Plant Omics Journal POJ

POJ 3(6):204-209 (2010) ISSN:1836-3644

Quantification of allelopathic substances and inhibitory potential in root exudates of rice (*Oryza sativa*) varieties on Barnyardgrass (*Echinochloa crus-galli* L.)

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Abstract

Present study was conducted to analyze six phenolic acids by HPLC method in three rice cultivars root exudates (Dollar, IR60 and Dashti sard). The selected cultivars were grown in hydroponic system to be qualified and quantified by HPLC method. The inhibitory effects of exudates on barnyardgrass were also investigated. Among the studied cultivars IR60 had the highest phenolic acid content (9.078 mg/l), meanwhile vanillic and *p*- coumaric acid only detected in IR60 (0.04 and 8.83 respectively), whereas, ferulic acid has not detected among cultivars. Overall morphological traits (root, shoot and total lengths, total fresh and dry weights and germination rate and percentage), IR60 exudates showed the higher suppressive activity in comparison other two cultivars. Also exudates of Dashti sard showed the least inhibitory effect on all mentioned traits. The nitrogen content of barnyardgrass seedlings which was treated with exudates was measured by Total Kjeldahl Nitrogen (TKN) method. These results suggest that, vanillic acid and *p*-coumaric acid could play the key roles as allelochemicals in rice exudates.

Keywords: Barnyardgrass, HPLC, Inhibitory, Nitrogen content, Rice

Abbreviations: RL, Root length; SL, Shoot length; TL, Total length; TFW, Total fresh weight; TDW, Total dry weight; GR, Germination rate; GP, Germination percentage; NC, Nitrogen content; P-h, *P*- hydroxy benzoic acid; V, Vanilic acid; P-c, *P*-coumaric acid; F, Ferulic acid; M-c, *M*-coumaric acid; C, Cinnamic acid HPLC, High performance liquid chromatography; EC, Electrical conductivity.

Introduction

Rice (*Oryza sativa* L.) is one of the most important crops in the world and weeds are the most significant biological constraint to rice production (Kato-Naguchi and Ino, 2001). Its production is characterized by heavy use of herbicides and fungicides, which may cause environmental problems in the paddy ecosystem (Chung et al., 2001; Kim and Kim, 2000), and human health problems, make it necessary to diversify weed management options. Barnyardgrass (*Echinochloa crus-galli*) is a widely distributed weed and the world's main weed of rice and several cases of resistance to herbicides with different modes of action have been achieved for this plant (Rahman et al., 2010). Thus, the best way to control barnyardgrass in an environmentally acceptable and sustainable approach is to develop natural compounds like allelochemicals (Olofsdotter, 1998). Allelopathy was defined as any process involving secondary metabolites produced by plant, algae, bacteria, and fungi that influences the growth and development of agriculture and biological system (International Allelopathy Society, 1993) or sometimes the substances which originated from plant could play as a crop protection role, Ambang et al. (2010) suggested that crude extracts from *Thevetia peruviana* seeds are efficient biocide substances with antifungal activity. On other hand weeds could be also controlled by using crops, which have been developed to exert their own weed control by releasing chemicals into the soil. Further, more crops have an allelopathic activity on weeds, like wheat, maize, sorghum, barely, sunflower and rice. Wu et al. (1999) evaluated wheat seedling allelopathy against annual ryegrass (*Lolium rigidum*) in a collection of 453 wheat accessions originating from 50 countries. The effectiveness of different accessions in their ability to inhibit root growth of ryegrass was ranged from 10% to 91%. Wu et al. (2001) have implicated 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one as an important allelochemical in wheat. Kato-Noguchi (1999) found six substances with inhibitory activity in the acetone extract of germinating maize seedlings. One of these substances, identified as 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) was higher in light-grown maize than in dark grown. Roth et al. (2000) found that prompt tillage of the mature sorghum stalks delayed development of the following wheat crop but did not affect grain yields, probably because allelopathic compounds degraded in the soil. Minorsky (2002) stated that barley (*Hordeum vulgare*) should be considered as a depressive prior crop for both durum wheat and bread wheat in a field cropping sequence. Moreover, sunflower (*Helianthus annuus*) showed effective weeds suppression and strong inhibitory effects on physiological processes of test plants (Bernat et al., 2004). Secondary plant metabolites such as terpenoids, steroids, phenols, coumarins, flavonoids, tannins, alkaloids, and cyanogenic glycosides, and their degradation products have been known to be involved in allelopathic phenomena, and are important in all agroecosystems (Seigler, 1996). These compounds could play a valuable role in an integrated weed management system, potentially reducing the amount of synthetic herbicides required for weed control (Seal

et al., 2004). Among these substances, phenolic compounds are the most widely studied with regard to their phytotoxicity (Putnam and Tang, 1986). He et al. (2004) 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 with GC-MS. The observed chemicals included of: Terpenoids, phenols or quinones, aldehydes or ketones, heterocycles, alcohols, ethers, and hydrocarbons. There were differences in quantity, content and chemical composition between the two rice accessions. They suggested that the interaction among the compounds resulted in a variable allelopathic effect, which ranged from enhancement to inhibition. The specific objectives of this study were to evaluate the allelochemicals rate in exudates from rice cultivars and inhibitory effects of these exudates on barnyardgrass seed and seedlings properties.

Materials and methods

Collecting rice root exudation (Hydroponics culture)

The mature seeds of three rice cultivars (Dollar, IR60 and Dashti sard) collected according to the results of previous studies (Asghari et al., 2006) from Rice Research Institutes of Iran in 2009 (RRII, Rasht, Guilan). The pure seeds were collected after removing the trash and defected seeds by floating them in distilled water. The 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. Then seeds were allowed to imbibe on moistened paper towels for 2 h. Filter paper (Whatman No. 42) containing 100 seeds were placed in sterilized 9 cm Petri dishes at 25ºC with 12 h photoperiod in a germinator (Iran khodsaz, IKHRH, Iran) for germination. For each cultivar, 100 uniformly grown rice seedlings (With 2 mm radical length) were selected and transferred into a sheet of plastofoam (35.5 × 45.5 cm) which was allowed to float on distilled water (24l) inside a PVC container (36 × 56 × 20 height cm). Also for oxygen requirements, air pumps (Resun Ac-9906, China) prepared for each container. The container was placed in growth chamber (With 27/20°C day/night temperature, 70% RH and 14000 lux light intensity). This method is adapted from Kato- Noguchi and Ino (2005) with a slight modification. The seedlings in each container were nourished by Yoshida (1981) rice nutrient solution every five days until harvesting time. pH and EC were kept at 5.5 and 2.0 dS/m respectively. After 35 days of germination the water of each container was collected separately to be used in qualification allelochemicals by HPLC method and for bioassay experiment.

HPLC instrumentation for Quantification of phenolic acids in root exudates

HPLC analyses were conducted on samples of water collected after harvesting rice seedling of cultivars. A Perkin-Elmer model Lambda 25 (USA), double beam UV/Vis spectro-photometer equipped with 10 mm matched silica cells was used for analytes spectra recording. A chromatographic system consisted of an Agilent Technologies 1100 series HPLC (USA), 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 25ºC. Chemstation software was used for data processing. Analytical grade cinnamic acid, vanilic acid, ferulic acid, *p*-coumaric acid, *m*-coumaric acid, *p*-hydroxybenzoic and 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 4ºC. A gradient elution was used in this study. The initial mobile phase composition was a mixture of methanol, acetonitrile and 10 mM orthophosphoric 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. Root exudates of three cultivars from hydroponics were injected to HPLC system.

Table 1. Retention times of phenolic acids under proposed experimental conditions.

Analyte	Retention time (min)
p-hydroxybenzoic 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

Seed preparations

Barnyardgrass *(Echinochloa crus-galli* L.*)* seeds were collected from paddy fields. Before the bioassay, the pure seeds were collected and defected by floating them in distilled water. The 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. Then seeds were allowed to imbibe on moistened paper towels for 2 h. Filter paper (Whatman No. 42) containing 100 seeds were placed in sterilized 9 cm Petri dishes. Ten millilitres of solution (rice root exudates) was added to each Petri dish and distilled water was used as a control. All Petri dishes were placed in a germinator (with 27/20°C day/night temperature and 70% RH). Rate of germination was calculated by dividing the number of germinated seeds in each day by the number of days and pluralize the values. Also germination percentage was determined (By counting of germinated seeds at the end of seventh day and subtracted from whole seeds), the inhibition percentage determined as follows (equation 1): Inhibition percentage (%) = [Sample extracts – Control)/Control] × 100 [Equation 1]

Seedling preparations

The seeds were pre-germinated in germinator, and 10 uniformly seedlings were placed on each filter paper separately

Table 2. Rice root exudates phenolics content (mg/l).

Cultivar	Phenolics						
	P-h	V	P-c	F	M-c	C	sum
Dollar	0.01	ND	ND	ND	0.37	0.090	0.47
IR-60	0.12	8.83	0.04	ND	0.051	0.037	9.078
Dashti sard	0.016	ND	ND	ND	ND	ND	0.016

ND, not detection

Table 3. The inhibitory percentages of three cultivars exudates in two concentrations on seedling growth and seed germination parameters of barnyardgrass.

Traits Treatments	Inhibition (%)							
	RL	SL	TL	TFW	TDW	GR	GP	NC
Cultivars (A)								
Dollar	42.01^{b}	14.74	26.96^{b}	23.87^{b}	18.74^{b}	34.91	25.49^{b}	16.93
Dashti sard	33.66^{c}	10.17	20.11^{c}	12.61^{c}	12.00^{c}	28.06	24.29^{b}	14.93
IR60	47.99^{a}	20.26	32.29^{a}	31.83^{a}	23.63^{a}	47.92	31.91^{a}	19.15
LSD	2.58	2.09	2.27	3.06	3.15	4.27	2.47	0.62
Concentration (B)								
50%	32.37^{b}	8.20	18.83^{b}	19.26^{b}	13.47^{b}	28.14	2152^{b}	14.65
100%	50.07^{a}	21.90	34.09^{a}	26.28^{a}	22.78^{a}	45.78	32.95^{c}	19.35
LSD	2.07	1.71	1.86	2.50	2.57	3.49	2.02	0.51
S.O.V								
A	**	**	**	**	**	**	**	**
B	**	**	**	**	**	**	**	**
A×B	ns	*	ns	ns	ns	*	ns	**
CV (%)	5.81	13.26	8.19	12.82	16.57	11.01	8.66	3.50

The data with the same letter are not significantly different.
ns, *, ** not significant, significant at probability levels 0.05 and 0.01, respectively**.**

(Whatman No. 42) laid in a sterilized 9-cm Petri dish. Root exudates of each cultivar were prepared at two concentrations (50% and 100%). Each Petri dish was treated with 10 ml of these concentrations and then placed in a germinator. The experiments replicated four times. Ten days after germination, the seedling RL and SL, TL and TFW and TDW were measured. The seedlings were dried at 70°C for 4 h in oven (B.M.P Incubator, Iran), then weighed. TDW, TFW, TL, RL and SL inhibition of seedlings (compared to control), were determined by using the following equation:

Trait inhibition = [1 – (samples) / control] ×100 [Eq. 2]

Total Nitrogen Content Determination

Nitrogen content was measured by Total Kjeldahl Nitrogen (TKN) method (Isaac and Johnson, 1976), (Kjeltec Auto1030 Analyzer, Foss Tecator AB, Hoganas, Sweden). Total reduced nitrogen was determined by using a micro Kjeldahl procedure with sulphuric acid, digestion catalyst and conversion of organic nitrogen into ammonium form according to the Total Kjeldahl Nitrogen (TKN) method.

Statistical analysis

Experiment was evaluated in a factorial arrangement based on completely randomized design with two factors and four replications. Analysis of variance was performed for barnyardgrass seedling traits by using the general linear model (PROC GLM) procedure in Statistical Analysis System (SAS) program (SAS Institute, 1997). Means were separated using the LSD test and statistical significance was evaluated at $P = 0.05$.

Results

Detecting of phenolic acids in root exudates by HPLC

After optimization of the chromatographic conditions, various concentrations of analytic standards were injected to HPLC for obtaining calibration curves. Retention times of each phenolic acid are presented in Table 1. Among of cultivars, vanillic acid and *p*-coumaric acid was detected only in IR60 exudates but ferulic acid was not found, *m*-coumaric and cinnamic acid was observed in root exudates of all cultivars except Dashti sard (Table 2). Results indicated that the highest and lowest contents of *p*- hydroxybenzoic acid were related to IR60 and dollar exudates, respectively. Meanwhile, IR60 had the highest phenolic acid content among three studied cultivars (9.078 mg/l) and the lowest amount was belonged to Dashti sard (0.016 mg/l). Furthermore, among all detected phenolics, vanillic acid content was considerable (Based on Table 2), and total Phenolic acid content also had positive correlation with all inhibition percentage of all parameters.

Effect of rice root exudation on barnyardgrass

The various inhibitory effects were observed for studied traits (root, shoot and total lengths, total fresh and dry weights, germination rate and percentage and nitrogen content). The results of analysis variance showed differences among cultivars

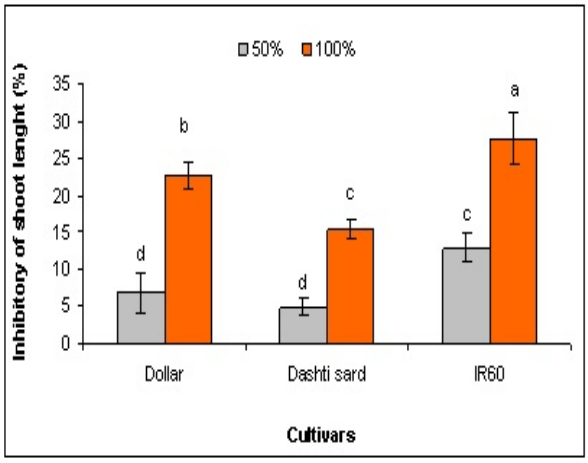

Fig 1. Interaction effects of root exudates of rice cultivars and their concentrations on shoot length of barnyardgrass seedlings.

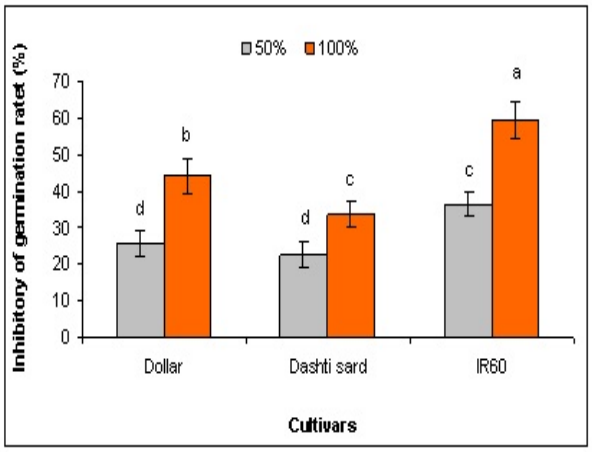

Fig 2. Interaction effects of root exudates of rice cultivars and their concentrations on germination rate of barnyardgrass seeds.

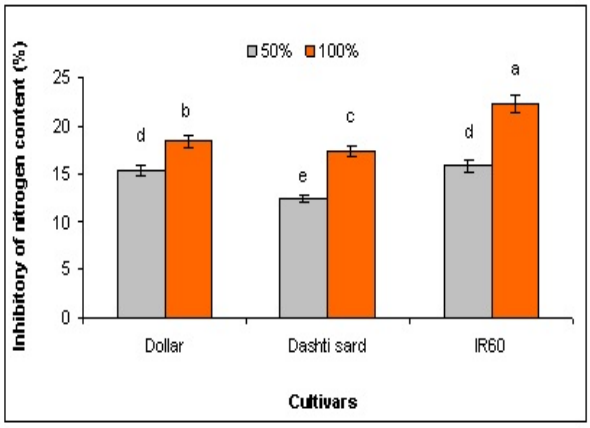

Fig 3. Interaction effects of root exudates of rice cultivars and their concentrations on nitrogen content of barnyardgrass seedlings

and concentrations (Table 3). Inhibitory activity of rice root exudates was increased by increasing concentration. Mean values (Table 1) indicated that, exudates of IR60 (With 47.99% inhibition) had the highest inhibitory effect on root length of barnyard grass and the lowest amount was belonged to Dashti sard cultivar (33.66% inhibition). Also, in terms of other traits such as total length, fresh and dry weight and germination percentage, root exudates of IR60 showed the highest inhibitory effect (39.29, 31.83, 23.63 and 31.91% respectively) and the lowest was observed in Dashti sard (20.11, 12.61, 12.00 and 24.29% respectively). Interaction effects (cultivar × concentration) were significant on shoot length, germination rate and nitrogen content of barnyardgrass. Exudates from IR60 at the higher concentration (100%) were more suppressive on shoot length of barnyardgrass than other exudates (Fig 1). In terms of germination rate, mean values were different between cultivars, so that Inhibitory percentage was ranged from 22.45% in Dashti sard to 59.32 % in IR60. Also the highest suppressive cultivar in both concentrations was IR60 and the lowest was Dashti sard (Fig 2). According to the results (Fig 3), nitrogen content of Barnyardgrass seedlings were more inhibited by IR60 exudates (22.27 %) in comparison other two cultivars. In addition, the lowest amount of inhibition was related to Dashti sard exudates in lower concentration (with 12.49 % inhibition).

Discussion

Assessment of allelopathy potential of rice cultivars is difficult and delicate task because of multiplicity of reactions between plants and environments. Rice allelopathic potential and determining rice hull extracts effects on growth inhibition of weeds have been widely studied (Olofsdotter et al., 1995; Mattice et al., 1998; Olofsdotter, 2001; Takeuchi et al., 2001; Ahn and Chung, 2000; Chung et al., 2002, 2003, 2005, 2006). But rice root exudates effects on growth factors of weeds especially nitrogen content of seedlings has been concerned in few studies. In this study our approach was to compare allelopathic potential of rice root exudates and their qualities by use of cultivars from previous studies (Chung et al., 2002; and Asghari et al., 2006) with the results obtained from the root exudates tested by high performance liquid chromatography (HPLC). There were significant variations on the growth inhibition of barnyardgrass seedlings treated with rice cultivars root exudates (Table 3). Root exudates of IR60 showed the highest inhibitory activity in all studied traits of barnyardgrass seedlings (Table 3), these cultivars may release more allelopathic substances in paddy fields during rice growth (Table 2). Therefore, it maybe provides stronger allelochemicals such as vanillic and *p*-coumaric acid to suppress weeds growth in paddy fields. According to the results, phenolics content had positive correlation with all measured inhibitions (which means, growth parameters has been suppressed more with increasing in total phenolic contents). These results are in agreement with results of Mattice et al. (1998), who suggested that allelopathy of rice against weeds is correlated with the amounts of phenolic acids released by living rice roots. Rice can release many kinds of secondary metabolites through its root tissues. These metabolites in rice exudates possess multiple functions on the chemical interactions among organisms in the environment (Bacilio et al., 2003), and allelopathy is one of multiple functions among

metabolites (Kato-Noguchi and Ino., 2001), also, to exert phytotoxic effects on other plant species, chemicals may have to move to the roots of the target plant (Inderjit, 2006). Our results are in agreement with results of Chung et al. (2002) who suggested that Ferulic, *p*-hydroxybenzoic, vanillic, *p*-coumaric, and *m*-coumaric acids were the most active compounds and caused the greatest inhibitory effect on seed germination, germination rate, and total seedling dry weight of barnyardgrass. They recommended that these compounds may be, at least, a key factor in rice allelopathy on barnyardgrass, and may contribute to the development of naturally occurring herbicides. The amount of available nitrogen is often limiting factor in crop production. According to allelopathy definition, it is so evident that allelochemicals could affect all phases of nitrogen cycle that involve plant or microorganisms, such as, biological N fixation, mineralization, and nitrification. When plants take up nitrate, they must use energy to convert it to ammonium form before it can be used (Reigosa et al., 2006). Growth reduction due to missing of energy could be an argument for nitrogen reduction in seedlings which were treated with root exudates, or loosing of nitrogen content could be occurred by limiting or reducing some key factors in nitrogen metabolism such as nitrate reductase and glutamine synthetase (Nie, 2005).

Conclusion

According to the results, if a blend of compounds are responsible for the observed growth inhibition of weeds, phenolic acids could play key roles in such a mixture, particularly vanillic and *p*-coumaric acid, as these compounds were detected exclusively in IR60 in this experiment.

Acknowledgements

The authors would like to thank the RCRI (Rice and Citruses Research Institute) for providing the grant to conduct this research and head of institute Dr. Ghorban Ali Nematzade for his support and helpful comments in this study.

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