text{l/L}$  ethanol, and various concentrations of glucose. Ethanol concentrations were quantified by gas chromatography after 48 h of incubation. Each treatment was replicated four times. In A, +NIR and -NIR treatments were significantly different ( $P < 0.05$ ) at all concentrations except 1,000  $\mu\text{l/L}$ , according to *t* test at each concentration. In B, glucose concentration had a significant effect ( $P < 0.05$ ), according to analysis of variance.

Few studies have examined the effect of spermosphere or spermoplane microbes on seed volatiles. *Trichoderma harzianum* Rifai and *Enterobacter cloacae* reduced the concentration of ethanol and acetaldehyde produced by pea seeds during germination (6). This reduction was not detected until 24 h after imbibition, but in this work, reductions were observed as early as 2–4 h after imbibition. Harman et al (8) also demonstrated that *Pseudomonas* spp. and *Phomopsis* spp., when inoculated onto the seed, could use more than 80% of the aldehydes from the aerial solution. In a taxonomic study by Stanier et al (33), over 90% of strains of *P. putida* biotypes A and B could use ethanol as a sole carbon source. Fluorescent pseudomonads can break down a wide variety of organic compounds. This catabolic versatility, combined with their fast growth rate, makes them ideal spermosphere competitors.

Competition for volatile seed exudates by spermoplane bacteria apparently has not been previously postulated as a mechanism of biological control of damping-off pathogens. Bacteria that are efficient users of volatiles might interfere with the early germination signal from the seed, thus reducing the density of germinated pathogen propagules. In addition, these volatiles may be used as a nutrient source by the pathogen for saprophytic growth to the spermoplane. Reduction of seed volatile concentrations through competition may reduce the inoculum potential of individual propagules and reduce seed infection. This work suggests that biological control may be mediated through competition for seed volatiles, which might serve both an inductive and nutritional function in the pathogenesis of *Pythium* damping-off. However, numerous other factors may influence the interaction between seed volatiles and spermoplane bacteria, including host, seed age, temperature, moisture level, time of imbibition, and other spermoplane and soil microbes. Glucose and other reducing sugars exuded by the seed might inhibit the production of alcohol dehydrogenase and the catabolism of ethanol, although this inhibition was only seen at higher glucose levels in our work. Clearly, further work is needed in order to unravel some of these complexities.

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