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The role of *Pseudomonas* spp. and competition for carbon, nitrogen and iron in the enhancement of appressorium formation by *Colletotrichum coccodes* on velvetleaf

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Abstract

Colletotrichum coccodes is currently being investigated as a mycoherbicide against the weed velvetleaf (*Abutilon theophrasti*). Two isolates of *Pseudomonas* spp. (Ps2 and Ps5) reduced the percentage of germ tubes and increased appressorial formation of *C. coccodes* on detached leaves of velvetleaf. A study was conducted to see whether this effect could be attributed to competition for nutrients or iron between *C. coccodes* and *Pseudomonas* spp. Ps2 and Ps5 had no effect on early spore germination, but reduced the percentage of germ tubes at 24 and 30 h, compared to the nontreated control. This reduction was diminished by the addition of nutrients but not Fe^{3+}. Ps2 and Ps5 stimulated the formation of dark-coloured appressoria without germ tubes (AWGT), but this stimulation was diminished by the addition of nutrients or Fe^{3+}. Germ tube branching at 30 h was also inhibited by the bacteria, but was not diminished by the addition of nutrients or iron. EDTA stimulated conidial germination at 10 h, which was reduced by the addition of Fe^{3+}. However, EDTA did not stimulate the formation of appressoria (AWGT). These results suggest that the reduction in the percentage of germ tubes and the increase in the percentage of appressoria induced by the bacteria may be due to the competition for carbon or nitrogen. Iron competition may also be involved in the stimulation of appressorial formation, but not in the reduction in germ tube percentage and branching. Phylloplane bacteria may compete for carbon, nitrogen and iron, limiting the saprophytic phase of the pathogen on the phylloplane and accelerating the development of the parasitic phase. This may enhance the field efficacy of *C. coccodes* as a biocontrol agent against velvetleaf.

Introduction

The fungus *Colletotrichum coccodes* (Wallr.) Hughes is a promising mycoherbicide against velvetleaf (*Abutilon theophrasti* Medic.), a weed in corn and soybean production. This fungus causes leaf blight and leaf spot. Like many mycoherbicides, this fungus requires a prolonged dew period (18–24 h) for maximum infection, which may limit its efficacy in the field [Wymore *et al.*, 1988]. Recent research [Fernando *et al.*, 1994] demonstrated that *Pseudomonas* spp. isolated from the phylloplane could enhance disease and appressorial formation when coinoculated with the fungus. One hypothesized mechanism for this phenomenon is that appressorial formation may be stimulated by nutrient competition and stress. Leaves exude simple sugars and amino acids [Godfrey, 1979; Tukey, 1971], which may stimulate the germination and growth of nutrient-dependent fungi on the phylloplane. Bacteria such as *Pseudomonas* can rapidly remove amino acids from the phylloplane, and may affect the germination of plant pathogens [Blakeman and Brodie, 1977]. Nutrient stress may also stimulate the formation of appressoria [Emmett and Parbery, 1975].

Another hypothesis is that phylloplane bacteria may compete for iron and influence the infection process. Fluorescent *Pseudomonas* spp. produce pyoverdine, a siderophore that chelates iron [Loper and Buyer, 1991]. Siderophores are low molecular weight compounds that are produced under iron limiting conditions, chelate ferric iron (Fe^{3+}) with a high specific activity, and serve as vehicles for the transport of Fe^{3+} into the microbial cell [Neilands, 1981]. Iron competition via siderophores may stimulate disease development and appressorium formation [Swinburne, 1981]. For example, the iron-chelating agent ethylenediaminetetraacetic acid (EDTA) stimulated the formation of lesions by *Botrytis cinerea* on leaves of *Vicia faba* [Brown and Swinburne, 1982]. Slade *et al.* [1986] demonstrated that a siderophore purified from a *Pseudomonas* spp. stimulated germination and appressorium formation by *Colletotrichum acutatum* on strawberry stolons. However, Fe^{3+} chelated with the siderophore, and free Fe^{3+} inhibited germination and appressorium formation.

In this study, we investigated the role of *Pseudomonas* spp. and competition for carbon, nitrogen, and iron in the acceleration of *C. coccodes* infection of velvetleaf. A simple sugar (glucose) and two amino acids (glutamine and alanine) were applied to detached leaves alone or in combination with bacteria, followed by inoculation with *C. coccodes*. In separate experiments, the iron chelator EDTA or bacteria were applied to leaves, with or without an excess of Fe^{3+}. Germ tube and appressorium formation of the pathogen were examined during a 30-h period after inoculation.

Materials and methods

Preparation of fungal inoculum. A culture of *C. coccodes* isolate AG-3 [Gotlieb *et al.*, 1987] was used in all experiments. This fungus was maintained on potato dextrose agar (PDA) at 26 °C. Two 0.5-mm plugs from the actively growing margins of the colony were transferred to 100 ml of Richard's modified V-8 broth medium [Walker, 1980], and incubated at 25 °C for 7 days on a rotary shaker (250 rpm). The spores were harvested by filtering the cultures through four layers of cheesecloth and centrifuging the filtrate at 5000 g for 10 min. The supernatant was decanted and the spore pellet suspended in sterile distilled water. Spores were counted using a haemocytometer and adjusted to the desired concentration.

Preparation of bacterial inoculum. Two isolates of *Pseudomonas* (Ps2 and Ps5) which increased appressorial formation of *C. coccodes*, early disease expression, and disease severity were selected for the following experiments. The cultures were stored in nutrient broth (Difco Co, St. Louis) + 10% glycerol (NBGly) at −80 °C. They were grown on nutrient agar plates amended with 10% glycerol (NAGly). A loop of bacteria was transferred to 100 ml of NBGly and incubated on a rotary shaker overnight at 150 rpm. The bacterial suspension was centrifuged at 3500 g for 15 min and the supernatant was decanted. The cell pellet was resuspended in 0.1 M $MgSO_4$ and centrifuged again. The pellet was resuspended in 0.1 M $MgSO_4$ and adjusted to the desired concentration based on absorbency at 640 mm and by comparison to a standard curve. Isolate Ps2 was used in the nutrient experiments and isolates Ps2 and Ps5 were used in the iron competition experiments.

Preparation of iron chelator. All glassware used in the iron experiments was soaked in 1 N hydrochloric acid for 12 h and rinsed in demineralized water. Ethylenediaminetetraacetic acid (EDTA) was prepared using demineralized sterile distilled water. Stock solutions were prepared at 10 times the required concentration and stored at 4 °C. The concentration used in the experiments was 5×10^{-4} M. Prior to the experiment, 1 ml of the stock solution was diluted 10-fold with sterile distilled water. To prepare EDTA + Fe, equimolar solutions of EDTA and $Fe_2(SO_4)_3$ (1×10^{-3} M) were mixed together (1:1 vol) so the final concentration of each was 5×10^{-4} M.

Iron competition experiment. Plants were grown in a growth chamber at 24 °C with incandescent and fluorescent lighting for 16 h/day. The first true leaves were detached from 12-day-old velvetleaf plants. The following treatments were applied by dipping detached leaves in the following solutions: Control (water); EDTA only (5×10^{-4} M); EDTA + Fe (equimolar concentrations of EDTA and $Fe_2(SO_4)_3$ (5×10^{-4} M); Ps2 *Pseudomonas* isolate Ps2 (10^8 cells/ml); Ps2 + Fe (*Pseudomonas* isolate Ps2 (10^8 cells/ml) and $Fe_2(SO_4)_3$ (5×10^{-4} M), Ps5 (*Pseudomonas* isolate Ps5 (10^8 cells/ml); and Ps5 + Fe (*Pseudomonas* isolate Ps5 (10^8 cells/ml) and $Fe_2(SO_4)_3$ (5×10^{-4} M). Detached leaves were then inoculated with *C. coccodes* by misting fungal inoculum (10^7 conidia/ml) onto the detached leaves.

Nutrient competition experiment. Detached leaves were dipped in a suspension of Ps2 (10^8 cells/ml) for the bacterial treatment or water for the treatments without bacteria. Ten μl of nutrient solution or water were then applied to the upper surface of the detached leaves. The following treatments were tested: control (water); Ps2 only (10^8 cells/ml); glucose only (5×10^{-5} M); glutamine only (5×10^{-5} M), alanine only (5×10^{-5} M); Ps2 (10^8 cells/ml) + glucose (5×10^{-5} M); Ps2 (10^8 cells/ml) + glutamine (5×10^{-5} M); and Ps2 (10^8 cells/ml) + alanine (5×10^{-5} M). Immediately after the nutrient and bacterial treatments were applied, a conidial suspension of *C. coccodes* (10^7 conidia/ml) was misted onto the leaves.

Experimental conditions. Detached leaves from both experiments were placed on moist filter paper inside 100-mm diameter plastic Petri dishes and incubated in the dark in an enclosed plastic box with moist paper towels at 24 °C. After 4, 10, 24, and 30 h, the detached leaves were removed and cleared using the methods of Fernando *et al.* [1993]. The leaves were transferred to a 1:1 mixture of glacial acetic acid and 95% ethanol. Pieces of absorbent cotton were soaked in the same mixture and placed in plastic Petri dishes (100-mm diameter). A filter paper was placed on the top of the cotton. The leaves were placed on the filter paper and the plates were sealed with Parafilm (American Can Co., Greenwich, CT). After 24–48 h, the cleared detached leaves were mounted on glass slides and stained with 0.05% cotton blue in lactoglycerine. A total of 100 fungal structures were counted on each detached leaf, and there were five replicate detached leaves per treatment. These structures were, in the order of development: nongerminated conidia, conidia with germ tubes with or without appressoria, germ tubes with appressoria, and dark appressoria without germ tubes (AWGT). AWGT were the most mature stage and resulted from lysis of the original connecting germ tube. The percentage of germ tubes, germ tubes with appressoria, or AWGT were calculated by dividing the number of germ tubes, germ tubes with appressoria, or AWGT by the total number of fungal structures (nongerminated conidia + conidia with germ tubes + germ tubes with appressoria = appressoria without germ tubes), and multiplying by 100. The percentage of germ tubes with branches was counted at 30 h.

Data analysis. All experiments were arranged in a completely randomized design, with five replicates, and performed twice. The percentage data were arcsine-square root transformed and analyzed with one-way analysis of variance (ANOVA). The means were separated using the Duncan's Multiple Range Test, $P = 0.05$. There was no difference between the variances of the two trials, as tested by Bartlett's test for homogeneity of variances [Steel and Torrie, 1980]. There were no significant interactions between trials and any of the variables measured, so similar trends and conclusions could be drawn from both trials. The results of the first trial are presented as a representative example.

Results

Nutrient competition experiment. Bacterial and nutrient treatments did not significantly affect the percentage of germ tubes at 10 h compared to the control (Fig. 1). However, after 24 and 30 h, Ps2 applied alone reduced the percentage of germ tubes (Fig. 1). The addition of glucose or glutamine to the bacterial treatment on detached leaves increased the percentage of germ tubes at 24 h but not at 30 h, compared to the treatment with Ps2 alone. Glucose or glutamine applied alone also reduced the percentage of germ tubes at 30 h, but not as much as Ps2 alone.

Ps2 + alanine stimulated appressorial formation on detached leaves at 10 h. At 24 h, glucose or glutamine added with Ps2 significantly enhanced the formation of appressoria at the ends of germ tubes (Fig. 2). However, most of the germ tubes with appressoria had converted to appressoria without germ tubes (AWGT) by 30 h, due to germ tube lysis (Fig. 3). Most bacterial and nutrient treatments reduced the percentage of germ tubes with appressoria at 30 h, but the percentage in most treatments was <10%.

Treatment of detached leaves with Ps2 alone stimulated the formation of appressoria without germ tubes (AWGT) at 24 and 30 h (Fig. 3). The addition of glucose or glutamine to detached leaves treated with bacteria reduced or delayed appressorial formation at 24 and 30 h, compared to the treatment with Ps2 alone (Fig. 3). However, the addition of alanine with the bacterium stimulated appressorial formation at 24 and 30 h on detached leaves, when compared to non-treated detached leaves. The addition of nutrients alone had no effect on formation of AWGT at any time.

On detached leaves, germ tube branching was present only in the untreated controls, where 7% of the

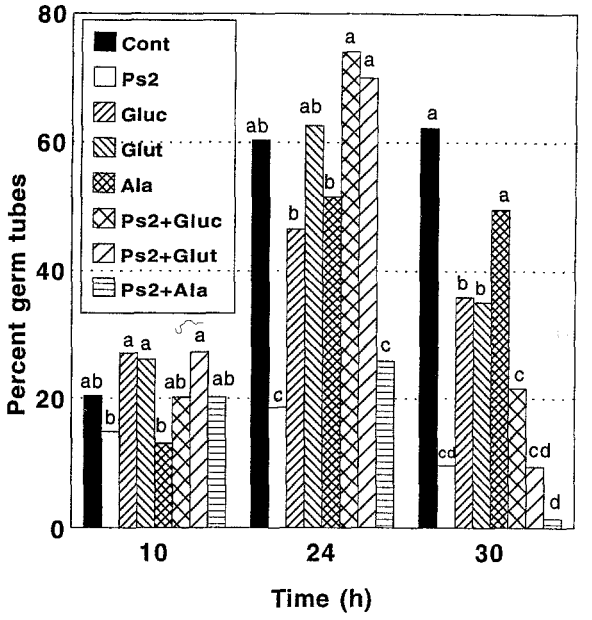

Fig. 1. Effect of *Pseudomonas* isolate Ps2, glucose, glutamine, and alanine on the percentage of conidia with germ tubes with or without appressoria on detached leaves of velvetleaf. Percentage of conidia with germ tubes = number of conidia with germ tubes / total number of fungal structures (nongerminated conidia + conidia with germ tubes + germ tubes with appressoria + appressoria without germ tubes) × 100. Detached leaves were fixed, cleared, and stained after 4, 10, 24, and 30 h. Bars within the same sampling time with the same letters are not significantly different, according to Duncan's Multiple Range Test, $P = 0.05$.

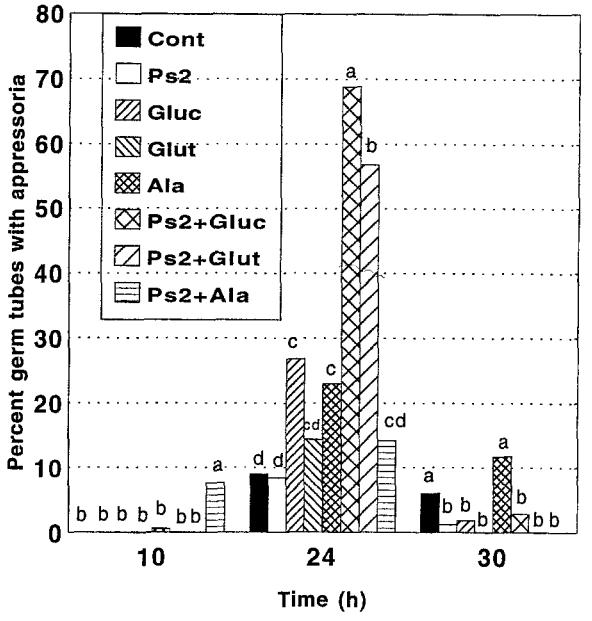

Fig. 2. Effect of *Pseudomonas* isolate Ps2, glucose, glutamine, and alanine on the percentage of germ tubes with appressoria on detached leaves of velvetleaf. Percentage of germ tubes with appressoria = number of germ tubes with appressoria / total number of fungal structures (nongerminated conidia + conidia with germ tubes + germ tubes with appressoria + appressoria without germ tubes) × 100. Detached leaves were fixed, cleared, and stained after 10, 24, and 30 h. Bars within the same sampling time with the same letters are not significantly different, according to Duncan's Multiple Range Test, $P = 0.05$.

germ tubes had branched. No branching was observed on leaves treated with either bacteria or nutrients.

Iron competition experiment. Over 60% of the conidia germinated on the leaves treated with water (CONT) after 24 h (Fig. 4). Compared to the control, the addition of EDTA increased germination at 10 h, but the addition of iron to EDTA reduced this stimulation (Fig. 4). EDTA had no effect on the percentage of germ tubes with appressoria (Fig. 5). After appressoria formed at the end of germ tubes, the germ tubes lysed, resulting in appressoria without attached germ tubes (AWGT). EDTA had no effect on the formation of AWGT (Fig. 6).

Both bacterial isolates Ps2 and Ps5 significantly reduced the percentage of germ tubes on leaves at 24 and 30 h (Fig. 4). The addition of iron to the bacterial isolates did not affect this trend. Ps2 and Ps5 increased the percentage of AWGT on detached leaves, and this effect was diminished by the addition of iron (Fig. 6). On leaves not treated with bacteria, the percentage of AWGT was <10%, but was >50% when Ps2 or Ps5 were added. In control treatments without bacteria, 28% of the germ tubes had branched at 30 h. However, the addition of both bacteria, with or without iron, completely inhibited germ tube branching. The addition of EDTA or EDTA + Fe significantly reduced germ tube branching to 11 and 15%, respectively. The addition of iron had no significant on germ tube branching in any treatment.

Discussion

In this research, we examined the effect of *Pseudomonas* spp., nutrients, and iron on formation of appressoria by *C. coccodes*. Both isolates of bacteria stimulated the formation of appressoria without germ tubes (AWGT), confirming the previous work of Fernando *et al.* [1994]. These dark, thick walled appressoria are initially formed at the end of the germ tubes, but the germ tubes quickly lyse, leaving isolated

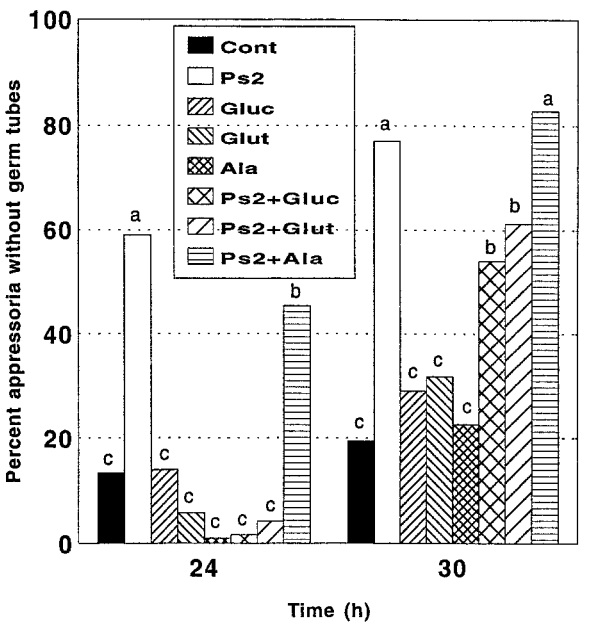

Fig. 3. Effect of *Pseudomonas* isolate Ps2, glucose, glutamine, and alanine on the percentage of dark appressoria without germ tubes (AWGT) on detached leaves of velvetleaf. Percentage of AWGT = number of AWGT / total number of fungal structures (nongerminated conidia + conidia with germ tubes + germ tubes with appressoria + appressoria without germ tubes) × 100. Detached leaves were fixed, cleared, and stained after 24 and 30 h. Bars within the same sampling time with the same letters are not significantly different, according to Duncan's Multiple Range Test, $P = 0.05$.

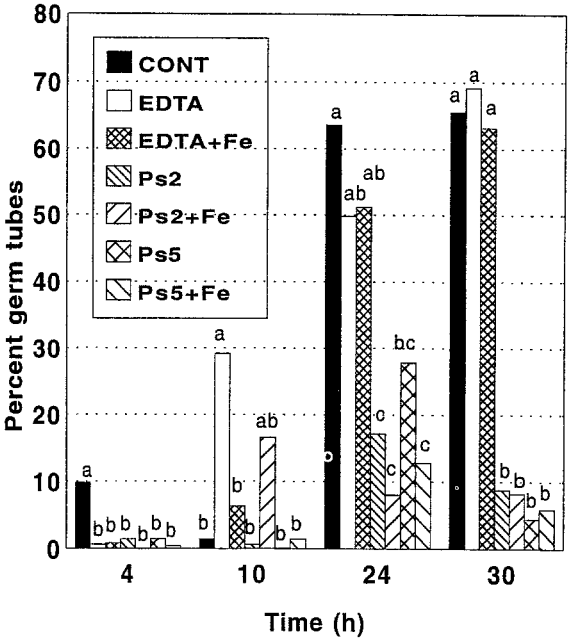

Fig. 4. Effect of EDTA, *Pseudomonas* isolate Ps2, Ps5, and Fe on the percentage of conidia with germ tubes with and without appressoria on detached leaves of velvetleaf. Percentage of germ tubes = number of conidia with germ tubes / total number of fungal structures (nongerminated conidia + conidia with germ tubes + germ tubes with appressoria + appressoria without germ tubes) × 100. Detached leaves were fixed, cleared, and stained after 4, 10, 24, and 30 h. Bars within the same sampling time with the same letters are not significantly different, according to Duncan's Multiple Range Test, $P = 0.05$.

appressoria. These melanized appressoria are resistant to lysis by antagonistic bacteria [Lenne and Parbery, 1977] and may also be important in the short term survival of the fungus under adverse environmental conditions, especially when dew periods are interrupted by periods of leaf dryness. The same phenomena of bacteria stimulating appressorial formation has been reported for other species of *Colletotrichum*, including *C. acutatum* [Blakeman and Parbery, 1977], *C. dematium* [Blakeman and Brodie, 1977] and *C. truncatum* [Schisler *et al.*, 1991].

This stimulation in appressorium formation may arise from a change in nutrients (carbon and/or nitrogen) or iron, resulting from growth of *Pseudomonas* on the phylloplane. We hypothesized that nutrient competition by phylloplane bacteria may accelerate the infection process of this pathogen, leading to enhancement of disease and better biological control of the target week. Exogenous carbon and nitrogen are required for the saprophytic growth of many foliar pathogens prior to penetration and infection [Blakeman, 1985]. Epiphytic bacteria can compete for these nutrients, and reduce the germination of conidia of pathogens such as *Botrytis cinerea* [Sztejnberg and Blakeman, 1973]. However, for specialized necrotrophic pathogens like *Colletotrichum*, excessive nutrients may suppress or delay the development of pathogenic structures [Mercer *et al.*, 1970]. The availability of iron in the phylloplane may also affect appressorial formation and the infection process [Slade *et al.*, 1986].

In the process of infection by *Colletotrichum*, there are morphologically distinct but developmentally related steps leading to formation of appressoria. Conidial germination will give rise to formation of germ tubes which in turn will form appressoria. Appressoria may be seen at the end of the germ tube or without a germ tube attached (AWGT). Bacteria may have caused lysis of germ tubes through production of antifungal compounds (heterolysis) or nutrient deprivation (autolysis). In our study, the addition of glucose or glutamine delayed the loss of germ tubes. Blakeman and Parbery [1977] suggested that autolysis of germ tubes may be induced by nutrient deprivation.

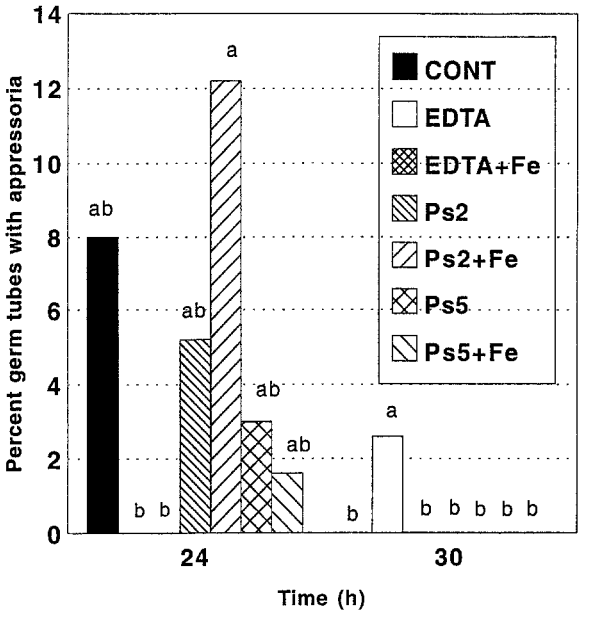

Fig. 5. Effect of EDTA, *Pseudomonas* isolate Ps2, Ps5, and Fe on the percentage of germ tubes with appressoria on detached leaves of velvetleaf. Percentage of germ tubes with appressoria = number of germ tubes with appressoria / total number of fungal structures (nongerminated conidia + conidia with germ tubes + germ tubes with appressoria + appressoria without germ tubes) × 100. Detached leaves were fixed, cleared, and stained after 24 and 30 h. Bars within the same sampling time with the same letters are not significantly different, according to Duncan's Multiple Range Test, $P = 0.05$.

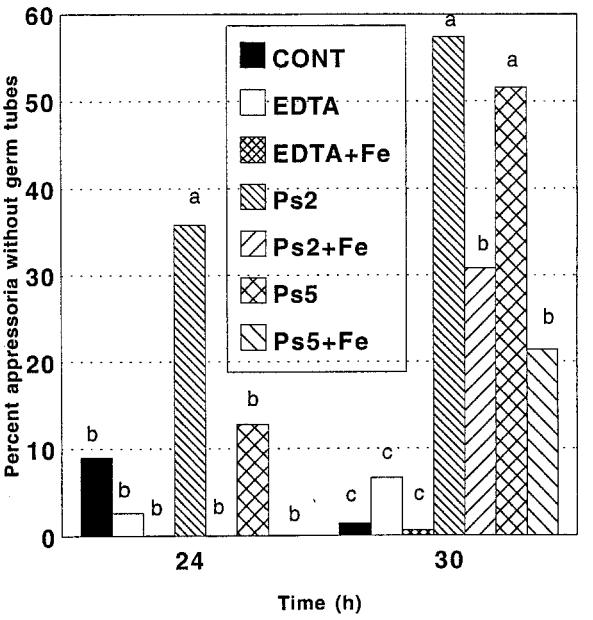

Fig. 6. Effect of EDTA, *Pseudomonas* isolate Ps2, Ps5, and Fe on the percentage of dark appressoria without germ tubes (AWGT) on detached leaves of velvetleaf. Percentage of AWGT = number of AWGT / total number of fungal structures (nongerminated conidia + conidia with germ tubes + germ tubes with appressoria + appressoria without germ tubes) × 100. Detached leaves were fixed, cleared, and stained after 24 and 30 h. Bars within the same sampling time with the same letters are not significantly different, according to Duncan's Multiple Range Test, $P = 0.05$.

Appressorial formation, especially dark coloured, thick walled appressoria without germ tubes (AWGT), was stimulated by both Ps2 and Ps5 (Fig. 3, 6). The increase in AWGT triggered by the bacterium was partially diminished by the addition of glucose or glutamine with the bacterium. Nutrient deprivation may lead to autolysis of germ tubes and trigger formation of appressoria. The rapid induction of appressoria explains the increase in lesion number and disease severity we observed in leaves of velvetleaf [Fernando *et al.*, 1994]. Emmett and Parbery [1975] reviewed evidence suggesting that starvation initiates appressorium formation. Lenne and Parbery [1976] observed a similar effect of nutrients on the stimulation of appressoria of *C. gloeosporioides* by an unidentified bacterium. Blakeman and Parbery [1977] also observed that the addition of nutrients with an isolate of *Pseudomonas* completely negated the stimulatory effect on appressorium formation in *C. acutatum*. However, two common phyllosphere yeasts, *Sporobolomyces roseus* and *Cryptococcus laurentii* var. *flavescens*, had no effect on conidial germination, superficial mycelial growth or appressorial formation by *Colletotrichum graminicola*, under continuous nutrient stress [Williamson and Fokkema, 1985]. In our study, the addition of nutrients alone had no effect on appressorium formation, similar to the results of Saad [1993] with glycine and asparagine. On the contrary, Blakeman and Parbery [1977] found that a mixture of glutamine and glucose reduced the number of appressoria of *C. acutatum*.

EDTA did not stimulate the formation of appressoria on detached leaves. The formation constant of EDTA is 10^{25} [Lindsay, 1979], while the formation constant of bacterial siderophores is estimated at 10^{30} or higher [Neilands, 1981b]. Thus the bacteria may have been more effective at iron competition than the chelator. In every case, the bacterial stimulation of appressorium formation was diminished by the addition of iron, strongly suggesting the involvement of a siderophore. The same phenomenon was observed by Slade *et al.* [1985] with purified siderophores from a *Pseudomonas* sp. However, iron competition did not seem to be involved in reducing the percentage of germ tubes. The ability of iron to diminish the stimulation

of appressorial formation but not the lysis of germ tubes indicates that the mechanisms involved in these two phenomenon are probably different, but may act in conjunction with each other. Nutrient completion for sugars and amino acids or antibiosis may result in autolysis or heterolysis of germ tubes and cessation of saprophytic growth (reduction in germ tube percentage and branching) on the leaf surface. Nutrient competition and the production of siderophores by these fluorescent pseudomonads may stimulate the formation of dark pigmented melanized appressoria without germ tubes, which may be resistant to adverse environmental conditions. The formation of dark coloured appressoria seems to hasten the infection process, and act as survival structures or initiate latent infections that could continue to colonize the leaf at a later time. In any case, infection of the target weed by the mycoherbicide *C. coccodes* may be enhanced by manipulation or augmentation of phylloplane bacteria.

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