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ALLELOPATHY OF YELLOW FIELDCRESS (*Rorippa sylvestris*): IDENTIFICATION AND CHARACTERIZATION OF PHYTOTOXIC CONSTITUENTS

A. YAMANE,[1] H. NISHIMURA,[2] and J. MIZUTANI[1,*]

[1]*Department of Agricultural Chemistry, Faculty of Agriculture*
Hokkaido University, Sapporo 060, Japan
[2]*Department of Bioscience and Technology*
Hokkaido Tokai University
Sapporo 005, Japan

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Abstract—Both the neutral and acidic fractions of the acetone extract of yellow fieldcress (Kireha-inugarashi, *Rorippa sylvestris* Besser) inhibited lettuce seed germination. Salicylic, *p*-hydroxybenzoic, vanillic, and syringic acid were identified in the acidic fraction. In the neutral fraction, hirsutin (8-methylsulfinyloctyl isothiocyanate), 4-methoxyindole-3-acetonitrile, and pyrocatechol were identified. Bioassay using a root exudate recirculating system showed *R. sylvestris* during flowering inhibited the lettuce seedling growth. Hirsutin (13 μg/plant/day) and pyrocatechol (9.3 μg/plant/day) were the major compounds released into the rhizosphere. Several combinations of pyrocatechol, *p*-hydroxybenzoic acid, vanillic acid, and hirsutin reduced lettuce seedling growth. These compounds seemed to be allelochemicals.

Key Words—Allelopathy, *Rorippa sylvestris*, weeds, salicylic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, hirsutin, 4-methoxyindole-3-acetonitrile, pyrocatechol, root exudate.

INTRODUCTION

A cruciferous weed species, *Rorippa sylvestris* Besser (yellow fieldcress, Kireha-inugarashi in Japanese) has been naturalized in Hokkaido since the 1950s and is considered to be one of the worst weeds in wet fields and pastures (Morita, 1981). The weed has strong propagative power and invades the territories of other plant species. It may be allelopathic against neighboring plants.

*To whom correspondence should be addressed.

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R. sylvestris contains hirsutin in the roots, which inhibits lettuce growth (Kawabata et al., 1989). Isothiocyanates in cruciferous plants play an important role as inhibitors of plant growth in addition to their antimicrobial activity (Harborne, 1988). These sulfur-containing compounds are usually found in macerated tissues due to mixing of the precursor glucosinolates and the hydrolytic enzyme thioglucosidase (Olsen and Sørensen, 1981).

Allelopathic inhibition of seed germination and plant growth typically occurs by the joint action of several allelochemicals. Additive or synergistic effects have been reported with combinations of monoterpenes, organic acids, and several classes of phenolic compounds (Einhellig, 1987; Williams and Hoagland, 1982).

Many studies of possible allelopathic effects on germination and growth have been dealt with using crude plant extracts or soil leachates, which make it difficult to discern the active principle in plant–plant interactions. For this reason, we used a bioassay with a root exudate recirculating system to explore the inhibitory constituents in the rhizosphere of *R. sylvestris* against lettuce growth. Inhibitory activities of pure compounds, singly and in combination, against lettuce seedling growth were also tested.

METHODS AND MATERIALS

Plant Collection and Extraction. Fresh roots of *Rorippa sylvestris* Besser (yellow fieldcress) were collected on the Hokkaido University campus in August 1988. The fresh roots (5.4 kg) were extracted with acetone for one month at room temperature. The filtered extract was concentrated under reduced pressure, and the aqueous concentrate was acidified with HCl (1 N), followed by extraction with ethyl acetate. The ethyl acetate extract was further divided into an acidic and a neutral fraction. From the aqueous residue, a basic fraction was obtained in the usual way. The basic fraction did not show any significant activity against lettuce.

Bioassay of Inhibitory Activity against Lettuce. A lettuce (*Lactuca sativa* L.) seed germination bioassay was used to examine the inhibitory activity of each extract, chromatographic fraction, or isolated compound. An appropriate amount of each of the samples was dissolved in 0.5 ml of acetone, and the solution was poured into a 6-cm Petri dish with two sheets of filter paper (No. 3, 5.5 cm, Toyo Roshi Co., Ltd.) placed on the bottom. Acetone was evaporated and 2 ml of water containing 100 ppm (w/v) Tween-80 was poured into the Petri dish. The dishes were covered and kept in the dark for 24 hr at room temperature to allow samples to dissolve completely and avoid light reaction. Thirty lettuce seeds were placed on the filter paper and incubated at 23°C in the dark. Germination percentages were recorded after 24 hr incubation. A

control with water containing 100 ppm (w/v) Tween-80 was included in each experiment. Each experiment was duplicated.

A lettuce seedling growth bioassay was used to determine the activity of the isolated compounds. The compound was dissolved in 150 μl of acetone and mixed into 15 ml of hot agar solution (0.35% w/v) in a Petri dish. The agar solution was cooled and 20 germinated lettuce seedlings (24 hr after germination) were placed in each dish. The dishes were covered and kept in a growth chamber (23°C, 12 hr dark and 12 hr light). The hypocotyl and root lengths for each seedling were measured at the end of one week. Mean hypocotyl and root lengths were determined for 20 seedlings (two replications). A control was included in each experiment.

Fractionation and Identification of Acetone Extract. The acidic fraction was subjected to silica gel column chromatography using hexane, ethyl acetate, and methanol as eluents. The hexane–ethyl acetate (1:1, v/v) eluate inhibited lettuce seed germination by 79% at 200 ppm (w/v). Further purification of this fraction by silicagel preparative thin-layer chromatography (TLC) using chloroform–methanol (2:1, v/v) as a mobile phase yielded compound I. Compound I was characterized by EI-MS, [^{1}H]NMR, and IR spectra, which were identical with those of authentic salicylic acid.

The hexane–ethyl acetate (2:3,v/v) eluate inhibited lettuce seed germination by 32% at 200 ppm (w/v). Compounds II, III, and IV were isolated from this eluate by silica gel preparative TLC. Compound II had a mp of 220–221°C and EI-MS, [^{1}H]NMR, UV, and IR spectra that were identical with those of authentic *p*-hydroxybenzoic acid. Compound III had a mp of 215°C and EI-MS, [^{1}H]NMR, UV, and IR spectra that were identical with those of authentic vanillic acid. Compound IV (colorless needles, 2.0 mg) had EI-MS, [^{1}H]NMR, and IR spectra that were identical with those of authentic syringic acid.

The neutral fraction including phenolics, which contained hirsutin (compound VII) (Kawabata et al., 1989), inhibited lettuce seed germination by 90% at 1000 ppm. Compound V (buff-colored needles, 45.9 mg) was isolated by silica gel preparative TLC (R_f = 0.78, developed with $CHCl_3$–MeOH, 20:1, v/v) and had a mp of 143°C. FD-MS *m/z*: 186 (M^+, 100%), EI-HR-MS *m/z*: M^+, 186.0797 ($C_{11}H_{10}N_2O$ requires 186.0793); EI-MS *m/z* (%): 186 (M^+, 90), 171 (100), 155 (8), 143 (13), 130 (4), 116 (13), 101 (4), 89 (12), 75 (4), 63 (7), 51 (4). The [^{1}H]NMR, [^{13}C]NMR, UV, and IR spectra were identical with those of authentic 4-methoxyindole-3-acetonitrile. Compound VI (colorless needles, 9.7 mg) was also isolated by silica gel preparative TLC (R_f = 0.46, developed with $CHCl_3$–MeOH, 10:1, v/v) and had a mp of 103.5°C. Compound VI had FD-MS, EI-MS, [^{1}H]NMR, UV, and IR spectra, which were identical with those of authentic pyrocatechol.

Bioassay Using Root Exudate Recirculating System. A bioassay using a root exudate recirculating system was performed following the method of

Stevens and Tang (1985). Donor pots containing *R. sylvestris* and acceptor pots containing lettuce seedlings were made from 1-gal brown glass bottles with the bottoms cut off. Both donor and acceptor pots contained coarse grade silica sand as the medium, with a 10-cm layer of small rock (2 cm size) under the sand. The sand in acceptor pots was topped with 1 cm of vermiculite to maintain moisture. Nutrient solution (Tadano and Tanaka, 1976) from the donor pot was lifted through 8-mm-diameter glass tubing to the acceptor pot using an air pump. Containers and glass tubings were wrapped with aluminum foil to exclude light. The solution was circulated at a rate of about 0.8 liters/hr. In the resin control pot, hydrophobic allelochemical compounds were removed from the recirculating solution using an XAD-4 column (18 × 120-mm glass column packed with 10 g Amberlite XAD-4 polymeric adsorbent resin). Treatments consisted of *R. sylvestris* as a donor species, *R. sylvestris* with a column containing XAD-4 (resin control), and a donor pot without the weeds (pot control). *R. sylvestris* plants (fresh weight ca. 50 g) were established in donor pots, and 30 lettuce seedlings (24 hr after germination) were planted in acceptor pots. Nutrient levels were maintained, and pH kept at 5.5 with H_2SO_4. Recirculating solution was not changed during the experiment except for replenishing water and nutrient solution. Lettuce seedlings were removed 14 days after beginning the bioassay, and plant heights, root lengths, and fresh weights of aerial parts and subterranean parts (two replications) were determined.

Quantification of Allelochemical Candidates in Recirculating Solution. Hydrophobic substances (100 μg) trapped with XAD-4 resin were eluted with acetone. Residual water was removed azeotropically by several additions of methanol. After 200 μl of pyridine and 100 μl of *N,O*-bis(trimethylsilyl)acetamide were added to the sample, the glass tube containing the reaction mixture was sealed with a polyethylene stopper and heated in an oil-bath at 80°C for 30 min (Schulz and Herrmann, 1980). 1-Octanol (15 μl) was then added to the sample as an internal standard and the trimethylsilylated sample was analyzed with gas chromatography [1.5% Silicone OV-17 on Uniport HP (80–100 mesh), 1 m].

Hirsutin was determined by adding 50 μl of methylene chloride and 3 ml of 20% ammoniacal ethanol (1 part concentrated NH_4OH and 4 parts anhydrous ethanol) to a portion (1 mg) of the total substances trapped with XAD-4 resin and heating the mixture for 2 hr at 50°C in a water bath (Wetter and Young, 1976). After cooling, the optical density was measured at 230, 259, and 304 nm (λ_{max} values of hirsutin–phenylthiourea). The blank was 50 μl of methylene chloride in 3 ml of 20% ammoniacal ethanol. On TLC chromatogram, hirsutin was found to be only one isothiocyanate.

Bioassay of Combined Compounds. Six combinations of two compounds (pyrocatechol plus *p*-hydroxybenzoic acid, pyrocatechol plus vanillic acid, pyrocatechol plus hirsutin, *p*-hydroxybenzoic acid plus vanillic acid,

p-hydroxybenzoic acid plus hirsutin, and vanillic acid plus hirsutin) with the concentration of each component 0.9×10^{-4} M, four combinations of three compounds (pyrocatechol plus *p*-hydroxybenzoic acid plus vanillic acid, pyrocatechol plus *p*-hydroxybenzoic acid plus hirsutin, pyrocatechol plus vanillic acid plus hirsutin, and *p*-hydroxybenzoic acid plus vanillic acid plus hirsutin) with the concentration of each component 0.6×10^{-4} M, and all compounds combined (pyrocatechol plus *p*-hydroxybenzoic acid plus vanillic acid plus hirsutin) with the concentration of each component 0.45×10^{-4} M were tested to determine their effects on lettuce seed germination. This total concentration (1.8×10^{-4} M) used in the bioassay represents a reasonable biological test level for the comparison of naturally occurring phytotoxic chemicals.

Analysis of Data. Differences among means were calculated using Duncan's multiple range test at the 5% level of probability. This allowed the effect of each compound, combined compounds, and the recirculating solution, including substances trapped with XAD-4 resin, to be examined.

RESULTS AND DISCUSSION

Both the neutral and acidic fractions of the acetone extracts inhibited lettuce seed germination (data not shown). Salicylic, *p*-hydroxybenzoic, vanillic, and syringic acid were isolated from the acidic fraction (Figure 1). At 200 ppm,

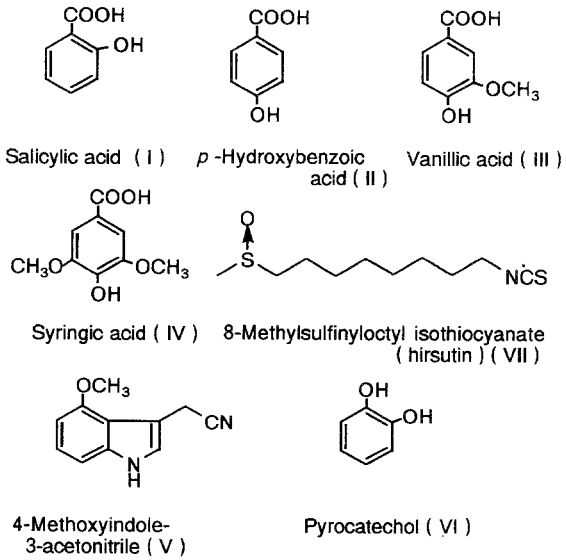

FIG. 1. Chemical structures of allelochemical candidates in the text.

these phenolic acids inhibited germination at 100, 100, 93, and 57%, respectively. Many phenolic compounds are inhibitory to seed germination or plant growth (Kuiters, 1989). In some instances, these compounds affect fundamental plant processes such as hormonal balance, protein synthesis, photosynthesis, chlorophyll production, plant–water relations and membrane permeability (Einhellig, 1986).

In addition to hirsutin, 4-methoxyindole-3-acetonitrile and pyrocatechol were isolated from the neutral fraction (Figure 1). 4-Methoxyindole-3-acetonitrile and pyrocatechol inhibited the germination at 200 ppm (w/v) by 12% and 100%, respectively. 4-Methoxyindole-3-acetonitrile had been isolated from the clubroots of Chinese cabbage (*Brassica pekinensis*) (Nomoto and Tamura, 1970), but this is the first report of this compound in *R. sylvestris*.

We have examined the effect of removing the hydrophobic substances using XAD-4 resin from the root exudates of *R. sylvestris* on lettuce seedling growth. Differences between the resin control and the pot control (no *R. sylvestris*) were presumed to be the results of inhibition by hydrophilic root exudates, while differences between the resin control and *R. sylvestris* were presumed to be caused by hydrophobic root exudates. Differences between *R. sylvestris* and the pot control represent the total allelopathic effect.

Root exudates inhibited lettuce seedling growth in June, July and August (Table 1). These were the flowering stages of *R. sylvestris*. However, for March

TABLE 1. INHIBITION OF LETTUCE GROWTH FROM EXPOSURE TO YELLOW FIELDCRESS ROOT EXUDATES

	Lettuce growth[a]								
	Aerial parts				Subterranean parts				
	Plant height		Fresh weight		Root length		Fresh weight		Germination inhibitory activity[c]
Period	vs Cont.	XAD-4[b]	vs Cont.	XAD-4[b]	vs Cont.	XAD-4[b]	vs Cont.	XAD-4[b]	
March	NS		NS		NS		NS		−
June	**		**		**		**		++
July	**	**	**	**	*	*	**		++
August	**	**	**	**	*	NS	NS	NS	+
September	*		*		NS		NS		−
November	NS		NS		NS		NS		−

[a] Significance levels: **, 0.01 level of probability; *, 0.05 level of probability; NS, not significant.
[b] Difference between yellow fieldcress and resin control (effect of hydrophobic root exudates).
[c] Lettuce germination inhibitory activity of hydrophobic substances in recirculating solution (400 ppm, 24 hr). Inhibitory activity: ++, 100%; +, ≥70%; −, <70%.

and November bioassays, lettuce seedling growth was not inhibited (Table 1). These periods were the vegetative growing stages of *R. sylvestris*. Aerial parts of the lettuce plant were more sensitive than the subterranean parts to the inhibition (Table 1). These responses were probably caused by the accumulation of toxic root exudates, as nutrient solution was not changed during the experiments.

Hydrophobic root exudates (XAD-4 versus control) inhibited growth of acceptor lettuce seedlings in the July and August bioassays (Table 1). The rate of hirsutin, pyrocatechol, vanillic acid, and *p*-hydroxybenzoic acid released from *R. sylvestris* roots in July and August was 13.0, 9.3, 1.1, and 0.7 μg/plant/day, respectively (data not shown). Recently, we (Mizutani and Yamane, 1991) reported that the content of precursor glucosinolates (glucohirsutin and deoxyglucohirsutin) increased during the vegetative growing stage and the flowering stage of *R. sylvestris*, suggesting that the content of glucosinolates may relate to the content of hydrophobic root exudate (hirsutin) and the bioassay results on this report. Deoxyhirsutin, which has no activity against lettuce, is another possible isothiocyanate, but we could not detect deoxyhirsutin in the hydrophobic root exudate.

Tang and Takenaka (1983) reported that for 2-month-old *Carica papaya* L. the rate of benzyl isothiocyanate release from the root system was 2–3 μg/tree/day in a continuous root exudate trapping system. This herbicidal secondary metabolite was released from the undisturbed plant root system.

Einhellig (1987) reported that allelopathic inhibition of germination and plant growth typically occurs from the joint action of several allelochemicals. Thus, the potential impact of an allelochemical on plant growth should be evaluated with regard to both the presence of associated allelopathic compounds and the influence of other chemical and physical conditions in the environment.

Several combinations of pyrocatechol, *p*-hydroxybenzoic acid, vanillic acid, and hirsutin reduced lettuce seedling growth (Table 2). Pyrocatechol plus *p*-hydroxybenzoicacid, pyrocatechol plus vanillic acid, pyrocatechol plus hirsutin, and *p*-hydroxybenzoic acid plus hirsutin significantly reduced lettuce hypocotyl elongation. From the combination effect (total inhibition rate of the single test divided by constituent number of combination), pyrocatechol plus *p*-hydroxybenzoic acid significantly reduced hypocotyl elongation, while the other combinations produced slight or insignificant effects.

On the other hand, several combinations showed significant effects on root elongation of lettuce seedlings. The combinations of pyrocatechol plus *p*-hydroxybenzoic acid and pyrocatechol plus vanillic acid, pyrocatechol plus *p*-hydroxybenzoic acid plus vanillic acid, and the combination of all four compounds increased root elongation. Among these, the combination of pyrocatechol plus *p*-hydroxybenzoic acid at 230% over the control was the most

TABLE 2. EFFECT OF COMBINATIONS OF POTENTIAL ALLELOPATHIC COMPOUNDS ON LETTUCE GROWTH

Combination[a]				Length (%)[b]	
Pyrocatechol	*p*-Hydroxybenzoic acid	Vanillic acid	Hirsutin	Hypocotyl	Root
+	−	−	−	58 e	70 e
−	+	−	−	96 a	54 f
−	−	+	−	89 b	50 f
−	−	−	+	44 h	66 e
+	+	−	−	51 fg	230 a
+	−	+	−	54 efg	170 b
+	−	−	+	49 g	69 e
−	+	+	−	90 b	52 f
−	+	−	+	53 efg	70 e
−	−	+	+	56 ef	63 e
+	+	+	−	63 d	180 b
+	+	−	+	56 ef	93 d
+	−	+	+	55 ef	97 d
−	+	+	+	69 c	90 d
+	+	+	+	65 d	110 c

[a] The pluses in each row indicate which chemicals were combined, of the four listed at the top. See text.

[b] Percent lengths (percent of the control growth) for a single test or a combination test, followed by the same letter are not significantly different at the 0.05 level of probability as determined by Duncan's multiple range test.

effective. These data suggest an auxin effect observed in the lettuce root growth bioassay at low concentrations of phytotoxic compounds (Manners and Galitz, 1985).

Our investigation clearly shows several phenolic compounds and hirsutin to be potent phytotoxins to developing lettuce seedlings and suggests that these compounds are allelochemical compounds in plant–plant interactions.

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