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Phenol-mediated suppression of soil-borne root-infecting fungi in mungbean

Imran Ali Siddiqui[1] and S. Shahid Shaukat[2]

[1]P.A.F. Intermediate College, Korangi-Creek, Karachi-75190, Pakistan
[2]National Nematological Research Centre, University of Karachi, Karachi-75270, Pakistan

Summary. Under field conditions, there is a variety of phenolic acids as well as other toxic and non-toxic organic compounds that interact with plant seeds and roots; but in laboratory bioassays, with few exceptions, only single phenolic acids are usually tested. In this study, the effect of various concentrations of two phenolics (caffeic acid and *p*-hydroxybenzoic acid) on the wilt-inducing fungus *Fusarium solani* and the damping-off fungus *Rhizoctonia solani* was tested in pot experiments. The effect of these phenolics on the biocontrol efficacy of *Pseudomonas aeruginosa*, a plant growth-promoting rhizobacterium, was also evaluated. Caffeic acid and *p*-hydroxybenzoic acid significantly suppressed *F. solani* and *R. solani* infection in mungbean. However, high concentrations of the phenolic acids interfered with plant growth. *P. aeruginosa* in the rhizosphere declined in the presence of caffeic acid and *p*-hydroxybenzoic acid.

Key words: caffeic acid, *p*-hydroxybenzoic acid, phenolic acids, allelopathy, soil-borne fungi.

Introduction

Allelopathy is an important ecological mechanism in natural and managed ecosystems (Rice, 1995; Inderjit, 1998). The term allelopathy was coined in 1937 by Molisch to designate plant-plant and plant-micro-organism biochemical interactions. Rice (1984), defined allelopathy as "any direct or indirect, harmful or beneficial effect by one plant (including micro-organisms) on another through production of chemical compounds that escape into the environment". Many studies of allelopathy have focused only on interactions in which one organism is detrimentally affected by the association, and only infrequently has reference been made to beneficial or stimulatory interactions (Halbrendt, 1996). Of potential allelopathic compounds, phenolic acids are the most frequently identified phytotoxins. Recent studies have demonstrated the toxic effects of phenolic compounds (Shafer and Rholf, 1995; Blum, 1996; Burhan and Shaukat, 2000). A major concern regarding the role of phenolic acids as allelopathic agents in no-till systems pertains to the fact that concentrations of individual phenolic acids recoverable from field soils are well below the levels required for inhibition of germination and growth *in vitro* (Blum *et al*., 1991; Blum, 1996). Furthermore, under field conditions a variety of allelochemicals are encountered which not only interfere with the growth of crop but also exert a det-

Corresponding author: I.A. Siddiqui
E-mail: imran_75850@yahoo.com

rimental effect on plant-associated micro-organisms, including beneficial ones.

Since soil-borne root-infecting fungi are recognized as economically important pathogens, many studies have focused on fungal-suppressive crops as a potential management tools in cropping systems. The prospect of exploiting naturally occurring allelochemicals for fungal control has advantages over the current use of toxic chemicals, and there have been many attempts to utilize this approach either by rotation, intercropping, or green manure treatments. Potential allelopathic compounds identified in living and decomposing tissue of small grain-cover crops include phenolic acids (Liebl and Worsham, 1983; Barnes *et al*., 1986; Blum *et al*., 1991), hydroxamic acids (Nair *et al*., 1990; Gagliardo and Chilton, 1992), other organic acids (Patrick, 1971; Chou and Patrick, 1976; Lynch, 1977) and volatile substances (Buttery *et al*., 1985; Bradow, 1991). Among these, phenolic acids have been most frequently identified as phytotoxins.

The present paper focuses on the effect that various concentration of two phenolic acids (caffeic acid and *p*-hydroxybenzoic acid) have on *Fusarium solani*, a wilt-inducing fungus, and *Rhizoctonia solani*, a damping-off fungus on mungbean. The impact of these phenolics alone or in combination on the biocontrol potential of *Pseudomonas aeruginosa*, a plant growth-promoting rhizobacterium (PGPR), is also examined.

Materials and methods

The soil used for the experiment was obtained from the experimental field of the Department of Botany, University of Karachi. The soil (sandy-loam, pH 8.1, moisture holding capacity 40%) was passed through a 2-mm sieve to discard non-soil particles. The soil was naturally infested with 3000-cfu g^{-1} of soil of a mixed population of *Fusarium* spp., (*F. oxysporum* and *F. solani*) as estimated by the soil-dilution technique (Nash and Snyder, 1962) as well as with 5–7% colonization of *Rhizoctonia solani* on sorghum seeds used as bait (Wilhelm, 1955). The soil was also infested with 100–150 larvae ×250 g soil of a mixed population of *Helicotylenchus brassicae, Rotylenchulus reniformis* and *Tylenchorhynchus brassicae*. Eight mungbean *Vigna radiata* (L.) Wilczek seeds were sown in 8-cm-diam. plastic pots containing 350 g soil. Before planting, the potted soil was drenched with 20 ml of caffeic acid (a derivative of cinnamic acid) and *p*-hydroxybenzoic acid (a derivative of benzoic acid) to give acidic concentrations of 1, 2 and 4 μg g^{-1} soil. Soil drenched with 20 ml sterile distilled water served as control. Concentrations of the phenolic compounds greater than 4 μg g^{-1} have been found to be severely toxic to mungbean (Shaukat and Siddiqui, unpublished). In the combination treatments, the concentration of each acid was reduced by half (0.5, 1 and 2 μg g^{-1} soil and control). Treatments were replicated four times and arranged in a randomized complete block design.

The plants were harvested 45 days after emergence and growth parameters (plant height and f wt of shoot) were recorded. To determine the incidence of fungi, the roots were washed in running tap water, cut into 5-mm long pieces, surface-sterilized and placed on potato dextrose agar (PDA) plates supplemented with penicillin (100,000 units l^{-1}) and streptomycin sulphate (0.3 g l^{-1}). One-week after incubation at 28°C, the incidence of the root-infecting fungi was estimated using the following formula:

$$\% \text{ Infection} = \frac{\text{No. of plants infected with a fungus}}{\text{Total No. of plants}} \times 100$$

In another experiment, after soil amendment with single phenolic acid, plant growth-promoting rhizobacterium *Pseudomonas aeruginosa* (Schroeter) Migula was also introduced into the soil. Inoculum was produced by transferring two loopfuls of the bacterium from a 5-day-old culture on King's B medium (amended with 100 ppm of streptomycin) at 28°C to 100-ml KB liquid medium and incubated at room temperature on a shaker (150 rpm) for 48 h. Bacterial cells were centrifuged (4,500 *g*, 15 min) at 4°C, the supernatant was discarded and the pellet resuspended in sterile $MgSO_4$ (0.1 M). The soil was drenched with 25-ml aqueous cell suspension of IE-6S$^+$ (3.5×10^8 cfu ml^{-1}). Soil drenched with 25-ml $MgSO_4$ (0.1 M) served as controls. Plants were uprooted 45 days after emergence and growth parameters and incidence of root-infecting fungi recorded.

Rhizosphere populations of *P. aeruginosa* were reisolated by the modified method of Pillay and Nowak (1997) in which root samples with adhering soil were placed in 250-ml Erlenmeyer flasks each containing 10-ml of a 0.1 M $MgSO_4$ solution

(pH 6.5) plus 0.02% Tween-20 and shaken vigorously. Ten-fold serial dilutions of the suspension were prepared and 50-µl aliquots from the 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated onto KB medium supplemented with streptomycin. The plates were incubated at room temperature (25±3°C) for 48 h and the number of cfu was recorded. Plants grown in soil not inoculated with *P. aeruginosa* were also examined for fluorescent pseudomonads.

The data were subjected to factorial analysis of variance (FANOVA) followed by the least significant difference test (LSD) in accordance with Sokal and Rohlf (1995). Bacterial population data were transformed to $\log_{10}$ (x+1) before analysis.

Results

FANOVA revealed that both benzoic and caffeic acids caused significant (P<0.01) suppression of *F. solani* and *R. solani* (Table 1). In general, *F. solani* was reduced more than *R. solani*. Benzoic acid was more effective against *F. solani* than against *R. solani,* while caffeic acid was effective against both fungi, and was slightly more effective than benzoic acid. Caffeic acid (4 µg g^{-1} soil) and benzoic acid (2 µg g^{-1} soil) used together produced the greatest suppression (92% more than controls) of *F. solani,* whereas caffeic acid (4 µg g^{-1} soil) mixed with either 1 or 4 µg g^{-1} soil of benzoic acid caused the maximum inhibition (72% more than controls) of *R. solani*. Benzoic acid was more effective in suppressing root-infecting fungi when it was combined with caffeic acid. Whether used singly or in combination, phenolics significantly (P<0.05) affected plant height and fresh weight of the shoots. Plant growth was reduced with increasing phenol concentration. The two phenolics applied together had greater phytotoxic effects than when each was applied singly.

The two phenolics with *P. aeruginosa* significantly (P<0.001) suppressed *F. solani* and *R. solani* compared with the controls (Table 2). Caffeic acid (4 µg g^{-1} soil) mixed with either 2 or 4 µg g^{-1} soil of benzoic acid without the biocontrol bacterium gave an 87% suppression of *F. solani* compared to the control, while with *P. aeruginosa*, caffeic acid and benzoic acid (both at 4 µg g^{-1} soil) used together completely suppressed *R. solani*. Plant growth decreased with the increasing phenolic concentration. In general, however, application of *P. aeruginosa*

Table 1. Effect of mixtures of phenolic acids on *Fusarium solani* and *Rhizoctonia solani* and on growth of mungbean.

Treatment (µg g^{-1} soil)		Infection (%)		Plant height (cm)	Shoot weight (cm)	Root weight (g)
Caffeic acid +	Benzoic acid	*F. solani*	*R. solani*			
0	0	81	91	17.1	0.6	0.4
0	1	91	81	21.9	0.8	0.4
0	2	75	100	23.2	0.7	0.4
0	4	38	69	19.1	0.7	0.3
1	0	56	78	19.3	0.6	0.4
1	1	75	56	21.3	0.6	0.4
1	2	75	63	17.5	0.6	0.3
1	4	31	44	18.2	0.5	0.3
2	0	75	81	21.3	0.7	0.3
2	1	38	56	22.2	0.7	0.4
2	2	75	44	16.6	0.5	0.4
2	4	25	31	15.3	0.5	0.3
4	0	63	38	19.1	0.5	0.3
4	1	25	25	16.9	0.4	0.3
4	2	6	31	13.9	0.4	0.2
4	4	38	25	12.6	0.3	0.2
$LSD_{0.05}$	Caffeic acid	16.8	19.2	0.8	0.1	0.04
	Benzoic acid	16.8	19.2	0.8	0.1	0.04

Table 2. Effect of mixture of phenolic acids on *Fusarium solani* and *Rhizoctonia solani*, on growth of mungbean and on populations of *Pseudomonas aeruginosa* in the rhizoshere.

Treatment (μg g^{-1} soil)		Infection (%)				Plant height (cm)		Shoot weight (cm)		Root weight (g)		Bacterial rhizosphere colonization Log cfu g^{-1} fresh wt
		F. solani		*R. solani*								
Caffeic acid	+ Benzoic acid	*Pa*-[a]	*Pa*+[b]	*Pa*-	*Pa*+	*Pa*-	*Pa*+	*Pa*-	*Pa*+	*Pa*-	*Pa*+	
0	0	94	50	100	75	15.9	17.3	0.5	0.8	0.4	0.4	4.48
0	1	100	69	75	75	20.5	23.9	0.6	0.8	0.4	0.4	4.59
0	2	75	81	81	38	21.2	21.8	0.6	0.9	0.4	0.5	4.36
0	4	69	50	69	50	18.5	19.9	0.5	0.7	0.3	0.5	4.03
1	0	100	50	81	50	18.9	24.2	0.5	0.6	0.4	0.3	4.42
1	1	81	75	69	50	19.9	22.9	0.6	0.7	0.3	0.4	4.38
1	2	38	69	75	38	15.8	19.3	0.4	0.7	0.2	0.3	3.69
1	4	69	38	75	50	14.9	17.8	0.4	0.5	0.2	0.4	3.63
2	0	75	38	75	75	20.7	19.5	0.5	0.5	0.4	0.3	3.95
2	1	69	75	50	38	19.9	20.3	0.4	0.5	0.3	0.3	4.38
2	2	75	50	75	50	16.9	19.7	0.3	0.5	0.3	0.3	3.28
2	4	38	38	38	25	17.7	15.5	0.4	0.5	0.4	0.4	3.46
4	0	75	38	69	69	17.6	17.8	0.3	0.4	0.3	0.3	3.49
4	1	50	38	50	38	18.2	20.8	0.5	0.5	0.3	0.3	3.49
4	2	13	25	38	50	15.5	16.3	0.3	0.5	0.3	0.3	3.28
4	4	13	0	25	13	13.8	15.5	0.3	0.3	0.2	0.3	3.09
$LSD_{0.05}$	Caffeic acid	28.7	31.1	0.29	0.03	0.02	0.19					
	Benzoic acid	28.7	31.1	0.29	0.03	0.02	0.19					
	P. aeruginosa	21.5	23.8	0.19	0.02	0.01	-					

[a] *Pa*-, soil without *P. aeruginosa*.
[b] *Pa*+, soil with *P. aeruginosa*.

was associated with enhanced plant growth. Rhizosphere colonization by the bacterium declined sharply with caffeic acid in the soil, but when benzoic acid was added at 1 μg g^{-1} soil the bacterial population in the rhizosphere increased.

Discussion

The present study strongly suggests that allelopathy is an important phenomenon affecting the attack of root-parasitic fungi and plant growth. The addition of the caffeic acid and *p*-hydroxybenzoic acid individually or in combination significantly reduced infection percentage of mungbean by *F. solani* and *R. solani*. Whether the phenolic acids actually caused the observed suppression of the two root-infecting fungi was not established but was possible. The phenolics may have had an inhibitory effect on the fungi, preventing their colonization of mungbean. Phenol amendment was associated with an increase in fungal and bacterial populations, especially those with chitinolytic activity inhibiting *F. solani* and *R. solani*. The phenolics may also have resulted in a greater release of phytotoxins in the crop rhizosphere, thus suppressing the pathogens. Once absorbed in the root cells, these phenolic compounds may act as phytoalexins, inhibiting the fungi. Whatever the mechanism involved, the phenolics can be utilized in sustainable agriculture to control root-infecting fungi and other plant pathogens, including nematodes, bacteria etc.

In the present study, benzoic acid was more effective against soil-borne root-infecting fungi and had a greater phytotoxic effect when it was used in combination with caffeic acid. The effect of a phytotoxin on plant processes in the presence of other compounds, whether toxic or not can vary. If

the sites or action of two phytotoxins (e.g., phenolic acid mixtures) are similar, a combination of these phytotoxins could have a synergistic or additive effect on plant growth (Blum, 1996). A cinnamic acid derivative such as caffeic acid and a benzoic acid derivative such as *p*-hydroxybenzoic acid vary widely in their phytotoxicity, but all have the same mode of action. The primary effect of these phenolic acids on susceptible species is reduction in hydraulic conductivity and in the net nutrient uptake of the roots and hence of growth (Blum, 1995). In the present study, caffeic acid inhibited mungbean growth more than did *p*-hydroxybenzoic acid. Cinnamic acid derivatives are reported to be more phytotoxic than their corresponding benzoic acid derivatives. Relative toxicity to cucumber seedlings, for example, is ranked as follows: ferulic acid and sinapic acid > *p*-coumaric acid; vanillic acid and syringic acid > caffeic acid; and *p*-hydroxybenzoic acid > protocatechuic acid (Gerig and Blum, 1991).

Our results indicated that the two phenolics alone or in combination not only inhibited soil-borne root-infecting fungi of mungbean but also strongly reduced populations of *P. aeruginosa* in the rhizosphere. Similarly, in a previous report, eight phytotoxins isolated from *Polygonum aviculare* not only inhibited seed germination and/or seedling growth of a plant species but also inhibited some of the test strains of the nitrogen-fixing bacteria, *Rhizobium* and *Azotobacter* (Rice, 1979; Al-Saadawi and Rice, 1982). These allelochemicals thus exert a direct allelopathic effect on mungbean by producing phytotoxins, and an indirect allelopathic effect by inhibiting the beneficial micro-organisms, especially those known to produce phytohormones (pseudomonads) and other plant-beneficial substances. Furthermore, some of these chemicals may also change certain soil chemical characteristics. In nature, however, several plant interference mechanisms (allelopathy, resource competition, microbial nutrient immobilization, differential susceptibility to root-infecting fungi) may operate simultaneously and (or) sequentially (Inderjit and Del Moral, 1997).

The study of allelopathy is now widely recognized as a valid scientific discipline, and numerous aspects of allelopathy research have found a practical application in the control of pest problems. Mixtures of caffeic acid and *p*-hydroxybenzoic acid could be exploited to inhibit wilt-inducing fungi and damping-off fungi, though high concentrations of these acids may inhibit plant growth.

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