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# Promotion of Phytotoxic Bacteria in Rhizospheres of Leatherleaf Fern by Benlate DF[1]

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## ABSTRACT

Foliar damage symptoms to leatherleaf fern (*Rumohra adiantiformis* [Forst.] Ching) in Florida during widespread use of the fungicide Benlate DF could not be attributed to nutrition, cultural practices, environmental conditions, or new pathogens developing on the crop. The objective of this study was to assess the involvement of rhizosphere bacteria in the damage symptoms using bioassays to detect

[1]Benlate ® is a registered trademark of E.I. duPont de Nemours & Co. Product names are necessary to report factually on available data; however, the University of Georgia, Lincoln University of Missouri, or USDA neither guarantees nor warrants the standards of the product, and the use of the name implies no approval of the product to the exclusion of others that may be suitable.

phytotoxic activity. Rhizosphere bacteria were cultured from rhizospheres of leatherleaf ferns sampled from ferneries where Benlate DF was routinely applied and from check ferneries not receiving the fungicide. Using a lettuce seedling bioassay, the proportion of rhizosphere bacteria that was plant growth-inhibitory ranged from 7.5% and 11% for isolates from two check ferneries to 70% for those from a fernery previously treated with Benlate DF. Rhizosphere bacteria originating from Benlate DF-treated leatherleaf ferns caused most severe damage on seedlings with some isolates reducing root growth 70% compared to control seedlings. Other symptoms induced by these bacteria, which were mainly fluorescent and nonfluorescent pseudomonads, included necroses and inhibition of root hair development. Results suggested that Benlate DF affected the composition of bacteria in leatherleaf fern rhizospheres by promoting a bacterial component with phytotoxic properties toward plant growth.

## INTRODUCTION

Leatherleaf fern is a leading ornamental export crop from Florida and a popular cut foliage used by florists throughout the world. To assure production of high quality fronds, frequent applications of fungicides through foliar sprays or irrigation are recommended to control fungal disease development (Chase, 1982; Henley et al., 1981). Benomyl (methyl 1-[butyl carbamoyl]-2-benzimidazole carbamate) was one of the most widely used fungicide in leatherleaf



fern production (Stamps and Chase, 1984; Stamps and Chase, 1987). Benomyl formulated as Benlate DF was used by most leatherleaf fern growers from 1987 to 1991. During this same time period, symptoms of various distortions, discoloration, and stunting of marketable fronds occurred, which severely decreased quality and economic value. Although the damage symptoms were most widespread at production sites receiving repeated applications of Benlate DF, no direct relationship between symptomology and Benlate DF use could be found. Evaluation of plant material from Benlate DF-treated ferneries revealed that development of new plant pathogens that may have caused the observed symptoms did not occur (Hanson, 1992).

Previous studies with crop plants demonstrated that colonization of roots by certain bacteria was detrimental to plant development and was implicated as a significant factor in limiting crop growth (Elliott and Lynch, 1984; Gardner et al., 1984; Schroth et al., 1984; Suslow and Schroth, 1982). These phytotoxic rhizosphere bacteria likely induce damage through production of phytotoxins that are absorbed by plant roots. Symptoms induced by phytotoxic rhizosphere bacteria include plant growth suppression, foliar chlorosis, root distortions and necroses, and reduced seed germination (Schippers et al., 1986; Schroth and Hancock, 1982; Suslow and Schroth, 1982). This group of bacteria have been overlooked in the past due to their nonparasitic and relatively subtle nature of attack on plants, but now are considered an important group of bacterial phytopathogens (Schroth et al., 1984; Suslow and Schroth, 1982). Continuous use of soil-applied pesticides enhance root colonization of crop plants by specific groups of bacteria and fungi and increase

the development or severity of root injury (Altman and Campbell, 1977; Greaves and Sargent, 1986; Katan and Eshel, 1973).

Although fungi were not considered to be problematic in the injury symptoms of leatherleaf fern, other organisms in the soil and rhizosphere may be implicated. Phytotoxic rhizosphere bacteria are ubiquitous and likely common to all plant root systems (Suslow and Schroth, 1982). In previous laboratory and greenhouse studies, a relationship between benomyl-treated leatherleaf ferns and phytotoxic rhizosphere bacteria was observed (Mills et al., 1996). Therefore, a survey of ferneries was conducted to determine the existence of similar relationships in the field. Leatherleaf ferns were collected from ferneries where Benlate DF was previously routinely applied and from controls not receiving Benlate DF in an effort to determine how this fungicide influenced phytotoxic bacteria in the rhizosphere. The objectives were to identify and characterize rhizosphere bacteria occurring on leatherleaf fern treated with Benlate DF and to screen rhizosphere bacteria for phytotoxic activity.

## MATERIALS AND METHODS

### Isolation of Rhizosphere Bacteria

Intact leatherleaf fern plants and soil surrounding the rhizome/roots systems were collected from commercial ferneries in Volusia and Putnam Counties in Florida in 1993, 1994, and 1995. Plants and soils were carefully placed in plastic bags and shipped in coolers within 48 h to the Soil Microbiology Laboratory at the University of Missouri, Columbia for processing. Rhizome/root segments (2- to 3-cm long) were removed from each plant, shaken to remove loosely adhering soil, and weighed. Soil remaining attached to rhizome/root surfaces was considered as rhizosphere soil. Rhizome/roots were placed in milk dilution bottles (160-ml) containing 100 ml 0.01% Tween 40 and shaken on a rotary shaker at 500 rpm for 20 min. Serial 10-fold dilutions were made in sterile phosphate-buffered saline (10 mM $K_2PO4$-$KH_2PO_4$, 0.14 M NaCl [pH 7.0]) and plated on the medium of Sands and Rovira (SR) (Sands and Rovia, 1970). After 48 h of incubation at 28 C, bacterial colonies were counted; representative colonies were selected and subcultured by streaking onto SR, and then single colony isolates were characterized. The selection of bacteria was based on distinct types observed according to culture plate morphological characteristics including pigment, colony form, elevation, margin, texture, and opacity (Smibert and Krieg, 1994).



### Characterization of Rhizosphere Bacteria

Isolates of rhizosphere bacteria were characterized based on standard procedures (Smibert and Krieg, 1994) for Gram stain, oxidase reaction, motility, and morphology and on utilization of substrates in the API Rapid NFT diagnostic kit (Bio Merieux Vitek Inc., Hazelwood, MO).

### Lettuce Seedling Bioassay for Phytotoxicity

Each bacterial isolate was grown on SR at 22 C for 48 h. Inocula for bioassays were prepared by suspending bacterial growth from SR agar plates in 0.1 M $MgSO_4$ to yield suspensions of approximately $10^8$ cells per ml.

Lettuce (*Lactuca sativa* L. var. Blackseeded Simpson) seeds were surface-sterilized by immersion in 1.25% sodium hypochlorite for 4 min, rinsing in sterile water, immersing in 70% ethanol for 2 min, rinsing five times in sterile water, and blotting on autoclaved

paper towels. Surface-sterilized seeds were pregerminated on 1% agar for 12 h. After pregermination 10 seeds were place equidistantly on fresh water agar plates where they were treated with a bacterial suspension by applying 30 µl per seed. Two replicate plates were prepared for each bacterial isolate. Seeds in corresponding control plates received 30 µl sterile water per seed. Plates were incubated in the dark at 22 C for 48 h. Root length of each of 20 seedlings was measured to the nearest mm. Roots were also evaluated for injury on a scale of 0 to 4, where 0 = no injury evident and 4 = abundant necroses, severe stunting, little or no root hairs, seedlings dead or nearly so.

Results of bioassays were statistically analyzed as a completely randomized design using Fisher's least significant difference (p=0.05) for all treatment mean comparisons.

## RESULTS AND DISCUSSION

Bacteria isolated from rhizospheres of leatherleaf ferns collected at all sites were comprised primarily of fluorescent and nonfluorescent pseudomonads, *Erwinia herbicola*, *Flavobacterium* sp., and *Alcaligenes* sp. The fluorescent pseudomonads were predominantly *Pseudomonas fluorescens, P. putida, P. aureofaciens*, and *P. aeruginosa*; nonfluorescent isolates were mainly *P. cepacia, P. maltophilia*, and *P. stutzeri*. The presence of bacteria on rhizome and root surfaces was supported with more direct evidence obtained by scanning electron microscopy (Kremer et al., unpublished data), which indicated that bacteria often associated with rhizome/root surfaces in characteristic patterns of colonization. The total



populations of bacteria on rhizome/root surfaces from plants at all sites were similar, averaging about 106 cells per g rhizome/root.

The proportion of bacterial isolates that were growth inhibitory based on the lettuce seedling bioassay ranged from 7.5% and 11% for isolates originating from checks (Ferneries C1 and C2) to 70% for those from Fernery B4, which previously received Benlate DF (Figure 1). The greatest proportion of inhibitory bacteria was associated with the fluorescent and nonfluorescent pseudomonads. This is consistent with the distribution of deleterious bacteria occurring on roots of other plants (Elliott and Lynch, 1984; Gardner et al., 1984; Suslow and Schroth, 1982).

Seedling root bioassays were used to rapidly assess the impact of bacterial isolates from leatherleaf fern on plant growth and development. Lettuce seedling bioassays were used for preliminary evaluation of phytotoxicity prior to development of direct bioassay systems using leatherleaf fern. Bioassays in which root development is used as an index to identify potentially inhibitory substances are routinely used by the U. S. Environmental Protection Agency and the Food and Drug Administration (Wang, 1987). Lettuce seedlings are good indicators of effects of growth-inhibiting bacteria and bioassays based on lettuce seedlings have provided consistent results in repeated trials (Alstrom, 1987).

Representative data for individual bacterial isolates from a check fernery (Fernery C1) and a Benlate-treated fernery (Fernery B3) indicate the range of phytotoxicity detected based on lettuce seedling radicle growth inhibition (Figure 2). The greatest proportion of isolates inhibitory toward radicle growth and those exhibiting the

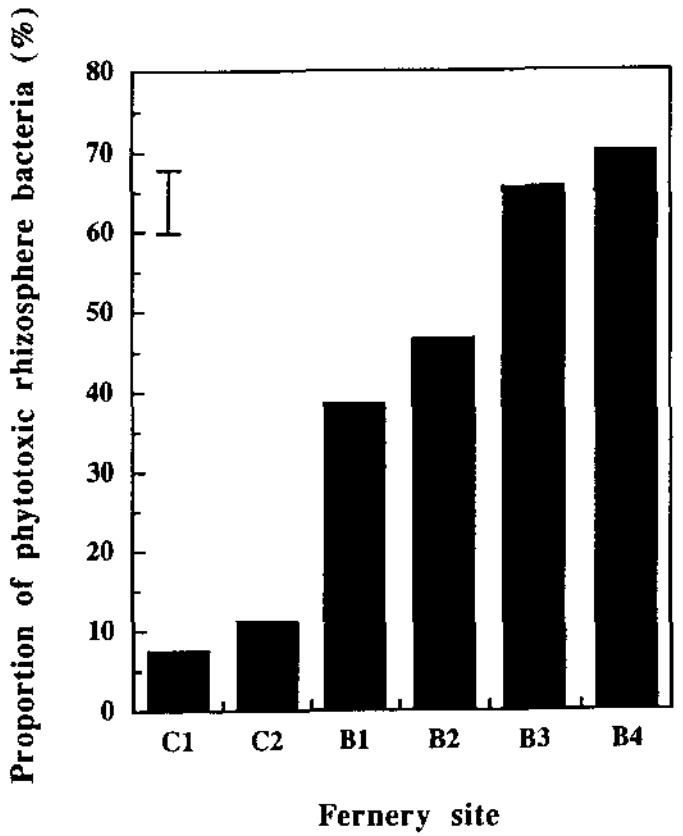


**Figure 1.** Proportion of total rhizosphere bacteria with potential phytotoxicity from leatherleaf fern collected at six ferneries in Florida in 1993, 1994 and 1995. C1 and C2 are check ferneries (no Benlate DF applied); B1 through B4 are ferneries with histories of Benlate applications. Vertical bar indicates LSD ($p<0.05$).



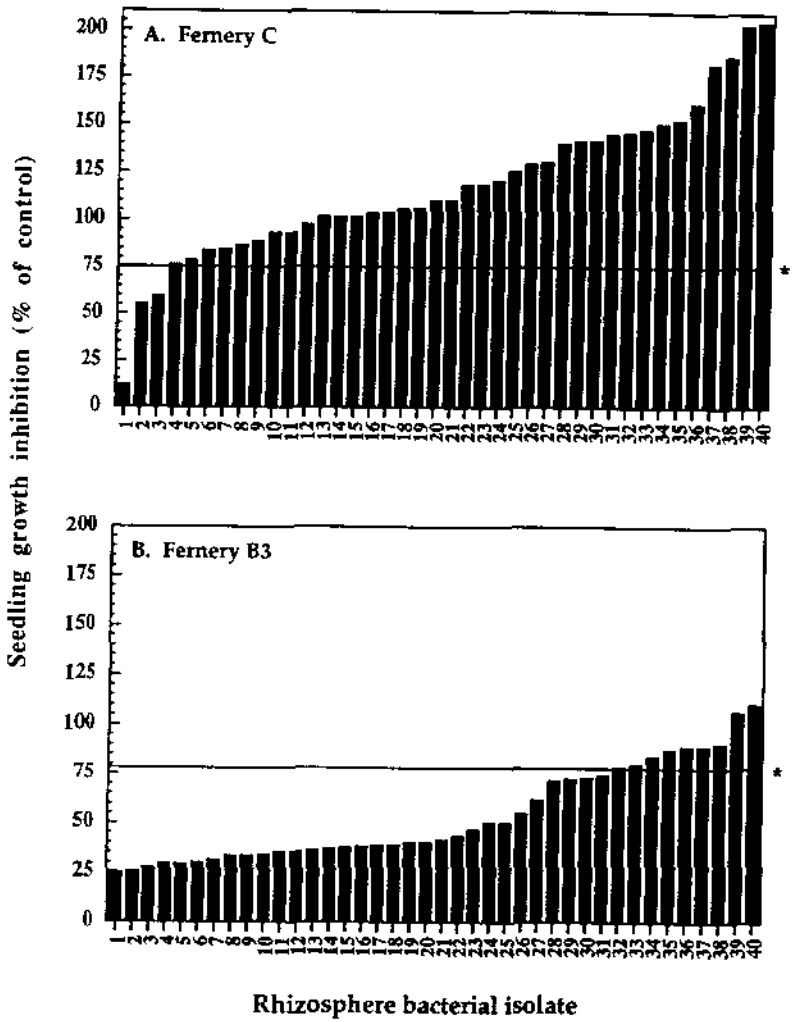


**Figure 2.** Effects on seedling growth by individual rhizosphere isolates from two ferneries. A. Check fernery; B. Benlate DF treated fernery. Significant ($p<0.05$) root growth inhibition indicated as percentage of control below horizontal line (*).

TABLE 1. Root development effects in lettuce seedling bioassays caused by rhizosphere bacteria isolated from leatherleaf ferns.

| Site | Bacterial isolate no. | Appearance of roots[a] | Root rating | Average root length[b] | Reduction (increase) in root growth[c] |
|---|---|---|---|---|---|
| | | | | mm | % |
| C1 | Jr2c1 | Long; root hair proliferation | 0 | 18.8† | (59) |
| | Jr4b1 | Long; root hair proliferation | 0 | 17.2† | (46) |
| | Jr4b2 | Same as control | 0 | 14.0 | (18) |
| | Jr1c1 | Same as control | 0 | 12.8 | (8) |
| | Jr3c | Stunted | 2.5 | 6.4* | 46 |
| B1 | 7rc1 | Same as control | 0 | 15.0 | (25) |
| | 7ra1 | Same as control; slight necrosis | 0.25 | 11.6 | 3 |
| | 9ra1 | Stunted; few root hairs; necrotic | 2.0 | 8.5* | 29 |
| | 9ra2 | Severely stunted; some dead | 3.8 | 6.0* | 50 |
| | 14rc1 | Stunted; no root hairs; necrotic | 3.5 | 3.5* | 71 |
| B2 | 51b | Same as control | 0 | 13.5 | 10 |
| | 13a | Long; few root hairs; necroses | 1.2 | 13.0 | 13 |
| | 22b | Stunted; necroses | 2.0 | 10.0* | 33 |
| | 32b | Stunted | 2.5 | 8.5* | 43 |
| | 42a | Stunted; no root hairs; necroses | 3.5 | 4.2* | 72 |
| B3 | 55a1 | Same as control | 0 | 16.6† | (38) |
| | 104a2 | Stunted | 1.0 | 10.0* | 44 |
| | 124b5 | Stunted; necrotic | 2.2 | 7.0* | 42 |
| | 134b3 | Stunted; no root hairs; necrotic | 3.2 | 4.2* | 65 |
| | 24c1 | Dead | 4.0 | 3.5* | 71 |
| B4 | 2rb1 | Long; root hair proliferation | 0 | 15.5 | (10) |
| | 27ra1 | Stunted | 2.0 | 8.5* | 39 |
| | 1rb1 | Stunted; necrotic root tips | 2.0 | 7.8* | 44 |
| | 2c4 | Stunted; necrotic | 2.5 | 5.8* | 58 |
| | 2a4 | Stunted; entire root necrotic | 2.8 | 4.5* | 68 |

[a]Descriptions relative to control roots on water agar.

[b]Within sites, significant (P=0.05) reduction or increase in root length denoted by * or †, respectively.

[c]% Reduction = [(Control root length - Isolate root length)/Control root length] X 100; % Increase = [(Isolate root length - Control root length)/Control root length] X 100.

most severe inhibition were clearly associated with Fernery B3 having a history of Benlate application.

An array of effects on seedling root growth was observed on the bioassay plates. Several isolates strongly inhibited root growth manifested by stunting, necroses, distortions, and poor root hair development (Table 1). Many isolates reduced root length without obvious plant cell or tissue damage. This is similar to previous reports first describing deleterious bacteria, which attributed reduced plant growth to rhizobacterially-produced toxins that were absorbed by roots (Fredrickson and Elliott, 1985; Fredrickson et al., 1987; Schroth and Hancock, 1982; Suslow and Schroth, 1982).

The highest incidence of root stunting, necroses, and inhibition of root hair development were associated with isolates obtained from Benlate-treated ferneries (Table 1). The severity of phytotoxicity exhibited by rhizosphere bacteria appeared to be associated with origin of the isolates (Figure 2 and Table 1). Isolates from leatherleaf fern grown at Benlate-treated ferneries were generally more phytotoxic than those from plants grown at a check fernery. In contrast, a distinct group of bacteria that significantly increased seedling growth occurred for check ferns, whereas very few of these bacteria were detected in samples from Benlate-treated ferns (Figure 2 and Table 1). This suggests that Benlate treatment may be associated with a decrease in the plant growth-promoting component of rhizosphere bacteria.

This study presents evidence that Benlate DF affected the composition of bacteria in the rhizosphere of leatherleaf fern resulting in promotion of a bacterial component, primarily pseudomonads, with phytotoxic properties toward plant growth. High frequencies of isolation of phytotoxic bacteria from rhizosphere soil suggest their



activity might affect crop yields (Curl and Truelove, 1986). A previous report on crown and root rot of sainfoin (*Onobrychis viciaefolia* Scop.) thought to be due to fungal pathogens were found to be the result of a complex of rhizosphere bacteria (Gaudet et al., 1980). Similarly, early investigations into causes of damage to leatherleaf fern focused on fungal pathogens were not successful, but the present study indicates the likely involvement of rhizosphere bacteria.

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