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 Fe³⁺. 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 Fe³⁺. 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 Fe³⁺. 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 coincoculated 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 [Gottlieb 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 nm 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 $5 \times 10^{-4}$ 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$_4$)$_3$ ($1 \times 10^{-3}$ M) were mixed together (1:1 vol) so the final concentration of each was $5 \times 10^{-4}$ 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 ($5 \times 10^{-4}$ M); EDTA + Fe (equimolar concentrations of EDTA and Fe$_2$(SO$_4$)$_3$ ($5 \times 10^{-4}$ M); Ps2 Pseudomonas isolate Ps2 ($10^8$ cells/ml); Ps2 + Fe (Pseudomonas isolate Ps2 ($10^8$ cells/ml) and Fe$_2$(SO$_4$)$_3$ ($5 \times 10^{-4}$ M), Ps5 (Pseudomonas isolate Ps5 ($10^5$ cells/ml); and Ps5 + Fe (Pseudomonas isolate Ps5 ($10^5$ cells/ml) and Fe$_2$(SO$_4$)$_3$ ($5 \times 10^{-4}$ M). Detached leaves were then inoculated with C. coccodes by misting fungal inoculum ($10^7$ conidia/ml) onto the detached leaves.