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CARFAX

Effect of Botanical Aromatic Compounds and Seed-surface pH on Growth and Colonization of Cotton Plant Growth-promoting Rhizobacteria

E. M. BAUSKE,[1] P. A. BACKMAN,[2] K. M. HARPER,[2] P. M. BRANNEN,[2] R. RODRÍGUEZ-KÁBANA[2] AND J. W. KLOEPPER[2]

[1]*Horticulture Department, Auburn University, Auburn, AL 36949, USA*; [2]*Department of Plant Pathology and Biological Control Institute, Auburn University, Auburn, AL 36949, USA*

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Citral (3,7-dimethyl-2,6-octadienal), furfural (2-furaldehyde) and benzaldehyde (benzoic adelhyde) previously demonstrated control activity against Meloidogyne incognita *and fungal diseases on cotton. Plant growth-promoting rhizobacteria (PGPR) applied to cotton were previously found to promote plant growth and reduce seedling disease. Studies were undertaken to determine if these compounds were compatible with PGPR. In tests with 12 PGPR strains, vapor of citral inhibited* in vitro *growth of most strains, and vapor of furfural and benzaldehyde, with one exception, killed all but the* Bacillus *spp. tested. When 0.35 ml* kg^{-1} *soil of each compound were applied to the soil 9–10 days prior to planting the cotton cultivar Deltapine 51, only furfural significantly reduced rhizosphere colonization across all strains from 4.70 colony-forming units (CFUs)/g of root to 4.42 CFUs/g root. In greenhouse studies, the low seed-surface pH (2.3) of commercial seed did not reduce root colonization, compared with colonization on roots from seed at pH 5.4. There were no synergistic interactions between seed-surface pH and any of the compounds. Although previous research indicated that application of both furfural and benzaldehyde increased the proportion of* Burkholderia *spp. in the soil, there is no indication that they increased cotton root colonization by the* B. cepacia *strain tested. These results indicate PGPR can be combined with citral and benzaldehyde in integrated management systems and that the low seed-surface pH of acid-delinted cotton will not limit their application.*

Keywords: *biocontrol, rhizobacteria, PGPR, seed-surface pH, nematicide, aromatic compounds*

INTRODUCTION

Cotton is a pesticide-intensive crop. Seed-treatment, hopper-box and in-furrow applications of fungicides are used for the control of pre- and post-emergence seedling damping-off diseases caused by *Pythium* spp., *Fusarium* spp. and *Rhizoctonia solani* Kühn. Nematicides

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are applied to control root-knot (*Meloidogyne* spp.) and reniform nematode (*Pratylenchus reniformis*). Recently, plant growth-promoting rhizobacteria (PGPR) have been applied to seed to promote growth and reduce seedling disease (e.g. Kodiak and Epic; Gustafson, Dallas, TX, USA). As emphasis shifts from chemical control to use of biological controls in bio-intensive integrated pest management (IPM) systems, biological components of the IPM system must be tested for compatibility with non-biological components.

Cotton seed is acid delinted in the US and therefore retains residual acid, resulting in a low seed-surface pH. This may limit the use of biological seed treatments. Mahaffee and Backman (1993) tested the effects of seed-surface pH and the fungicides pentachloronitrobenzene (PCNB) and metalaxyl on colonization of *Bacillus subtilis* strain GB03 on cotton seed and roots. They found that low seed-surface pH reduced colonization of seed after 60 h, but no significant effects could be detected after 2 weeks.

As part of an on-going study on the value of low-molecular weight, volatile compounds for control of plant parasitic nematodes, three compounds were identified which provide effective control of rootknot nematode (*Meloidogyne incognita*) on cotton in greenhouse studies and in field microplot tests (Bauske *et al.*, 1994a). The three compounds, citral, furfural and benzaldehyde, are naturally occurring botanical aromatics. Citral (3,7-dimethyl-2.6-octadienal) is the major constituent of lemon oil (Anon, 1976); furfural (2-furaldehyde) is found in many essential oils from plants, fruit juice and alcoholic beverages (Bauer *et al.*, 1990); and benzaldehyde (benzoic aldehyde) is the main component of bitter almond oil (Anon, 1976). Maximum control of *M. incognita* with all three compounds was achieved when rates as low as 0.25 ml kg^{-1} soil were applied 11 days prior to planting (Bauske *et al.*, 1994a).

Citral, furfural and benzaldehyde have potential as tools to control fungal diseases. The three compounds have demonstrated ability to control seedling disease on cotton caused by *Pythium* spp. in greenhouse tests with naturally infested field soil (Bauske *et al.*, 1994b). They also controlled disease caused by *Rhizoctonia solani* in soil artificially infested with the fungus (E. Bauske, personal communication). Canullo *et al.* (1992a,b) demonstrated that application of furfural to soil reduced severity of southern blight caused by *Sclerotium rolfsii* in lentil, reduced germination of sclerotia and reduced growth of the fungus. Other researchers tested the effects of these compounds on spore germination of several fungi. Generally, citral stimulated germination of *Penicillium* spp. whereas furfural and benzaldehyde reduced spore germination of several fungi including *Monilinia fructicola* and *Botrytis cinerea* (Flor, 1926; French *et al.*, 1978; Wilson *et al.*, 1987).

Previous research demonstrated that application of furfural and benzaldehyde causes both quantitative and qualitative shifts in the composition of the soil bacterial community (Bauske *et al.*, 1993). These changes could be detected 49 days after application of the compounds. In general, bacterial population counts decreased significantly 1 day after soil treatment and then increased after 1 week, remaining elevated when compared with the non-treated control throughout the 7 weeks of the test. One day after treatment, primarily *Bacillus* spp. were isolated from the soil. After 7 days, *Burkholderia* and *Pseudomonas* spp. predominated. These results suggested that these compounds might be used to manage indigenous microflora and may have an impact on PGPR applied in bio-intensive IPM systems.

Citral, furfural and benzaldehyde are appealing candidates for introduction into bio-intensive IPM systems on cotton because they are naturally occurring plant derivatives, and have a broad spectrum of activity against fungal diseases and nematodes. However, the effect of these compounds on PGPR is not known. The objective of this study was to determine if these compounds exerted a deleterious effect on PGPR growth and/or root colonization. Since the low seed-surface pH of acid-delinted cotton seed may affect root colonization, it was introdueed as a factor in these experiments.

MATERIALS AND METHODS

Bacterial Strains

The PGPR strains included in this study previously demonstrated biological control activity against *R. solani* and/or *Pythium ultimum* Trow as well as cotton root-colonization capacity (Table 1). They were selected by industrial laboratories and are in various stages of commercial development. Strains IPM 5 and 6 are *B. subtilis* strains GB07 and GB03 respectively and are commercially available as seed treatments (Epic and Kodiak; Gustafson). All strains were identified by gas chromatograph–fatty acid methyl ester (GC-FAME) analysis in combination with the Microbial Identification System (Microbial ID Inc., Newark, DE, USA). Bacteria were maintained for long-term storage at −80°C in tryptic soy broth (TSB; Difco, Detroit, MI, USA) with 25% glycerol. Spontaneous, stable mutants resistant to rifampicin (100 μg ml^{-1}) were generated for all strains, except IPM 3, 5, 6 and 12 for use in colonization studies. IPM strains 5 and 6 are *B. subtilis* strains which can be isolated on amended salt V-8 agar as described in the following. IPM 3 and 12 were not included in the greenhouse studies.

Effect of Botanical Aromatic Compounds on PGPR Growth *in vitro*

Bacterial cultures were initially grown in TSB for 48 h at 28°C and 150 rpm on a rotary shaker. Bacterial cells were pelleted by centrifugation for 10 min at 4000 × *g*, the supernatant was discarded, and the pellet was suspended in 10 ml of sterile potassium phosphate buffer (PB, 0.02 M, pH 7.0).

Because citral, furfural and benzaldehyde are volatile compounds, the effects of vapor on growth of the bacteria were determined. Each bacterial strain (0.10 ml) was spread on to the surface of tryptic soy agar (TSA) in glass Petri dishes. Technical grade citral, furfural and benzaldehyde were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Filter paper discs (Whatman International Ltd, Maidstone, UK; area = 9.6 cm^2) were dipped in each compound and placed on the lid of the inverted glass Petri dishes. There were two replications of each treatment. After 48 h, growth on test plates was compared to growth on non-treated control plates. If growth was equal to that of the control, the test plate was assigned '+'. If growth was less than control, the plate was assigned '< +'. If there was no apparent growth on the plates, an inoculation loop was passed over the surface and then on to a fresh TSA plate. The fresh TSA plate was checked for growth after 24 h. If there was no growth on the TSA plate, the test plate was assigned '−'. If there was growth on the TSA plate, the test plate was assigned '− +'. This experiment was repeated.

TABLE 1. Effects of citral, furfural and benzaldehyde vapor on growth of PGPR

Strain	Identification	Citral	Furfural	Benzaldehyde
IPM 1	*Enterobacter asburiae*	+[a]	−+[b]	−[c]
IPM 2	*E. asburiae*	+	−	−
IPM 3	*Pseudomonas syringae*	+	−	−
IPM 4	*Burkholderia cepacia*	−	−	−
IPM 5	*Bacillus subtilis* strain GB07	<+[d]	−+	−+
IPM 6	*B. subtilis* strain GB03	<+	−+	−+
IPM 7	*B. macerans*	−+	−+	−+
IPM 8	*B. pasteurii*	<+	−+	−+
IPM 9	*B. macerans*	<+	+·	−+
IPM 10	*B. pasteurii*	<+	+	−+
IPM 11	*B. macerans*	<+	−+	−+
IPM 12	*B. thuringiensis*	−	−+	−+

[a] + = Growth equal to that of the untreated control plate.
[b] + = no apparent growth on test plate, but growth after transfer to a fresh TSA plate.
[c] − = no apparent growth on test plate or after transfer to a fresh TSA plate.
[d] < + = apparent growth but less than that on the control plate.

Effect of Botanical Aromatic Compounds on PGPR Colonization

Eleven strains were evaluated for their capacity to colonize seedling roots of the cotton cultivar Deltapine 51 in three experiments. The design of each experiment was a $2 \times 2 \times 10$ factorial, arranged in a randomized complete block design with four replications of each of the 40 treatments. In each experiment the treatments consisted of two levels of seed-surface pH, the soil was either treated or non-treated with one of the botanical aromatic compounds, and the seed was treated or non-treated with the IPM strains 1, 2 and 4 to 11.

Seed-surface pH was determined by stirring 50 g of acid-delinted cotton seed in 100 ml of deionized distilled water for 30 min and then measuring the pH of the solution with a combination pH electrode. The seed-surface pH of the non-treated cotton seed was 2.3. Seed-surface pH was raised to 5.4 by mixing 300 g of cotton seed and 150 g of sodium carbonate in 350 ml of water while stirring for 10 min. Seeds were rinsed five times with tap water and air dried at room temperature.

A sandy loam field soil with pH 6.1, organic matter content $< 1.0\%$ (w/w) and a cation exchange capacity of < 10 mEq 100 g^{-1} was used in the study. Half of the soil in these experiments was treated with 0.35 ml kg^{-1} soil with one of the compounds (citral, furfural and benzaldehyde) 9 days prior to planting. Each compound was applied directly to 1 kg of soil in 3-l polyethylene bags and thoroughly mixed. The soil was then placed on plastic trays until use.

Bacterial strains were initially grown in TSB for 24 h at 150 rpm. Broth cultures were spread on TSA plates amended with 100 μg ml^{-1} rifampicin (IPM 1, IPM 2, IPM 4 and IPM 7–11) or on salt V-8 agar amended with polymyxin B (100 units ml^{-1}) and 200 μg ml^{-1} of cycloheximide (IPM5 and IPM 6) and incubated at 30°C for 24 h. Cells were harvested by adding 2.5 ml of PB and scraping the bacterial lawn into suspension. The suspension was added to 0.1 ml of 1.0% alginic acid (high viscosity; Sigma, St Louis, MO, USA) and was immediately applied to seed. This resulted in applications of 6.8 (± 0.5) log colony-forming units (CFUs)/seed and did not affect the pH of the seed (data not shown). Seeds were planted immediately after application. Four seeds were planted into each of four Conetainers (Ray Leach Nursery, Canby, OR, USA) per treatment. Plants were allowed to grow in the greenhouse for 10–12 days, after which the colonization level on the roots was determined. The root systems for each Conetainer were weighed and placed in Tekmar sterile lab bags (Tekmar, Co., Cincinnati, OH, USA) with 10 ml of PB. Samples were triturated for 1 min with a Tekmar Stomacher Lab Blender model 80 (Tekmar, Co.), serially diluted and plated with a spiral plater on salt V-8 agar or TSA amended with rifampicin as appropriate for each treatment. Plates were incubated at 30°C for 48 h and colonies were enumerated.

Statistical Analysis

All colonization data from greenhouse assays were transformed ($\log_{10}$ CFUs/g of root) prior to analysis. Data were analyzed using analysis of variance (ANOVA) with SAS (Littell *et al.*, 1991). When statistically significant ($P \leq 0.05$) interactions between factors were present, statistical differences between levels of one factor within the other were detected by use of the *t*-statistic.

RESULTS

Effect of Botanical Aromatic Compounds on PGPR Growth *in vitro*

Both furfural and benzaldehyde vapor adversely affected growth of all bacterial strains tested (Table 1). Although the *Bacillus* spp. (strains IPM 5–12) survived for 24 h in the presence of these compounds, there was no apparent growth on test plates. Benzaldehyde killed IPM 1–4 and furfural killed IPM 2, 3 and 4. In general, the strains were tolerant of citral vapor, although growth of several of the strains (IPM, 5, 6, 8, 9, 10, 11 and 12) was reduced when compared to that of the control, and growth of IPM 4 and 12 was completely inhibited. These results were consistent across both repetitions of the experiment.

TABLE 2. Results of ANOVA for three experiments with botanical aromatic compounds showing main effects and interactions of seed-surface pH (low pH of 2.3 and high pH of 5.4), soil application of a botanical compound (citral, furfural or benzaldehyde) and 10 PGPR strains and root colonization 10–12 days after planting

Source of variation	Citral	Furfural	Benzaldehyde
Block	*[a]	NS[b]	**
PGPR	**	**	**
pH	NS	NS	NS
Compound	NS	**	NS
PGPR*ph	NS	NS	NS
PGPR*compound	*	NS	*
pH*compound	NS	NS	NS
Three-way interaction	NS	NS	NS

[a]*, **Significant at $P \leq 0.05$ and $P \leq 0.01$ respectively.
[b]NS, not significant.

Effects of Botanical Aromatic Compounds on PGPR Colonization

Significant main effects of application of citral and benzaldehyde were not detected in experiments testing the effect of soil application of citral, furfural and benzaldehyde and seed-surface pH on colonization of rhizobacterial strains (Table 2). However, significant main effects of application of furfural were detected. When furfural was applied to the soil 9 days prior to planting, the level of colonization across all strains was slightly reduced ($P \leq 0.05$) from 4.70 CFUs/g of root to 4.42 CFUs/g of root. This reduction is apparent in Table 3. Although t-tests indicated a significant reduction in colonization of only two strains, colonization of PGPR (except IPM 2) was consistently lower when furfural was applied to the soil (Table 3).

Significant strain by compound interactions were detected in both the citral and benzaldehyde experiments, indicating that some bacterial strains are more sensitive than others. Application of citral reduced colonization of strain IPM 7 but increased colonization of IPM 11 (Table 3). Application of benzaldehyde reduced colonization of both IPM 7 and 11. Colonization of other strains was unaffected by application of the compounds.

Seed-surface pH did not affect root colonization by PGPR in any of the experiments (Table 2). No main effects of this variable were detected, nor were interactions with PGPR which would suggest that individual strains were more sensitive to seed-surface pH than others. Soil treatment with the botanical aromatics did not interact with seed-surface pH

TABLE 3. Effects of soil application of three botanical aromatic compounds on cotton root colonization by 10 PGPR strains 10–12 days after planting (expressed as $\log_{10}$ CFUs/g root tissue)

	Citral		Furfural		Benzaldehyde	
PGPR Strain	Treated soil	Untreated soil	Treated soil	Untreated soil	Treated soil	Untreated soil
IPM 1	4.75	4.85	4.63	4.77	4.91	4.73
IPM 2	4.77	4.61	4.69	4.68	4.59	4.75
IPM 4	4.67	4.83	4.53	4.74	4.73	4.45
IPM 5	3.55	3.71	4.19	4.37	4.13	4.37
IPM 6	3.73	3.45	3.43	4.14**	3.85	3.93
IPM 7	4.37	4.90**[a]	4.38	4.86*	3.85	4.63**
IPM 8	4.62	4.55	4.82	4.95	4.55	4.55
IPM 9	4.26	4.38	4.44	4.81	4.57	4.6
IPM 10	4.59	4.53	4.51	4.79	4.81	4.59
IPM 11	4.78	4.35**	4.72	4.94	4.11	4.52*

[a]*, **Significantly different from treated soil at $P < 0.05$ and $P \leq 0.01$ respectively.

and three-way interactions of aromatics, seed-surface pH and PGPR strain were not detected.

DISCUSSION

Soil application of furfural reduced colonization of the PGPR tested in this study. Although the reduction was not large (averaging 0.32 log CFUs/g of root), it may limit the integration of PGPR into a bio-intensive IPM system that includes the application of furfural for control of *M. incognita*. Citral and benzaldehyde had little impact on PGPR colonization. Only two of the 11 strains were sensitive to the application of citral and benzaldehyde: the *Bacillus macerans* strains IPM 7 and 11. Citral decreased colonization of IPM 11 whereas benzaldehyde increased colonization by this strain. Both compounds decreased colonization of IPM 7. Although furfural and benzaldehyde treatments increased the proportion of *Burkholderia* spp. in the soil (Bauske *et al.*, 1993), there is no indication that either compound significantly promoted colonization of the *B. cepacia* strain (IPM 4) on cotton roots.

The relatively low seed-surface pH (2.3) of commercial acid-delinted cotton seed did not reduce colonization of the strains tested, either alone or in combination with a soil treatment, and, hence, should not limit the utilization of these PGPR alone or with these botanical aromatic compounds. This expands the findings of Mahaffee and Backman (1993) who reported that low seed-surface pH reduced colonization of seed by strain GB03 after 60 h, but detected no effects of low pH after 2 weeks. Initial effects of seed-surface pH may decrease as bacteria colonize actively growing roots. It is also possible that the buffering effect of the soil solution may change the seed-surface pH. Regardless, there are no indications that low seed-surface pH of acid-delinted cotton plays a significant role in colonization of any of the 10 strains tested in these studies.

In vitro effects of citral, furfural and benzaldehyde on growth of the bacterial strains were not indicative of the effects of these compounds on rhizosphere colonization in greenhouse assays. Other researchers have reported similar inconsistencies between *in vitro* and greenhouse tests with fungicides and insecticides (Zablotowicz *et al.*, 1992). Benzaldehyde and furfural inhibited growth of all strains tested *in vitro* (Table 1). In greenhouse tests, where PGPR placed on cotton seed were not exposed to the compounds until 9 days after application to soil, only furfural significantly reduced colonization of the strains. Since all three compounds are volatile (Anon, 1976), the concentration to which the bacteria were exposed was probably much lower than that initially applied to the soil. In addition, benzaldehyde oxidizes in the air to benzoic acid. Benzoic acid and furfural are readily subject to biological degradation by soil microorganisms (Trudgill, 1984; Amador & Alexander, 1988).

The emphasis in crop protection in the US has shifted from traditional pest control techniques to IPM and reduced reliance on synthetic pesticides. This has opened the door for the introduction of bio-intensive IPM systems which incorporate multiple non-pesticide components into a single comprehensive control system. Each component of such a system must be tested for compatibility with all other components. Overall, the results presented here indicate that both citral and benzaldehyde, which control rootknot nematode and have activity against pathogenic fungi, may be used with PGPR in a bio-intensive IPM system.

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