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Screening for Iranian rice allelopathic varieties by HPLC and bioassays

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ABSTRACT

The study on rice allelopathy is very recent in Iran, hence, till now the Iranian varieties with allelopathic potential have not been identified and categorized. The effects of hull water extracts was evaluated on the seedling growth of arrowhead (*Sagittaria trifolia*) weed. The extracts of varieties 'Dinorado' and 'Neda' were most suppressive to arrowhead seedling growth than other varieties. High performance liquid chromatography (HPLC) was used to analyze six phenolic acids in rice hull water extracts. Among the tested varieties, Dinorado variety hull extract had maximum phenolic acids content. None of the six phenolic acids were detected in extracts of Line-229 , while, Dashti-sard had highest (*p*)-hydroxy benzoic acid content. Of the 46 tested varieties, 10 were chosen and grown in hydroponics system to study effects of their root exudates on seedling growth of arrowhead weed. Root exudates of Dinorado, Neda and IR-60 showed higher inhibitory activities against arrowhead seedling growth than other tested varieties. Both hull water extracts and root exudates of Dinorado proved most inhibitory to arrowhead weed growth. From Iranian rice germplasm, Neda cultivar had high allelopathic potential against arrowhead weed.

Key Words: Allelopathy, arrowhead, Bioassay, HPLC, phenolic acids, rice (*Oryza sativa* L.), *Sagittaria trifolia*.

INTRODUCTION

Allelopathy is defined as any direct or indirect harmful or beneficial effect by one plant on another through the production of chemical compounds released into the environment (47). Rice (*Oryza sativa* L.) is major food crop in Asia but heavy use of herbicides and fungicides causes environmental problems in paddy ecosystem (7,29,41). Current allelopathy research is focussed on 3 grass specie crops; wheat, rice, and sorghum. Field experiments have shown economic benefits from allelopathic rice varieties in terms of yield under weed competition and less need for herbicides (45). The allelopathy accounted for 34% of competitive ability in rice and for varieties with high allelopathic potential, allelopathy is the dominant factor determining the competitive ability (43). The use of allelopathic rice is a hope for new weed management in sustainable agriculture (41). Arrowhead (*Sagittaria trifolia* L.) is most harmful broad-leaf weed in paddies of Iran.

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Many rice varieties inhibits the growth of several weeds such as barnyardgrass (*Echinochloa crusgalli* L.) in Lab bioassays or under field conditions (3,4,10,22,25,41,44). The rice crop releases large amount of root exudates in soil that may play a role in rhizosphere interactions. The roots exudates of allelopathic rice varieties contains the allelochemicals to suppress the growth of neighboring weeds (34). Studies of allelochemicals in rice plants have led to identification of a range of phenolic compounds, including (*p*)-hydroxybenzoic, vanillic, (*p*)-coumaric and ferulic acids (7,19,20,28,38,42,48,49,50), flavones, diterpenes and other types of compounds like momilactones A and B (23,24,27,32,35). Many relatively simple cinnamic acid derivatives (c6-c3- and c6-c1 compounds or phenyl-propanoids) are widespread in plants and soils. Among the best known of these compounds are ferulic acid, (*p*)-coumaric acid, sinapic acid and vannillic acid. These compounds are putative allelochemicals and commonly found in allelopathic plants in low concentration (54). They exert their effects through a series of generalized pathways. Various phenolic acids from rice plants have shown allelopathic activity in laboratory bioassays on different weeds (52). Although, some researchers reported valuable results from their greenhouse/field experiments (2,37), the study on rice allelopathy is very recent in Iran and Iranian varieties with allelopathic potential have not been identified and categorized yet. Hull's extract bioassay on weed germination and seedling growth parameters is a quick and low cost method suggested for screening allelopathic rice accessions (1). Mattice *et al.,* (39) suggested HPLC method for identifying the allelopathic rice accessions based on their results from different chromatograms of allelopathic and non-allelopathic rice leaf extracts (PI312777 and Rexmont, respectively). The main objective of this study was to evaluate the allelopathic potential of Iranian rice varieties in comparison to IRRI's approved allelopathic ones, by assessing the effect of (i). Hull water extracts on Arrowhead seedlings growth as a screening method and (ii). Selected rice varieties root exudates from hydroponics on Arrowhead seedling growth. We also wanted to investigate the possible involvement of six phenolic acids in rice allelopathy on the tested weed seedlings (by HPLC method).

MATERIALS AND METHODS

I. Hull water extract. Forty two rice varieties (commercial and gremplasm in Iran) and 4 allelopathic varieties from IRRI (International Rice Research Institute) were grown in a field experiment at the Rice Research Institute of Iran, Rasht, Guilan (37°, 12´N, and 49°, 38´E). Rice plants ear-heads were harvested, threshed and the hulls of each cultivar were separated from the plants and dried at room temperature (22°C), powdered using a milling machine (TEFAL, Prepline.850. France) to pass through 2 mm mesh and stored in freezer (GFL- 6483 Zusatz, Germany) at -70°C until needed. Water extracts were obtained using Ahn and Chung (1) methodology with slight modifications. Distilled water (500 ml) was added to 100 g powdered hull of each variety and stirred with incubator shaker (Infors AG CH- 4103, Bottmingen, Germany) for 24 h at 25°C and final volume was made 1000 ml (1:10 w/v dilution). The solutions were filtered through four layers of cheesecloth to remove fiber debris followed by filtering through one layer of filter paper (Whatman 42), to obtain stock solution. The solution was used to determine allelochemicals by HPLC.

II. Effects of hull extract: Arrowhead weed seeds were collected from the paddy fields of Guilan University, Rasht, Guilan. The seeds were collected after removing the trash and defective floating seeds on distilled water. Before the bioassay, seeds were surface sterilized in a 1:10 (v/v) dilution of commercial hypochlorite bleach for 10 min and rinsed several times with distilled water. The Arrowhead seeds were pre-germinated in a germinator. The germination was 46 %. Uniformly germinated seedlings (with 1 mm radicule length) were placed on paper (Whatman no. 42) in sterilized 9-cm Petri dishes (20 seedlings/Petri dish). From each cultivar's hull powder, two concentrations (5% and 10% w/v) of each rice cultivar's hull extract were prepared from stock solution. The pH and electrical conductivity of extracts were determined using a digital pH meter (SIBATA 6200) and a conductivity meter (LF 325 WTW, Germany). A treatment with distilled water only was included as control. After placing the seeds, each Petri dish was irrigated with 10 ml extracts as per treatments and then kept in controlled laboratory conditions (25/20 °C day/night temperature and 10 h light) for seven days. Afterwards, seedling root length, shoot length and fresh weight were measured. Roots and shoots were dissected and oven dried at 65°C for 4 h and then weighed (1). All bioassays were repeated twice and all the measurements were expressed as percentage of control treatment, [(Aqueous extracts-Control)/Control] × 100, (1). In addition, the following equation was used to obtain an average estimation of the effects on seedling shoot and root growth:

$$\text{Average effect on shoot (or root)} = \frac{\text{Length} + \text{Dry weight} + \text{Fresh weight}}{3}$$

Where, length, dry weight and fresh weight are expressed as percentage inhibition/stimulation of control. Average seedling growth inhibition/stimulation was also obtained by calculating the average effects on shoots + roots.

III. Effects of rice root exudates: Ten rice varieties (Dashti-sard, IR-60, Musatarom, Amol-2, Alikazemi, USEN, Line-229, Neda, Sepidrood, and Dinorado) were chosen as representative of all varieties included in first bioassay study, according to their inhibitory/stimulation effects. Seeds of each cultivar were surface sterilized as mentioned in first bioassay study, then allowed to germinate on a sheet of moist filter paper (Whatman no. 42) in 9-cm Petri dishes at 25 °C with 12-h photoperiod in a growth chamber for 4 days. For each variety, uniformly grown rice seedlings (with 1 mm radicule length) were selected and grouped into collections of 100 seedling for transferring onto a sheet of plasto-foam (11.5×19.5 cm) which was allowed to float on distilled water (1000 ml) inside a PVC container (12×20×7 cm). The container was placed in a room with natural condition (with 25/20 °C day/night temperature and 10 hours daily light). This method is adapted from Kato-Noguchi and Ino (26) with a slight modification. Container water height was kept constant by adding distilled water daily. The root system of rice seedlings were allowed to grow inside water. After 15 days, rice seedlings were harvested and the water collected was used in bioassay and for determining the allelochemicals by HPLC. Arrowhead seeds were collected, surface sterilized and pre-germinated in a germinator as described for first bioassay study. Twenty uniformly germinated seedlings (with 1 mm radicule length) were placed on filter paper (Whatman no. 42) in each sterilized 9-cm Petri dish (20 seedlings/Petri dish). A treatment with distilled water was used as control. Each Petri dish was treated with 10 ml water collected from one of the rice varieties hydroponics. Petri dishes were kept in laboratory at (25/20 °C day/night temperature and 10 h light). Ten days after incubation, the seedling root and shoot length, and fresh weight were measured. Roots and shoots were dissected and oven dried at 65°C for 4 h (1), then weighed. The bioassay experiment was repeated twice and all the measurements were expressed as percentage of control treatment. Equations were the same for both bioassay experiments.

Statistical Analysis*:* A completely randomized design with four replications, in a factorial arrangement with two factors was used for first bioassay study. The factors included (i). 46 rice varieties and (ii). three concentrations (0, 5 and 10% w/v) of rice hull extracts. A completely randomized design with four replications also was used for the second bioassay study. The experimental unit was a single Petri dish. Analysis of variance for the effects of rice hull extracts and root exudates on seedling growth parameters of arrowhead was performed using the general linear model procedure of the statistical analysis system (SAS) program. Mean values were separated based on high significant difference (Tukey's HSD) at 0.01 and 0.05% Correlations were carried out by SPSS software (version 11.9).

Quantification of phenolic acids.

HPLC analysis was conducted on extract stock solutions obtained from the powdered hull, and on samples of water collected after harvesting rice seedlings in second bioassay study. A Perkin-Elmer model Lambda 25 (U.S.A), double beam UV/Vis spectrophotometer equipped with 10 mm matched silica cells was used for analytes spectra recording. A chromatographic system consisted of an Agilent Technologies 1100 series HPLC (U.S.A), equipped with a 20 µl sample loop, degasser, quaternary pump, column oven and diode-array detector, were used for phenolic acids analysis. Chromatographic separations were carried out on an Agilent ZORBAX C18 column (250 × 4.6 mm, i.d. 5 µm) at 25 ºC. Chemstation software was used for data processing. Analytical grade cinnamic acid, vanilic acid, ferulic acid, (*p*)-coumaric acid, (*m*)-coumaric acid, (*p*)-hydroxybenzoic and ortho phosphoric acid were purchased from E. Merck (Germany) for preparation of standards. HPLC grade methanol, acetonitrile and water were obtained from CALEDON Chemicals (Canada). All samples were filtered using 0.42 µm millipore filters before injection and 20 µl of each was injected directly to HPLC system. Stock solutions of phenolic acid standards (1000 µg ml^{-1}) were prepared by dissolving appropriate amounts of analytes in methanol, placed in dark bottles and stored in a refrigerator at 4 ºC. A gradient elution was used in this study. The initial mobile phase composition was a mixture of methanol, acetonitrile and 10 mM ortho-phosphoric acid solution (7:11:82 %V/V) for 8 min, then the mobile phase composition changed to (9:11:80 %V/V) and eluted the column for 13 min and in final step changed to (50:11:39 %V/V) for 10 min. Flow rate of mobile phase was 1.5 ml min^{-1} and diode array detector was set at 220 nm for vanilic acid, 254 nm for (*p*)-hydroxybenzoic acid, 274 nm for both of cinnamic and (*m*)-coumaric acids and 314 nm for both of (*p*)-coumaric and ferulic acids. Hull aqueous extracts of 15 rice varieties and root exudates of 5 varieties from hydroponics were injected to HPLC system as representatives regarding to their allelopathic potential in both bioassay experiments.

RESULTS AND DISCUSSION

Rice hull extracts.

The differences between the effects of rice hull extract concentrations and rice varieties and their interactions on arrowhead seedling growth were highly significant in all traits, except the effects of extract concentrations on average seedling shoot growth inhibition, shoot dry weight and shoot length (Table 1). The hull extracts from different rice varieties exhibited different allelopathic potential on arrowhead seedling growth (Table 2). Since the mean values were separated based on the high significant difference (Tukey's HSD), and according to interaction effects between two factors, Dinorado and Neda hull extracts, inhibited the seedling growth of arrowhead at both concentrations than other varieties (Table 2). Rice hull extracts were more inhibitory to root growth of arrowhead seedlings than shoot growth (Table 2). Neither pH nor electrical conductivity of rice hull extracts had meaningful correlations with arrowhead seedlings growth (r= -.059 and .07 for pH and EC, respectively.

Rice root exudates.

The water containing the root exudates inhibited as well as stimulated the seedling growth of arrowhead weed (Table 3). The effects of root exudates from the different rice varieties were highly significant in all traits. Although Line-229 root exudates did not affect the seedlings shoot fresh weight and dry weight but other growth parameters were stimulated (12.5-49 % of control). The Dinorado, Neda and IR-60 root exudate significantly inhibited (-63.2, -58.8, and -51.8 %) the seedling growth, respectively) (Table 3). Musatarum, Amol-2, Sepidrood and Alikazemi root exudates had little affect on growth of arrowhead seedlings. The exudate caused more inhibition in root growth than in shoot growth. However the pH of rice varieties root exudates did not influence the arrowhead seedling growth (r = 0.251).

Quantification of Phenolic Acids. After optimization of the chromatographic conditions, various concentrations of analyte standards were injected to HPLC for obtaining calibration curves. Retention times of analytes are summarized in Table 4. Dinorado hull extract had the highest phenolic acid content (97.461 µg/g) among 15 studied varieties (Table 4). None of the six phenolic acids were detected in hull extract of Line-229, while, Dashti-sard had the highest content of (*p*)-hydroxy benzoic acid (Table 4). The water containing root exudates of all 5 tested varieties hydroponics did not contain vanilic and ferulic acids (Table 5). Usen, Dashtisard and Neda root exudates had higher amounts of phenolic acids among tested varieties (Table 5). The exudated cinnamic acid quantity from line-229 roots was negligible, while the other five phenolics were not detected by HPLC. Vanilic and ferrulic acids were not detected in both root exudates and hull extracts of Dashtisard and Neda. A significant negative relationship was found between the total phenolic acid content of rice hull extracts from HPLC and the average arrowhead seedling growth treated with hull extracts (Fig. 1). Correlation between total phenolic content (µg/g) of rice hull extracts and arrowhead seedlings mean root growth inhibition is shown in Fig. 2 (r= 0.597*).

Table 1. Analysis of variance for rice hull extracts on arrowhead seedlings growth traits

		MS										
S.O.V	df	RFW	SFW	SDW	RDW	RL	SL	TL	TDW	TFW	AR	AS
Variety (A)	45	8.01**	71.7**	37.8**	8.74**	28.4**	57.7**	38.2**	23**	34.2**	19.5**	84.3**
Concentration (B)	1	49.9**	15.3**	0.52ns	16.6**	413**	3.58ns	141**	9.32**	49.5**	210**	0.72ns
AB	45	1.51*	7.01**	1.84**	1.83**	3.9**	3.42**	2.93**	1.56*	3.94**	2.61**	4.01**
E	276											
CV		2.67	11.47	9.91	5.78	7.17	16.2	27.9	5.56	3.46	3.27	22.11

**Highly significant at P<0.01 level, * Significant difference at P<0.05 level, ns= not significant, R= root, S= shoot, F= fresh, D= dry, W= weight, L= length, T= total, AR= average root growth, AS= average shoot growth.

Table 3. Effects of root exudates of 10 rice varieties on the seedling growth of arrowhead over the control.

Treatment	RL	SL	RFW	SFW	RDW	SDW	TL	TFW	TDW	ARG	ASG	AG
Dinorado	-83.0	-43.1	-68.0	-59.7	-56.8	-68.6	-63.0	-63.9	-62.7	-69.3	-57.1	-63.2
Neda	-81.5	-42.5	-54.3	-49.2	-62.5	-63.0	-62.0	-51.8	-62.8	-66.1	-51.6	-58.8
IR-60	-71.8	-26.8	-49.3	-63.0	-56.8	-42.8	-49.3	-56.1	-49.8	-59.3	-44.2	-51.8
Dashtisard	-26.5	-10.5	-66.8	-6.8	-51.5	3.0	-18.5	-36.8	-24.3	-48.3	-4.8	-26.5
USEN	-51.6	-29.3	-39.0	-30.0	-28.1	-28.8	-40.4	-34.5	-28.4	-39.6	-29.4	-34.5
Line-229	49.0	14.8	23.8	5.8	15.5	9.6	31.9	14.8	12.5	29.4	10.1	19.8
Musatarom	5.5	7.0	-15.8	7.5	-13.5	6.3	6.3	-4.1	-3.6	-7.9	6.9	-0.5
Amol-2	1.8	9.5	-29.0	5.8	-22.0	13.0	5.6	-11.6	-4.5	-16.4	9.4	-3.5
Sepidrood	1.8	10.5	-34.3	6.3	-27.3	1.5	6.1	-14.0	-12.9	-19.9	6.1	-6.9
Alikazemi	4.8	7.0	-21.0	6.3	-16.0	5.8	5.9	-7.4	-5.1	-10.7	6.4	-2.2
MST	727**	263**	73.9**	107**	102**	121**	1069**	103**	208**	342**	328**	340**
C.V	9.55	8.19	5.05	17.17	5.1	17.45	5.29	6.18	5.64	4.02	7.67	5.89
HSD	8.03	6.85	15.34	13.85	11.79	13.82	5	12.28	8.83	11.7	10	10.9

**Highly significant at P<0.01 level, RL= Root Length, SL= Shoot Length, RFW= Root Fresh Weight, SFW= Shoot Fresh Weight, RDW= Root Dry Weight, SDW= Shoot Dry Weight, TL= Total Length, TFW= Total Fresh Weight, TDW= Total Dry Weight, ARG= Average Root Growth, ASG= Average Shoot Growth, AG= Average growth., (-) indicates the inhibitory effect.

Table 2. Effects of rice hull extracts on the seedling growth of arrowhead as compared to the control

Variety	Conc. (%)	SL (%)	TDW (%)	Variety	Conc. (%)	SL (%)	TDW (%)
*Dinorado	5	-58.7	-51.1	Sangtarom	5	-3.6	-40.2
	10	-80.6	-70.4		10	-14	-34
Neda	5	-48.3	-59.2	IR-3441	5	-5.8	-34.1
	10	-52.3	-65.1		10	-12.8	-38.4
Mehr	5	-32.8	-28.8	Dasht	5	-5.5	-35.5
	10	-43.9	-34.7		10	-12.7	-43.7
*IR-60	5	-32.9	-57.4	IR-28	5	-7.9	-34.6
	10	-42.5	-56		10	-12.6	-42.9
Dashtisard	5	-13.7	-41.1	Zenit	5	-25.8	-43.8
	10	-41.3	-56.9		10	-12.4	-38.9
*Dular	5	-25.8	-47.1	Amol1	5	-2.3	-27.2
	10	-34.7	-48.5		10	-11	-33.6
IR-29	5	-33.4	-44.9	Musatarom	5	-1.3	-13.6
	10	-33.7	-55.5		10	-10.7	-13.6
IR-67	5	-12.9	-24.5	Sangju	5	-5.2	-11.5
	10	-33.5	-28.3		10	-10.5	-17.6
IR-36	5	-27.1	-42.8	Binam	5	4.4	-34.6
	10	-27.5	-48.8		10	-10	-34.3
Domzard	5	-7.4	-31.2	Bejar	5	-9.8	-49.4
	10	-25.2	-32.5		10	-6.7	-46.8
Kadus	5	-12.7	-46.9	*Taichung	5	-7.5	-48.7
	10	-24.7	-52.7		10	-4.4	-52.9
Line-213	5	-11.9	-46.9	Hasansarae1	5	-7.5	-43.5
	10	-24.5	-43.5		10	-21.1	-44.8
Amol3	5	-21	-32.2	Shahpasand	5	-7.5	-27.6
	10	-24.3	-28.7		10	-0.2	-29.9
Hasani	5	-15.3	-34.6	Alikazemi	5	-7.3	-28.1
	10	-23.1	-23.9		10	9.8	-38.6
Dorfak	5	-15.6	-37.1	IR-30	5	-4.7	-8
	10	-21.4	-38.9		10	0.1	-21.3
Khazar	5	4.2	-31.2	Champa	5	-3.3	-24.2
	10	-18.2	-35.9		10	2.9	-23.5
Gharib	5	2.3	-41	FAON-110	5	-3	-39.6
	10	-18	-42.1		10	8.8	-38
Nemat	5	7.9	-27.3	Amol2	5	-2.1	-37.1
	10	-17.2	-25.6		10	2.7	-35.3
Nemat	5	-5.1	-45.8	Sepidrood	5	0.1	-39.7
	10	-16.2	-43.9		10	2.6	-38.7
Gill1	5	-1.6	-28.5	Domsorkh	5	2.5	-31.6
	10	-16.1	-26.8		10	11.9	-22
NP-125	5	-8.3	-36	Hasansarae2	5	4.4	-43.6
	10	-15.7	-34.1		10	-1.1	-39
Anbarboo	5	-10.8	-47.8	Hashemi	5	6.3	-38.3
	10	-15.1	-54		10	6.5	-37.9
USEN	5	-2.8	-15.6	Line-229	5	6.4	-17.2
	10	-14.1	-17.3		10	29.3	-23.6
Cv		27.9	5.56			27.9	5.56
HSD for A (0.05)		14.3	13.7			14.3	13.7
HSD for B (0.05)		21.92	21.04			21.92	21.04

*Allelopathic varieties suggested by IRRI, (-) indicates the inhibitory effect. A= Cultivars, B= Concentration, SL= shoot length, TDW= total dry weight

Table 4. Rice hull extracts, phenolic contents (µg/g) and retention times under proposed conditions (min)

Cultivars	Phenolic compounds						
	P-hydroxy benzoic acid	Vanilic acid	P-coumaric acid	Ferulic acid	m-Coumaric acid	Cinnamic acid	Total
Dinorado	8.64	9.28	27.37	13.25	14.92	24.001	97.461
Sepidrood	1.74	1.58	6.41	2.87	3.64	5.24	21.48
Taichung	2.66	3.85	13.099	4.55	5.57	7.9	37.629
Dasht	2.882	2.71	24	4.64	4.87	5.93	45.032
Alikazemi	0.704	0.345	1.74	2.69	1.138	1.543	8.16
Usen	1.166	ND	5.251	1.654	2.85	4.19	15.111
Mehr	20.5	ND	2.282	ND	1.468	2.4	26.65
Neda	0.39	ND	0.64	ND	0.73	1.791	3.551
Anbarboo	0.614	ND	3.1	ND	0.92	1.135	5.769
Dular	0.25	ND	1.89	ND	0.67	0.9	3.71
Line-229	ND	ND	ND	ND	ND	ND	0
Dashti sard	38.2	ND	6.65	ND	0.31	1.76	46.92
Domsorkh	1.3	ND	9.24	ND	1.45	2.7	14.69
Musatarom	ND	ND	9.01	ND	ND	0.699	9.709
Amol-2	25.2	ND	7.11	ND	ND	1.53	33.84

ND= not detected, Retention times (min); P-hydroxy benzoic acid (5.47), Vanilic acid (6.51), P-coumaric acid (12.20), Ferulic acid (15.17), m-coumaric acid (18.40), Cinnamic acid (25.13).

Table 5. Concentrations of six phenolic acids in root exudates of rice (µg /l)

Cultivars	P-hydroxy benzoic acid	Vanilic acid	P-coumaric acid	Ferulic acid	m-Coumaric acid	Cinnamic acid	Total
Dashtisard	33.4	ND	101.3	ND	51.9	109.3	295.9
Usen	56.7	ND	149	ND	106.4	154.6	466.7
Line-229	ND	ND	ND	ND	ND	21.2	21.2
Neda	39.3	ND	97.4	ND	52.8	65.6	255.1
Alikazemi	19.6	ND	75.8	ND	42.5	45.1	183

ND= Not detected.

The hull extracts from tested rice varieties had variable effects on the arrowhead seedlings growth. These allelopathic potentials among rice germplasm and organs (1,7,8,9,10,11,13,16,21,30,40,42,51) may provide the genetic sources for selection of crop varieties with high allelopathic ability (53). Significant concentration differences among the rice hull extracts indicated that higher inhibitory activity depends on higher concentration (Table 1). The phenolic compounds interact with cell memberanes, alter ion fluxes and reduce the hydraulic conductivity and net nutrient uptake by roots (46).

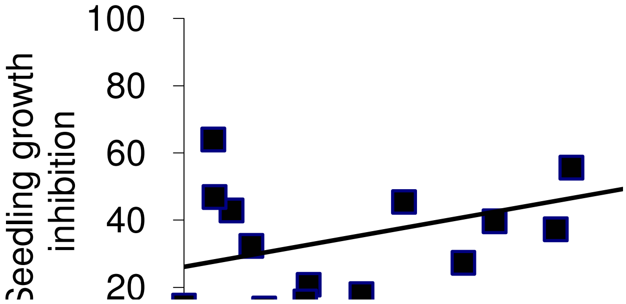

Figure 1. Correlation between total phenolic content (µg/g) of rice hull extracts and arrowhead seedlings growth inhibition over control.

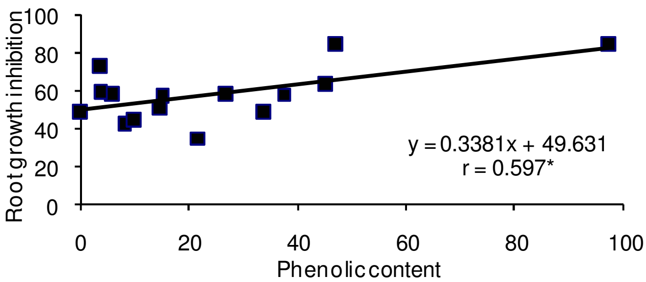

Figure 2. Correlation between total phenolic content (µg/g) of rice hull extracts and arrowhead seedlings average root growth inhibition over control.

Line-229 hull extracts and root exudates did not have detectable amounts of six analyzed phenolic acids, except 21.2 µg /l of cinnamic acid in root exudates. These results are in agreement with Ahn and Chung (1) and Rice (47) who found both stimulatory and inhibitory effects of allelopathic substances at low and high concentrations, respectively. Recent research suggests that allelopathic effects can be both positive and negative, depending upon the dose and organism affected (5). Allelopathic rice varieties hull extracts had more effects on the roots than on shoots of arrowhead seedlings. This may be due to the direct contact of root system to the extract solution in growth media. Significant differences between the effects of root exudate on arrowhead seedling growth parameters also indicate the variation in allelopathic potential of rice varieties. This result is in agreement with Dilday *et al.* (12), Chung *et al.* (6), Olofsdotter *et al.* (40), and Ahn and Chung, (1). HPLC studies detected little amounts of phenolic acid in rice root exudates. Phenolic inhibitory actions depend on their concentration (15). It has been suggested that allelopathy usually occurs at early growth stages (3 to 5-leaf stages) of plant species. Rice can release allelochemicals from its roots in to the paddy soil in early growth stages. These allelochemicals include phenolic acids, flavones, and terpenoids (34). The method that was used in the second bioassay study was adapted from Kato-Noguchi and Ino (26) designed especially to trap and assay Momilactones from 15-days old rice seedlings and might be not appropriate for determining the concentrations of phenolics, because of weak production and release of these compounds in this growth stage. Although detected phenolic acids did not have a significant correlation with results of root exudates bioassay, differences was observed in content and quantity of phenolics in chromatograms of tested rice varieties. He *et al.,* (18) studied the root exudates of two rice accessions (allelopathic rice PI312777 and non-allelopathic rice Lemont), and detected the ether extracts of the root exudates by GC-MS. They found differences in quantity, content and chemical composition between these two rice accessions. Olofsdotter *et al.,* (42) reported that phenolic acids are unlikely primary allelochemicals in rice. However, if a mixture of compounds is responsible for the observed growth inhibition of weeds, phenolic acids such as (*p*)-hydroxybenzoic acid could play a role in such a mixture. Seal *et al.,* (49) quantified the contents of 15 compounds from rice root exudates using GC/MS/MS and found that six of the seven most abundant compounds isolated were phenolic acids. Large amounts of 3 compounds [Trans-ferulic acid, (*p*)-hydrobenzoic acid and caffeic acids] of the 6 compounds, were detected in the exudates of allelopathic rice varieties. Further they determined that 5 phenolics [caffeic, (*p*)-coumaric, (*p*)-hydrobenzoic, syringic and vanillic acids] from the rice exudates were best correlated with the observed allelopathic effect on arrowhead (*Sagittaria montevidensis*) root growth with a multiple regression analysis (50). Despite the positive correlation of phenolic acids, they reported similar results as Olofsdotter *et al.,* (42) that the amount of phenolic compounds quantified in the exudates was much lower than the required threshold concentration for arrowhead inhibition. Since the hull extracts and root exudates of Neda showed high suppressing effect on arrowhead (studies 1 and 2) and barnyardgrass seedlings (3,4), this variety was introduced as a Iranian rice with high allelopathic potential among tested varieties.

We compared allelopatic potential of rice hull extracts derived from arrowhead seedlings bioassay with the results obtained from high performance liquid chromatograms. A strong correlation between the two procedures, on tested rice varieties can provide a reliable technique to use either of them. In this study, the highest amounts of phenolic acids were found in Dinorado hull extracts, which was similar to the hull extract inhibitory activities on arrowhead seedlings growth in laboratory bioassay. A significant negative relation was found between the total phenolic acid content of rice hull extracts and arrowhead average seedling growth (Fig. 1) treated with hull extracts, that resulted from significant correlation of three phenolic acids; vanilic acid ($r = -0.536^*$), (*m*)-coumaric acid ($r = -0.534^*$) and cinnamic acid ($r = -0.551^*$). The other three analyzed phenolic acids in detected concentrations did not have significant correlation with seedling growth traits. It seems that significant effect on average root growth only happens when a mixture of phenolics occur together, causing such a correlation. These compounds appear as a mixture and not a single substance and the contribution of phenolic compounds to allelopathy is probably never due to a single substance (15). The mixtures of allelochemicals have stronger inhibitory activity than individual compounds (33) and the interaction among the compounds resulted in a variable allelopathic effect, which ranged from stimulation to inhibition (18). Macias *et al.* (36) found that the activity depends on the lack of a hydroxyl group at C-2 position by correlating the phytotoxic activity of some benzoxazinones with their chemical structures (14). The results from the root exudates bioassay of different rice varieties did not have any significant correlation with their phenolic acid contents, probably because of the low concentration of six detected phenolics. Significant correlation at P<0.05 level (r=0.854**) between the results from the first bioassay study (Arrowhead seedlings treated with hull extracts) and the second bioassay study (Treated with root exudates) hull extracts and root exudates of allelopathic rice varieties suppressed the growth of arrowhead (Fig. 3).

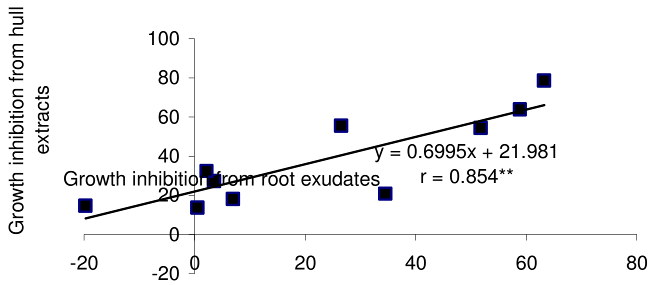

Figure 3. Correlation between results from the first bioassay study (Seedlings treated with hull extracts) and the second bioassay study (Treated with root exudates). (-) indicates the stimulation over control.

The phenolics may well be working together with much lower concentrations of other more phytotoxic substances passed over unnoticed in the usual complex mixture isolated from plant parts or exudates (48). Allelopathic activities of rice seem to be derived from the joint action of momilactones with phenolic compounds (17). Recent studies (23,24,25) have shown that the trace compound momilactone B is probably the true rice allelochemical, perhaps assisted by phenolic action. The natural activity level of momilactone B is as low as 3 micromolar. The recent studies also suggested that all rice varieties may produce momilactone A and secrete momilactone A into the culture solutions (27). In this case, it seems that the key factor of allelopathy in rice is still a mixture of phenolic compounds and momilactone B, because their variation in quality and quantity in various rice varieties has been verified. Insertion of two specific genes such as CA4H for (*p*)-coumaric acid and OsDTS2 for momilactone into one commercial rice cultivar by biotechnology will lead to the development of an allelopathic rice cultivar (31).

Further studies on the genetic control of rice allelopathy, the complete array of responsible chemicals in rice root exudates and their mode of action will shed more light on developing an allelopathic rice crop (31). It is possible that there are still other unknown allelochemicals in rice plants, especially in a few allelopathic rice varieties of numerous rice germplasm collections (8,12,32,40). At present, much remains unknown about the fate or persistence of allelochemicals in the soil or their effects on soil chemistry or micro-flora (45). Alleopathy alone is not likely to replace other weed control methods, but it surely functions as a component of integrated weed management technology.

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