

Elementary Identification of Phenolic Allelochemicals from Dwarf Lilyturf Plant (*Ophiopogon japonicus* K.) and Their Growth-Inhibiting Effects for Two Weeds in Paddy Rice Field

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Abstract : Dwarf lilyturf (*Ophiopogon japonicus* K.), used as a weed-suppressing cover crop and a medicinal plant, was suggested to be a promising natural herbicide to control weeds in the rice field through its allelopathic potential. Allelopathic chemicals from the dwarf lilyturf were identified and their growth-inhibiting effects on two major weeds in the rice field in Japan were examined. High pressure liquid chromatograph (HPLC) analysis showed the existence of at least six allelopathic chemicals, viz., salicylic acid, syringic acid, syringaldehyde, vanillic acid, *p*-hydroxybenzoic acid and sinapic acid in dwarf lilyturf plant. The chemical detected at the highest concentration was salicylic acid ($251.04 \mu\text{g g}^{-1}$), which occupied more than half of total allelopathic chemicals detected ($317.16 \mu\text{g g}^{-1}$), followed by syringic acid ($37.30 \mu\text{g g}^{-1}$), syringaldehyde ($13.30 \mu\text{g g}^{-1}$) and sinapic acid ($11.03 \mu\text{g g}^{-1}$). The chemicals detected at the lowest concentration was vanillic acid ($1.69 \mu\text{g g}^{-1}$). Salicylic acid displayed the most inhibitory effects on germination and growth of both barnyardgrass (*Echinochloa crusgalli* L.) and monchoria (*Monochoria vaginalis* P.). This compound might play a key role in dwarf lilyturf allelopathy.

Key words : Allelochemicals, Barnyardgrass, Dwarf lilyturf, HPLC, Monchoria.

In the past several decades, many studies have been made on allelopathic compounds and their inhibitory effects on the growth of various plants (Tinniin and Muller, 1971; Chou and Chung, 1974, Newman and Rovira, 1975; Miller, 1996). Recently, much attention has been paid to the allelopathic effects of plants as a means of biological control of weeds (Klein and Miller, 1980; Einhellig, 1995; Macias et al., 1998; Putnam, 1988) and some allelopathic plants have been identified to have inhibitory or herbicidal effects on some weeds (Yoshino et al., 2000; Chung and Miller, 1995; Tsuzuki et al., 1999; Leather, 1983; Teasdale, 1988). In addition, the pellet after centrifugation (Tsuzuki et al., 1999), and the residue after evaporation (Chung and Miller, 1995) of alfalfa and kava powder (Ogushi et al., 2000) were suggested to be natural herbicides. Secondary metabolites and degradation products such as terpenoids, steroids, phenols, coumarins, flavonoids, tannins, alkaloids, and cyanogenic glycosides are known to have toxic effects on some plants (Seigler, 1996). However, phenolic chemicals have been studied most intensively with regard to phytotoxicity and allelopathy of plants (Putnam, 1985).

Dwarf lilyturf (*Ophiopogon japonicus* K.) is used as

a weed suppressing cover crop in Japan and also as a medicinal plant for a sore throat and physiological thirsty (Kishima, 1963; Izawa, 1967). In 2001, Lin (unpublished data) found that dwarf lilyturf plant had strong allelopathic effect on lettuce, radish and some fungi of crops. Recently, the possibility of the use of the powder of dried dwarf lilyturf as natural herbicides to control weeds in rice fields has been reported (Lin et al., 2003b). However, to the best of our knowledge, it is still not clear what chemical compounds are associated with allelopathy of dwarf lilyturf. The purpose of this study is to detect phenolic allelopathic chemicals in dwarf lilyturf and to evaluate their effects on the growth of two major weeds in rice fields.

Materials and Methods

1. Plant materials

Whole plants of one-year-old dwarf lilyturf (*Ophiopogon japonicus* K.) cultivated at the farm of the Agricultural Faculty of Miyazaki University, Japan were delved and divided into aerial (leaves and stems) and subterranean (roots and root-tubers) parts as described previously (Lin et al., 2003a,b). In this study, we used only the subterranean part which was reported to have medical effects (Kishima, 1963; Izawa,

1967), allelopathic effects on some plants (Lin, 2001, unpublished data) and fungi (Lin et al. 2003a) and inhibitory effects on weeds in rice fields (Lin et al., 2003b). The subterranean part was washed well with distilled water and dried in oven at 50°C for 48hr. The dried samples were cut into 1cm-long pieces with sterilized scissors and ground in a juice mixer for 15 min. The dried powder was vacuum packed and kept at -20°C until extraction.

2. Preparation of methanol extracts

Forty gram of dried powders was soaked in 200ml of 99.7% HPLC-grade methanol (Nacalai Tesque Inc., Japan) for 24 hr at 5°C. Extracts were filtered through one layer of No.2 filter paper (Toyo Roshi Kaisha Ltd., Japan). The filtrate was evaporated to dryness in a rotary vacuum evaporator (Tokyo Rikakikai Co. Ltd., Japan) at 40°C. Then 50ml methylene chloride (Wako Pure Chemical Industries Ltd., Japan) was added to the residue and stirred well for 15 min. The suspension was evaporated to dryness in rotary vacuum evaporator (Tokyo Rikakikai Co. Ltd., Japan) at 40°C again, re-dissolved in 70ml of 99.7% HPLC-grade methanol (Nacalai Tesque Inc., Japan) and centrifuged at 3000rpm for 15min. Finally, an aliquot of the solution was concentrated to 10 ml and filtered through 0.20 μm HYDROPHILIC filter (Toyo Roshi Kaisha Ltd., Japan) just before HPLC analysis.

3. HPLC analysis

A 5 μl portion of the filtrate was injected into an HPLC analysis system consisting of two LC-10Adv pumps, a SPD-10Avp UV-VIS detector, an DGU-12A degasser, an CTO-2A column oven and a C-R6A CHROMATOPAC (all from Shimadzu, Japan). The ZORBAX ODS column (4.6 \times 250mm, 5 μm particle; Agilent Technologies, USA) was used. The column oven temperature was set at 40°C. The wavelength of the UV detector was 254 nm. The mobile phase consisted of 2% (v/v) HPLC grade acetic acid (A) (Wako Pure Chemical Industries, Ltd., Japan) and 99.7% HPLC grade methanol (B) (Nacalai Tesque Inc., Japan). Elution was performed with the linear gradient as follows: 15% to 40% B from 0 to 23min and maintain for 4 min, 40% to 62% B from 28min to 42min, 62% to 99% B from 42 min to 63min as described by Fujii et al. (1991). The flow rate was 1.8 ml/min. The retention times of the phenolic compounds were compared with those of authentic reagents and concentrations were calculated by comparing peak areas of samples with those of the standards.

Standard Chemicals: Twenty-one phenolic compounds, namely, gallic acid, coumalic acid, vanillin, syringaldehyde, benzoic acid, salicylic acid, syringic acid and *trans*-cinnamic acid all purchased from Wako Pure Chemical Industries Ltd., Japan, and

protocatechuic acid, catechin, chlorogenic acid, β -resorcylic acid, vanillic acid, *m*-hydroxybenzoic acid, ρ -coumaric acid, ferulic acid and sinapic acid all purchased from Sigma Chemical Co., Germany, and ρ -chlorobenzonic acid, ρ -hydroxybenzoic acid, caffeic acid and *o*-coumaric acid purchased from Nacalai Tesques Inc., Japan, were used as standards for HPLC analysis.

4. Bioassay of identified phenolic compounds

The growth-inhibiting effects of detected phenolic compounds were evaluated with barnyardgrass (*Echinochloa crusgalli* L.) and monchoria (*Monochoria vaginalis* P), which are two major weeds in the rice field in Japan. water-soluble salicylic acid, syringic acid, syringaldehyde, vanillic acid, and ρ -hydroxybenzoic acid were dissolved in distilled water of the concentration at 125, 250, 500 and 1000ppm. Water-insoluble sinapic acid was dissolved in 0.625%, 1.25%, 2.5% and 5% methanol, to prepare 125, 250, 500 and 1000ppm solutions, respectively. Dormancy-broken weed seeds were surface-sterilized in 75% alcohol solution for 30 sec and washed well with distilled water. Then 30 healthy seeds were sown on one layer of filter paper (diam: 90mm, No.2, Toyo Roshi Kaisha Ltd., Japan) in each Petri dish (90mm-diam) containing 10ml test solution and kept in a growth chamber under 4000 lux artificial light (7:00–19:00) at 25°C. Germination percentage, lengths of shoot and root were measured 7d after sowing in barnyardgrass and 10d after sowing in monchoria. The control seeds were sown on the filter paper with distilled water instead of phenolic solution although the control seeds for sinapic acid treatment were treated with methanol solution at the concentration used for respective treatment. Fungal contamination was not observed throughout the experimental period All treatments were arranged in a completely random design with three replicates.

5. Statistical analysis

Variance analysis was performed for all data except germination percentages. Comparisons between treatments were made at a 0.05 probability level using Duncan's multiple range tests. Inhibition percentage (%) was calculated as [(control value-treatment value)/control value] \times 100.

Results

1. Phenolic allelopathic chemicals detected in dwarf lilyturf plant

HPLC analysis clearly showed the existence of at least six phenolic compounds (Figure 1), namely, salicylic acid, syringic acid, syringaldehyde, vanillic acid, ρ -hydroxybenzoic acid and sinapic acid in the methanol extracts from the power of dwarf lilyturf plant. The compound detected at the highest

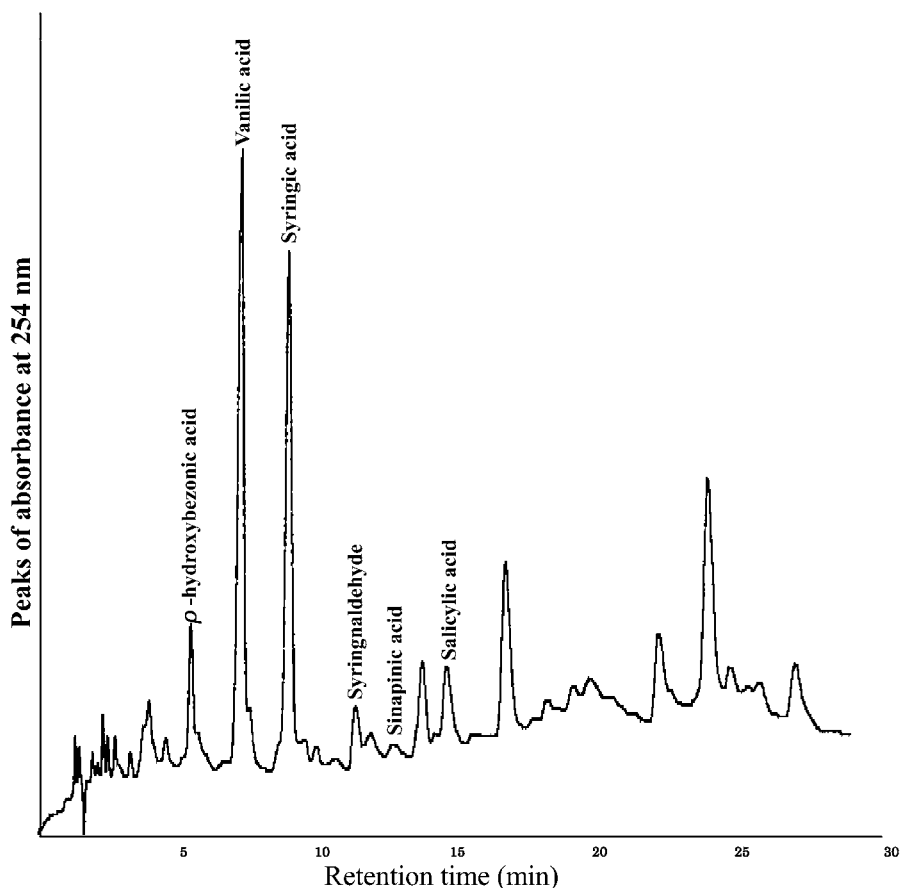


Fig. 1. Phenolic chemicals detected in methanol extracts of dwarf lilyturf separated by HPLC analysis.

Table 1. Contents ($\mu\text{g g}^{-1}$) of phenolic chemicals detected in dwarf lilyturf plant.

ρ -hydroxybenzoic acid	Salicylic acid	Sinapic acid	Syringic acid	Syringaldehyde	Vanillic acid	Total
2.80	251.04	11.03	37.30	13.30	1.69	317.16

concentration was salicylic acid ($251.04 \mu\text{g g}^{-1}$), which occupied more than half of total phenolic contents ($317.16 \mu\text{g g}^{-1}$) (Table 1). The concentrations of syringic acid, syringaldehyde, sinapic acid, ρ -hydroxybenzoic acid and vanillic acid, were $37.30 \mu\text{g g}^{-1}$, $13.30 \mu\text{g g}^{-1}$, $11.03 \mu\text{g g}^{-1}$, $2.80 \mu\text{g g}^{-1}$, $1.69 \mu\text{g g}^{-1}$, respectively. However, the HPLC analysis indicated the presence of a large number of unknown compounds (Figure 1), which need to be identified in further work.

2. Growth-inhibiting effects of detected phenolic compounds on two weeds in rice fields

The effects of five water-soluble phenolic compounds detected in dwarf lilyturf at concentrations of 125, 250, 500 and 1000ppm on the germination and initial growth of barnyardgrass (*Echinochloa crusgalli* L.) and monchoria (*Monochoria vaginalis* P.) are shown in Table 2. The effects of water-insoluble sinapic acid are shown in Fig.2.

(1) Barnyardgrass

All the six phenolic compounds at concentrations of 125, 250 and 500ppm had no or little inhibitory effects on germination of barnyardgrass except salicylic acid and sinapic acid which significantly inhibited the germinations at 250-500ppm and 500ppm, respectively. All the compounds at 1000ppm obviously hampered germinations, except ρ -hydroxybenzoic acid which allowed 91.7% germination even at 1000ppm. These results showed that salicylic acid had the most inhibitory effect on germination and sinapic acid (Figure 2) was the second most effective.

All compounds significantly inhibited the root elongation of barnyardgrass dose-dependently except for syringic acid and syringaldehyde at 125ppm and sinapic acid at 250ppm, which had rather stimulatory effects. Similarly, all phenolic compounds inhibited the shoot growth of barnyardgrass except sinapic acid at all concentrations tested and ρ -hydroxybenzoic acid at 250ppm, which had no inhibitory or little stimulatory

Table 2. Inhibitory effects of five water-soluble phenolic chemicals detected in dwarf lilyturf on germination and initial growth of barnyardgrass and monchoria.

Concentration (ppm)	Barnyardgrass			Monchoria		
	Germination (%)	Root length (cm)	Shoot length (cm)	Germination (%)	Root length (cm)	Shoot length (cm)
<i>salicylic acid</i>						
Control	100(0)	4.31(0)a	3.43(0)a	92.2(0)	0.81(0)a	0.74(0)a
125	100(0)	0.5(88.4)b	2.84(17.2)b	88.9(3.6)	0(100)b	0.21(71.6)b
250	23.4(76.6)	0(100)c	1.12(67.4)c	0(100)	0(100)b	0.15(79.7)c
500	0(100)	0(100)c	0(100)d	0(100)	0(100)b	0.11(85.1)c
1000	0(100)	0(100)c	0(100)d	0(100)	0(100)b	0(100)d
<i>syringic acid</i>						
Control	100(0)	4.31(0)a	3.43(0)a	92.2(0)	0.81(0)a	0.74(0)a
125	100(0)	4.83(-12.1)a	3.08(10.2)b	85.6(7.2)	0.04(95.1)b	0.47(36.5)b
250	98.4(1.6)	2.43(43.6)b	3.05(11.1)b	66.7(27.7)	0.01(98.8)b	0.32(56.8)c
500	91.7(8.3)	0.21(95.1)c	2.55(25.7)c	54.4(41.0)	0(100)c	0.11(85.1)d
1000	78.4(22.6)	0(100)c	1.46(57.4)d	50.0(45.8)	0(100)c	0.11(85.1)d
<i>p-hydroxybenzoic acid</i>						
Control	100(0)	4.31(0)a	3.43(0)a	92.2(0)	0.81(0)a	0.74(0)a
125	100(0)	4.21(2.3)a	3.41(0.6)a	86.7(6.0)	0.04(95.1)b	0.39(47.3)b
250	98.4(1.6)	3.24(24.8)b	3.59(-4.7)a	84.4(8.5)	0(100)c	0.22(70.3)c
500	95.0(5.0)	0.26(93.9)c	3.09(9.9)b	74.4(19.3)	0(100)c	0.12(83.8)d
1000	91.7(8.3)	0(100)c	1.97(42.6)c	52.2(43.4)	0(100)c	0.11(85.1)d
<i>vanillic acid</i>						
Control	100(0)	4.31(0)a	3.43(0)a	92.2(0)	0.81(0)a	0.74(0)a
125	100(0)	3.17(26.5)b	3.03(11.7)b	85.6(7.2)	0.03(96.3)b	0.28(62.2)b
250	100(0)	1.82(57.8)c	2.94(14.3)b	81.1(12.1)	0.01(98.8)b	0.13(82.4)c
500	88.4(11.6)	0.06(98.6)d	2.06(39.9)c	71.1(22.9)	0(100)c	0.12(83.8)c
1000	73.4(26.6)	0(100)d	1.26(63.3)d	54.4(41.0)	0(100)c	0.11(85.1)c
<i>syringaldehyde</i>						
Control	100(0)	4.31(0)a	3.43(0)a	92.2(0)	0.81(0)a	0.74(0)a
125	100(0)	4.83(-12.1)b	3.03(11.7)b	83.3(9.7)	0.19(76.5)b	0.63(14.9)b
250	95.0(5.0)	3.05(29.2)c	3.04(11.4)b	64.4(30.2)	0.04(95.1)c	0.34(54.1)c
500	80.0(20.0)	1.14(73.5)d	2.78(18.9)b	52.3(43.3)	0.01(98.8)c	0.18(75.7)d
1000	78.4(21.6)	0(100)e	1.92(44.1)c	0(100)	0(100)c	0.02(97.3)e

Numbers in parentheses are the inhibition percentage (%) compared with controls. Values with the same letters in a column are not significantly different at the 0.05 probability level determined by Duncan’s multiple range tests.

effects. Note that salicylic acid reduced the root growth by 88.4% at even 125ppm. Thus, salicylic acid exhibited the greatest inhibition on root and shoot growth of barnyardgrass, followed by vanillic acid. By contrast, sinapic acid had the weakest effect.

(2) Monchoria

All six phenolic compounds detected in dwarf lilyturf inhibited the germination of monchoria dose-dependently. Note that salicylic acid at 250ppm completely inhibited the germination of monchoria (100%) and sinapic acid at 125ppm inhibited the

germination to 52.4% of the control.

All compounds markedly inhibited the root and shoot growth of monchoria seedlings dose-dependently except sinapic acid at 125ppm and 250ppm, which promoted the root growth. Moreover, all phenolic compounds other than sinapic acid inhibited root growth more than 76.5% for root and more than 14.9% for shoot even at 125ppm.

Discussion

In this study, we detected at least six phenolic

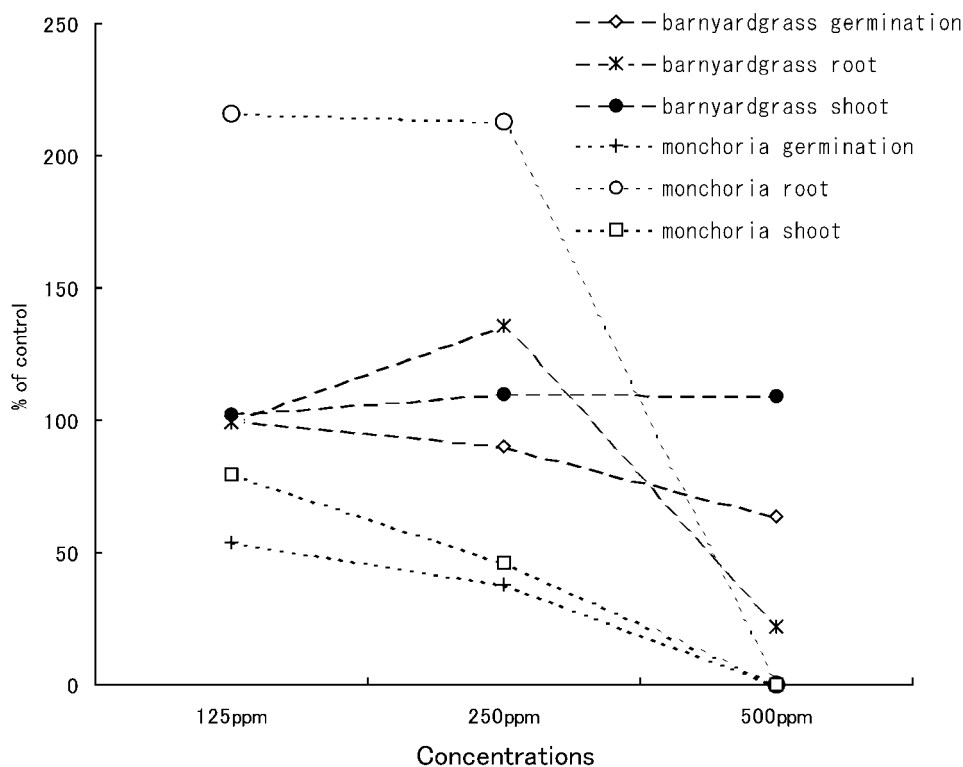


Fig 2. Effects of sinapic acid (water-insoluble) on germination and initial growth of barnyardgrass and monchoria. Data at 1000ppm could not be calculated because control solutions containing 5% methanol completely inhibited germination and initial growth of two weeds.

allelopathic chemicals, i.e., salicylic acid, syringic acid, syringaldehyde, vanillic acid, *p*-hydroxybenzoic acid and sinapic acid, in dwarf lilyturf plant by HPLC analysis. The compound detected at the highest concentration was salicylic acid ($251.04 \mu\text{g g}^{-1}$) which occupied more than half of total phenolic chemicals detected ($317.16 \mu\text{g g}^{-1}$), followed by syringic acid ($37.30 \mu\text{g g}^{-1}$), syringaldehyde ($13.30 \mu\text{g g}^{-1}$) and sinapic acid ($11.03 \mu\text{g g}^{-1}$). As a whole, salicylic acid displayed the greatest inhibitory effects on germinations and initial growth of both barnyardgrass (*Echinochloa crusgalli* L.) and monchoria (*Monochoria vaginalis* P.) among six phenolic compounds detected. Recently, Lin et al. (2003b) reported that dwarf lilyturf powder and its water extract inhibited emergence and growth of weed species in rice field and suggested that dwarf lilyturf powder can be used as natural herbicide for transplanted rice. It may be deduced that salicylic acid play a key role in inhibiting on weed growth and allelopathy judging from its strong inhibitory effect at low concentrations.

Salicylic acid, *p*-hydroxybenzoic acid and syringic acid at 10^{-3}M (Chung et al., 2001) and sinapic acid, vanillic acid, salicylic acid, *p*-hydroxybenzoic acid and syringic acid at even $10^{-3}\sim 10^{-5}\text{M}$ (Chung et al., 2002) markedly reduced the germination and seedling growth of barnyardgrass. Based on our results that phenolic chemicals present in dwarf lilyturf plant

markedly inhibited the growth of two major weeds in rice fields, natural phenolic chemicals isolated from allelopathic plants can be used to control weeds in rice. Although many researchers confirmed that numerous allelopathic phenolic chemicals inhibit the growth of some plants especially lettuce (*Lactuca sativa* L.) (Mizutani, 1999), information on the effect on the weeds in rice fields is limited. Thus, further studies on inhibitory or herbicidal effects of these phenolic chemicals on weeds in rice fields are needed.

In conclusion, this study showed that phenolic compounds especially salicylic acid present in dwarf lilyturf plants have inhibitory effects on the growth of on weeds in the rice field. Perhaps it is difficult to entirely control weeds in the rice field by a single allelopathic plant or allelopathic chemical because it might have a selective or limited inhibiting effect on weeds. Combining various allelopathic potentials of different plants may be necessary for complete control, and further screening and selection of plants with strong allelopathic effects on individual weeds in rice field are needed. In addition, it is necessary to elucidate whether there exist non-phenolic chemicals responsible for dwarf lilyturf allelopathy.

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