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Allelopathic interference of plant-water relationships by para-hydroxybenzoic acid

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Abstract. Soybean seedlings were used as the test species to investigate the effect of *p*hydroxybenzoic acid (*p*HBA) on growth and plant-water relationships. Plants were grown in nutrient solution under greenhouse conditions and were subjected to *p*HBA through amendments to the growth medium. Treatments were initiated 10 days after germination and continued for either 14 or 28 days with stomatal conductance, water potential, and water use monitored periodically. At harvest, effects on growth were determined and, in the 28-day study, the carbon isotope ratio (^{13}C : ^{12}C) of leaf tissue was analyzed as an indicator of integrated effects on plant-water status. Soybean growth was significantly reduced by 0.5 mM *p*HBA, or higher concentrations, with the degree of inhibition being concentration dependent. Plants treated with 0.75 mM *p*HBA had significantly lower stomatal conductance, lower water potential, and less discrimination against ^{13}C. Similar trends were apparent in 0.5 mM *p*HBA-treated plants. Interference with plant-water balance appears to be one mechanism of action of *p*HBA causing a reduction in plant growth.

Keywords: Allelochemical; Allelopathy; Carbon isotopes; *p*hydroxybenzoic acid; Soybean; Water status.

Introduction

The benzoic acid derivatives produced by higher plants have been frequently implicated in allelopathy (Rice, 1984). Para-hydroxybenzoic acid (*p*HBA; 4-hydroxybenzoic acid) has been isolated from a variety of crop residues and agricultural soils (Whitehead, 1964; Guenzi and McCalla, 1966; Chou and Patrick, 1976; Blum et al., 1991). It is one of several allelopathic *Sorghum* compounds found in aqueous extracts of plant material and associated with decomposing crop residues, germinating seeds, and root exudates (Abdul-Wahab and Rice, 1967; Hussain and Gadoon, 1981; Lehle and Putnam, 1983; Alsaadawi et al., 1986; Panasiuk et al., 1986; Einhellig and Rasmussen, 1989). Other plants implicated in allelopathic release of *p*HBA include *Camelina alyssum* and its influence on flax (Grummer and Beyer, 1960), several members of the genus *Althaea* (Gude and Bieganowski, 1990), and the grass *Imperata cylindrica* (Hussain and Abidi, 1991). Some of the reduction in root and coleoptile growth of wheat seedlings by wild oat (*Avena fatua*) root exudate is attributed to *p*HBA (Perez and Ormeno-Nunez, 1991).

Although several physiological effects of *p*HBA have been reported, its primary action affecting growth is obscure. Lee and Skoog (1965) found the hydroxybenzoic acids caused inactivation of indoleacetic acid. Effects on respiratory metabolism have been suggested, as *p*HBA inhibited succinate hydrogenase and altered oxidation of NADH (Hulme and Jones, 1963; Lee, 1966). Glass (1973, 1974, 1975) demonstrated the inhibitory action of hydroxybenzoic acids on potassium and inorganic phosphate absorption of barley roots and related work established that membrane depolarization occurred (Glass and Dunlop, 1974). Stomatal effects from *p*HBA interaction with abscisic acid have been suggested (Purohit et al., 1991). Our previous work established that related phenolic compounds caused stomatal closure and other constraints on plant water relationships (Barkosky and Einhellig, 1993; Barkosky et al., 1999; Barkosky et al., 2000). Mechanism of action models proposed by Einhellig (1986) have suggested that membrane perturbations are probably a primary action of many phenolic allelochemicals.

This study was designed to determine the impact of *p*HBA on plant-water relationships and to evaluate the correspondence between these actions with effects on plant growth. As a part of the data collection, the relative concentration of stable carbon isotopes in leaf tissue was used as an indicator of water stress over time (Farquhar et al., 1982; Roeske and O'Leary, 1984; O'Leary, 1988; Berry, 1989; Tieszen, 1991). This analytical theory is based on the fact that several plant factors and environmental conditions, including water stress, contribute to the extent to which plants discriminate against carbon-13 (Farquhar et al., 1982, 1989; Ehleringer, 1989; Guy et al., 1989).

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Materials and Methods

Soybean [*Glycine max* (L.) Merr. var. Wells II] seedlings were used as the test species in these experiments. Soybeans have been shown to be sensitive to several phenolic allelochemicals and indices of water stress are conveniently measured in these seedlings (Einhellig and Rasmussen, 1979; Patterson, 1981; Colton and Einhellig, 1980; Einhellig and Schon, 1982; Einhellig and Eckrich, 1984; Barkosky and Einhellig, 1993). Soybeans were germinated and grown in a greenhouse which experienced the usual variations of summer greenhouse conditions.

Seeds were germinated in vermiculite flats and after 7 days the seedlings were individually transplanted to opaque plastic vials containing 120 mL of nutrient medium. The seedlings were supported upright by a hole in the lid of the vial. The nutrient medium was a modified Hoagland's solution (Hoagland and Arnon, 1950) containing 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 0.9 mM $NH_4H_2PO_4$, 0.1 mM $(NH_4)_2HPO_4$, standard Hoagland's micronutrients, and 72 µM iron supplied as sodium ferric diethylene-triamine pentaacetate (Sequestrene 330). Transplanted seedlings were allowed to acclimate three days before treatments with *p*HBA were initiated (day 10).

Treatments were initiated by replacing the nutrient medium with nutrient solution amended with *p*HBA. Subgroups of plants in the first experiment were treated over a 14-day period with zero, 0.25 mM, 0.5 mM, and 1.0 mM *p*HBA. These results provided data demonstrating the threshold concentration at which *p*HBA inhibited soybean seedling growth. The intent of the second experiment (28-days treatment) was to subject seedlings to longer-term exposure to *p*HBA levels near the inhibition threshold. The treatments in this 28-day experiment were 0.50 mM *p*HBA, 0.75 mM *p*HBA, and a control.

On the day of treatment, a subset of six seedlings was harvested to establish leaf area and dry weight baseline data. Adequate numbers of seedlings were used in each treatment group to provide a minimum of six plants per group at the final harvest after the sacrifice of some seedlings for water potential determinations during the treatment period. On day 10 following treatment, plants were transferred into 350 mL containers, and in the 28-day experiment soybeans were subsequently transferred to 750 mL containers on day 15. The treatment solution was replaced every three days during the experiments to ensure the plants had a constant nutrient supply and allelochemical exposure. In the 28-day experiment, the amount of solution used was recorded.

In both experiments, stomatal conductance was measured every second day on six randomly chosen plants per treatment group. Measurements were obtained from the abaxial surface of the unifoliate leaves using a Li-Cor LI-1600 steady-state porometer. In the 28-day experiment, leaf water potential was obtained once a week from four plants in each *p*HBA level. A 7-mm diameter disk was punched from the center of a leaflet in the most fully developed trifoliate, leaf samples were equilibrated for 2 h in Wescor C-52 sample chambers, and water potential was determined using a Wescor HR 33 dewpoint microvoltmeter. Plants from which a leaf disk was cut were discarded. Stomatal conductance and water potential were obtained between 1300 and 1500 h.

At the termination of an experiment, plants were harvested by separating the leaves from the plant, obtaining the leaf area, and oven drying all tissue at 104°C for 48 h. These data were used to compute the leaf to plant weight ratio (LWR; mg mg^{-1}), specific leaf weight (SLW; mg cm^{-2}), and relative growth rate (RGR; mg mg^{-1} day^{-1}) (Evans, 1972; Bhowmik and Doll, 1983). Calculation of RGR over the treatment period utilized the initial plant weights from the subsample harvested at the time of treatment (T_1;W_1), compared to weights at harvest (T_2;W_2), using equation 1.

$$(1)\quad RGR = \frac{(\ln W_2 - \ln W_1)}{(T_2 - T_1)}$$

In the 28-day experiment, leaf tissue of the second and third trifoliate leaves was analyzed to determine the carbon isotope ratio, ^{13}C:^{12}C. Oven-dried tissue was ground in a Cyclone sample mill fitted with a 0.4 mm screen and the carbon isotope ratio was determined by mass spectrometry (Augustana College, Sioux Falls, SD). These results are expressed as the delta (δ) ^{13}C value in per mill (‰) units (Equation 2).

Table 1. Effects of *p*hydroxybenzoic acid (*p*HBA) on the growth of soybean after 14 days of treatment.[1]

Plant parameters	*p*HBA Treatment			
	Control	0.25 mM	0.50 mM	1.0 mM
Leaf Area (cm^{-2})	109.7 b	132.8 a	93.6 b	30.9 c
Leaf wt. (mg)	296 a	333 a	237 b	93 c
Plant wt. (mg)	743 a	856 a	642 b	267 c
SLW (mg cm^{-2})	2.71 ab	2.51 a	2.52 b	3.21 b
LWR (mg mg^{-1})	0.398 a	0.389 ab	0.369 bc	0.348 c
RGR (mg $mg^{-1}day^{-1}$)	97.8 ab	107.5 a	86.0 b	24.3 c

[1]Values (n=6) within a row not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's Multiple Range Test. SLW = specific leaf wt.; LWR = leaf wt. ratio; RGR = relative growth rate.

$$(2)\ \delta^{13}C = \frac{^{13}C/^{12}C_{(sample)} - {}^{13}C/^{12}C_{(standard)}}{^{13}C/^{12}C_{(standard)}} \times 1000$$

All the data were analyzed using one-way analysis of variance (ANOVA) with means separated by Duncan's Multiple Range Test using the Statistical Analysis System (SAS).

Results

Soybeans were slightly stimulated by 0.25 mM *p*HBA in the growth medium as evidenced by the leaf area and dry weight of plants after 14 days treatment (Table 1). Stunting of plants was noted after several days of treatment with 0.5 mM and 1.0 mM *p*HBA, and the latter were more severely affected. When harvested, these plants had significantly lower plant dry weight, leaf weight, leaf area, and RGR than controls (Table 1). Leaf growth was more markedly impacted than overall plant growth as evidenced by the smaller LWR. Soybeans exposed to 0.5 and 1.0 mM *p*HBA regularly had lower stomatal conductances (Figure 1).

Since the threshold for growth inhibition in the first trial was approximately 0.5 mM *p*HBA, the second experiment focused on effects of chronic treatments of *p*HBA at and slightly above this level. The data demonstrate that 0.5 mM *p*HBA caused even more deleterious effects over 28 days treatment, as compared to 14 days, with soybeans achieving only about half the leaf area, leaf weight, and plant weight of controls (Table 2). Other than their depressed growth, these plant did not show overt signs of damage from the *p*HBA treatment. Plants grown with 0.75 mM *p*HBA for 28 days achieved less than 20% of the leaf area and biomass of untreated seedlings, indicating the importance of the concentration response.

Throughout the 28-day treatment, rates of stomatal conductance were significantly depressed in soybeans treated with 0.75 mM *p*HBA (Figure 2). A significant reduction in stomatal conductance was also noted in 0.5 mM *p*HBA-treated plants on most measurements after the first week of treatment. The week by week water potentials in both

Table 2. Effects of *p*hydroxybenzoic acid (*p*HBA) on the growth of soybean after 28 days of treatment.[1]

Plant parameters	*p*HBA Treatment		
	Control	0.50 mM	0.75 mM
Leaf Area (cm^{-2})	1245.1a	614.4 b	181.4c
Leaf wt. (mg)	3383 a	1844 b	579 c
Plant wt. (mg)	8838 a	4716 b	1602 c
SLW (mg cm^{-2})	2.72 a	3.00 a	3.19 b
LWR (mg mg^{-1})	0.383 a	0.391 a	0.361b
RGR (mg $mg^{-1}day^{-1}$)	138.2 a	115.5 b	75.3 c

[1]Values (n=6) within a row not followed by the same letter are significantly different, P < 0.05, ANOVA with Duncan's Multiple Range Test. SLW = specific leaf wt.; LWR = leaf wt. ratio; RGR = relative growth rate.

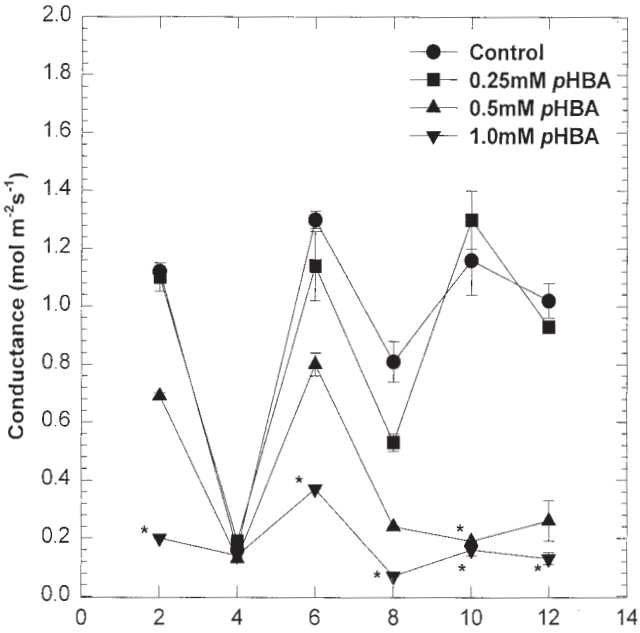

Figure 1. Effects of *p*Hydroxybenzoic acid (*p*HBA) on leaf conductance in soybean over 14 days of treatment. Each data point is the mean of measurements taken on six plants; *: indicates significance from controls (P<0.05, with Duncan's Multiple Range Test). Control (●); 0.25 mM *p*HBA (■); 0.5 mM *p*HBA (▲); 1.0 mM *p*HBA (▼).

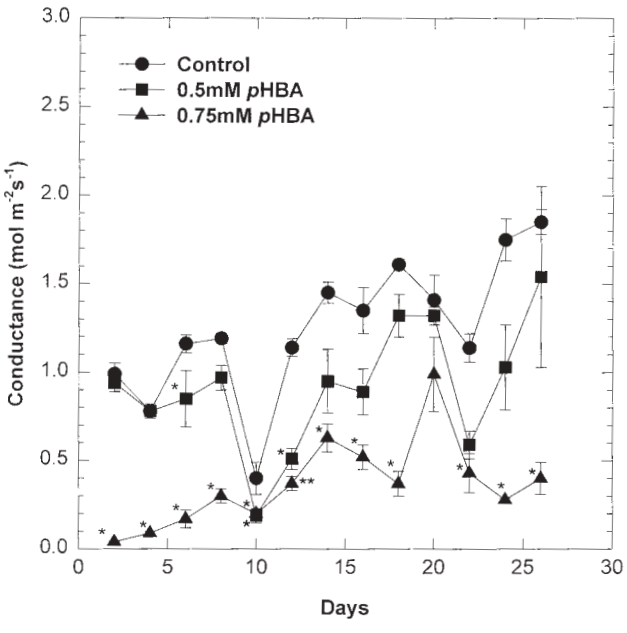

Figure 2. Effects of *p*Hydroxybenzoic acid (*p*HBA) on leaf conductance in soybean over 28 days of treatment. Each data point is the mean of measurements taken on six plants; *: indicates significance from controls (P<0.05, with Duncan's Multiple Range Test). Control (●); 0.5 mM *p*HBA (■); 0.75 mM *p*HBA (▲).

Table 3. Effects of *p*hydroxybenzoic acid (*p*HBA) on leaf water potentials (MPa) of soybean taken at regular intervals over 28 days of treatment. Mean values with standard errors in parentheses are provided.[1]

Treatment	Interval (days)			
	7	14	21	28
Control	-0.94 (0.05) a	-0.83 (0.05) a	-1.30 (0.15) a	-0.76 (0.06) a
0.05 mM *p*HBA	-0.97 (0.05) a	-1.05 (0.07) b	-1.26 (0.06) a	-1.03 (0.04) b
0.75 mM *p*HBA	-1.01 (0.07) a	-1.10 (0.06) b	-1.20 (0.08) a	-1.13 (0.06) b

[1]Values (n=4) within a column not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's Multiple Range Test.

*p*HBA treatments showed a trend toward lower values (Table 3). The water potentials in leaf samples from 0.5 and 0.75 mM *p*HBA-treated plants at the second week (14-day interval) and fourth week (28-day interval) were significantly below those of untreated plants. The fourth week water potentials were -0.27 and -0.37 MPa lower than controls for the 0.5 and 0.75 *p*HBA-treated plants, respectively.

Analysis of the carbon isotope ratio in leaf tissue at the termination of the 28-day experiment showed significantly less discrimination (less negative $\delta^{13}C$) had occurred against ^{13}C in plants grown with 0.75 mM *p*HBA and some indication of this effect was shown at the reduced *p*HBA treatment (Table 4). Conversely, the transpiration ratio computed over the duration of the treatments was significantly higher in the presence of 0.75 mM *p*HBA.

Discussion

The effects of *p*HBA on soybean growth fit the pattern of an allelochemical effect in that inhibition was concentration dependent and there was a slight stimulatory effect at a sub-toxic level. At the seedling growth-inhibition threshold of approximately 0.5 mM *p*HBA, plants treated for 28 days were more inhibited than those treated 14 day, achieving 86% and 53%, respectively, of the control biomass. Undoubtedly, both the duration of exposure to *p*HBA and variations in environmental conditions between the two experiments contributed to this difference. Due to periodic replacement of the growth medium, the longer treatment gave a higher total amount of *p*HBA exposure, a situation that influences the results in bioassays (Weidenhammer et al., 1987). Higher greenhouse temperatures and solar irradiance occurred during the 28-day experiment. These conditions resulted in more rapid growth in control plants and greater inhibition by *p*HBA. Einhellig and Eckrich (1984) reported that in combination with high temperature stress ferulic acid was more inhibitory to soybean growth.

Interestingly, even the highest concentrations of *p*HBA were not lethal. This may be due to the glucosylation of *p*HBA by enzyme systems within the plant. Scholten et al. (1991) found that cell cultures of *Datura innoxia* and *Scopolia carniolica* are able to glucosylate hydroquinone, vanillin, and *p*HBA acid by an enzyme-mediated, concentration dependent, bioconversion. Tabata et al. (1988) revealed that certain plant cell cultures can glucosylate a wide variety of phenolics including *p*HBA. Reduction of toxicity by glycosylation could allow soybean survival even through their growth is suppressed.

In all *p*HBA treatments which inhibited growth, lower stomatal conductances indicate the plants had some stomatal closure. Further evidence of water stress is shown by the reduced water potential values found in *p*HBA-treated plants in the 28-day experiment. Tissue from these plants also had relatively more ^{13}C than untreated seedlings, which supports an integrated view of *p*HBA resulting in higher stomatal resistances over time. Other phenolic acids allelochemicals, including *p*-coumaric, caffeic, ferulic, and salicylic acid, are known to cause water stress in plants (Einhellig et al., 1985; Booker et al., 1992; Barkosky and Einhellig, 1993).

The fact that higher transpiration ratios and thus lower water use efficiency was recorded under *p*HBA treatment is not readily explained. This apparent conflict with the data showing decreased stomatal conductance and enrichment of ^{13}C, typically associated with higher water use efficiencies, may be due to other actions of *p*HBA. Glass and Dunlop (1974) reported *p*HBA depolarizes membranes,

Table 4. Effects of *p*hydroxybenzoic acid (*p*HBA) on soybean water-use efficiency.[1]

Plant parameters	*p*HBA Treatment		
	Control	0.50 mM	0.75 mM
$\delta^{13}C$ (‰)	-28.07 a	-27.80 a	-26.82 b
Transpiration ratio (mL H_2O/g wt. gain)[2]	315 a	376 a	575 b

[1]Data are expressed as the magnitude of discrimination against ^{13}C. Values in a row not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's Multiple Range Test.

[2]Weight gain used to calculate transpiration ratio was obtained as the difference in weight between the mean dry weight and the final dry weight of a comparable set of soybean seedlings at the time of treatment and the final dry weight of treated plants. Water use over the period and the final dry weight were on an individual plant basis.

and it likely that membrane perturbations will have multiple effects on metabolism and the efficiency of plant processes.

The data from this study shows good correspondence between the changes in plant-water parameters and inhibition of plant growth, and we conclude that the impact of *p*HBA on water relationships is an important mechanism of action of this allelochemical.

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Para-hydroxybenzoic acid 以異體相克之方式干擾植物—水的關係

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大豆幼苗做為測試物種以檢驗 para-hydroxybenzoic acid (*p*HBA，一種常見之作為異物相克的化學份子) 對生長和植物—水關係的影響。大豆幼苗在溫室環境下培養於營養溶液 (不加或添加 *p*HBA)。*p*HBA 之處理自大豆種子發芽後 10 天開始，繼續 14 天或 28 天，並定期測定氣孔導電度 (stomatal conductance)、水勢 (water potential) 及水利用度。收穫時測定生長受阻程度，並且在 28 天之實驗裡棄組織之 (^{13}C:^{12}C) 之比率被測定作為植物—水狀態之 *p*HBA 之整合指標。結果顯示：大豆幼苗之生長顯著地受到 0.5 mM 或更高濃度之抑制 (效果和濃度成比例)；以 0.75 mM *p*HBA 處理之大豆幼苗顯著地與較低之氣孔導電度、水利用度，及驅除 ^{13}C 之能力；以 0.5 mM *p*HBA 處理者亦同。干擾植物—水之平衡似乎是 *p*HBA 抑制大豆幼苗生長的機制之一。

關鍵詞：異體相克之化學物；異體相克；碳同位素；*p*hydroxybenzoic acid；大豆；水平衡狀態。