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Study of Growth Factors, α-amylase and Peroxidase Activity in Various Cultivars of Rice (*Oryza sativa* L.) Under Vanillic Acid Stress

[1]O. Jazayeri, [1]T.A. Aghajanzadeh and [2]B. Sadeghpour Gildeh
[1]Department of Biology, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran
[2]Department of Statistics, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

Abstract: The effects of Vanillic Acid (VA) on germination, seedling and adult plant of rice (*Oryza sativa* L.) were investigated. Four cultivars, traditional (Taroom mahalli and Taroom deilamani) and improved (Shafagh and Onda) were studied. For germination, seeds were sterilized and then placed on Petri dish at 30°C at different concentrations (0, 10, 20 and 25 mM) for 7 days and growth factors of seedling were measured after 14 days. Seedling (10 days) planted in hydroponic medium including nutrient solution amended with 0, 25, 50 and 100 mg kg^{-1} VA. After 30 days, the growth factors were determined. α-amylase and peroxidase activities were assayed by Bernfeld and Maehly methods, respectively. Present results indicated that as a result of increasing concentrations of VA, germination percentage and germination rate as well as α-amylase activity markedly decreased except Onda in all cultivars. In all cultivars, seedling growth factors such as shoot length, fresh weight and dry weight as well as root have been reduced by increasing of VA. In adult plant, also shoot length, root length, shoot dry weight and root fresh weight were reduced by increasing concentrations of VA. Shoot fresh weight was decreased in Shafagh by increasing concentrations of VA; meanwhile, we have not observed any significant differences in the other cultivars. In the case of root dry weight, there were not significant differences in any cultivars. With increasing concentrations of VA, chlorophyll content reduced; on the contrary, peroxidase activity increased.

Key words: Vanillic acid, rice (*Oryza sativa* L.), growth factors, α-amylase, peroxidase

INTRODUCTION

Allelopathy is the direct influence of chemicals released from one plant on the development and growth of another plant (Olofsdotter, 1998). Phenolic acids are often mentioned as putative allelochemicals and are perhaps the most commonly investigated compounds among potential allelochemicals (Dalton, 1999). Vanillic acid (VA) is one of the phenolic compounds which involves in allelopathic phenomena. It is known that VA decreases the activity of root system in Chinese fir (Chen *et al.*, 2003). Other studies suggested that VA inhibits the availability of soil mineral nutrients including N, P and K (Chen *et al.*, 2002). It was reported that VA inhibits photosynthesis and protein synthesis in leaf cells of velvelleaf *Abutilon theophrasti* (Merise and Singh, 1993). Also; Herrig *et al.* (2002) described decreasing effect of vanillic acid on root length, fresh weight and dry in soybean. Rice is one of the most important crops in the world and one of the principle food crops in Iran. In our previous study, we observed that growth has been reduced in Iranian rice cultivars by their shoot extracts (Aghajanzadeh *et al.*, 2007) and present finding showed that there is an autoallelopathy phenomena in Iranian rice. From the quantitation work on rice root exudates, five phenolics including of p-hydroxybenzoic acid, caffeic acid, vanillic acid, p-coumaric acid and syringic acid identified as key components distinguishing allelopathic potential between rice varieties (Seal *et al.*, 2004). Thus, on the supposition that there is VA (as an allelochemical) in Iranian rice cultivars and regarding that little information is available about phytotoxic effects of VA on plants especially in rice, so we decided to investigate the role of VA on autoallelopathy phenomena in Iranian rice cultivars (traditional and improved cultivars). In this study effects of VA on physiological factors were studied in three stages(germination, seedling and adult plant). In the germination stage, germination rate, germination percentage and α-amylase activity were evaluated. In the seedling stage, physiological factors such as fresh weight, dry weight and length in both root and shoot were determined. In the adult plant stage, physiological parameters such as fresh weight, dry weight, length in root and shoot, peroxidase activity and chlorophyll content were assayed.

Corresponding Author: O. Jazayeri, Department of Biology, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran
Fax: +981125242161

MATERIALS AND METHODS

Plant material: Seeds of rice (*Oryza sativa* L.) were provided from rice research center in Amol two traditional cultivars (Taroom mahalli and Taroom deilamani) and two improved cultivars (Shafagh and Onda) were chosen. All works were done under laboratory condition. The seeds were surface sterilized in 02:10 (V/V) of commercial hypochlorite bleach for 10 min and rinsed several times with distilled water. All further manipulation carried out under sterile conditions. Ten seeds of rice were arranged on the filter paper in Petri dish and then VA was added at different concentrations 10, 20 and 25 mM. Distilled water was used as a control. Each treatment was carried out in four replications and all of the Petri dish were placed at 30°C in dark (Yang *et al*., 2002). Germination percentage and rate were determined after 7 days. Seedling root and shoot lengths were measured after 14 days. Seedlings were separated into roots and shoots then fresh weight and dry weight, oven-dried at 65°C for 4 h, were determined.

Seeds were germinated in Petri dish and after 7 days the seedling were individually transplanted to hydroponic medium. Transplanted seedling were allowed to acclimate 3 days before treatments with VA were initiated (10 days). Treatments were initiated by replacing the nutrient medium with nutrient solution amended with VA. Plants were treated for a 30-day period with 0, 25, 50 and 100 mg kg^{-1} VA. Four replications in each treatment and 6 seedlings in each replication were cultured. Planting were carried out at 30°C for 30 days in 12 h light. After ending 30-day period, growth factors such as length, fresh weight and dry weight of shoots and roots were determined. Chlorophyll was assayed by Arnon (1949) method.

Extraction and assay of α-amylase: The seeds were sterilized and sowed for 1 day in Petri dishes containing distilled water at 37°C in the dark. Uniformly germinated seeds were selected and transferred to Petri dishes containing two sheets of Whatman No. 1 filter paper moistened with distilled water or test solution. Each petri dish contained 10 germinated seeds. Each treatment was carried out in 4 replications. The germinated seeds were allowed to grow at 27°C in darkness and 3 mL of distilled water or test solution was added to each petri dish on day 3 (Lin and Kao, 1995).

Seeds were homogenized in a chilled (4°C) mortar and pestle with 0.2M sodium acetate (pH 5.4) containing 3 mM $CaCl_2$. All samples (3 mL each) were centrifuged at 4000 rpm for 15 min and supernatant was used for enzyme assay (Lin and Kao, 1995). Crude extract was used to determine α-amylase activity by dinitrosalicylic acid method. One unit (U) was defined as amount of enzyme which releases 1 μmol of reducing end group per minutes in 0.2M sodium acetate (pH 5.4) containing 3 mM $CaCl_2$ with 0.5% (W/V) soluble starch as substrate at 25°C (Bernfeld, 1955). D-maltose was used as standard.

Extraction and assay of peroxidase: Leaves were collected, weighed (1.0 g), immediately stored at -20°C until extraction. Frozen tissues were ground with mortar with pestle, suspended in 2 mL 0.1 mM Tris-Glycine buffer at pH 7.2.

The extract was centrifuged at 14000 rpm for 30 min at 4°C and the supernatant was used to determine the activity of peroxidase by Maehly and Chance method (1954).

Protein content was measured by Bradford (1976) method using BSA as standard.

Statistical analysis: All experiments were conducted in a completely randomized design with 4 replications. Analysis of variance was performed for all data by using SPSS 11.5 software by ANOVA (Tukey test).

RESULTS

Results showed that VA inhibited the germination rate and percentage of Taroom mahalli, Taroom deilamani and Shafagh, the inhibition was increased with increasing VA concentrations. But these effects were not observed in Onda. Activity of α-amylase was significantly decreased with increasing concentrations of VA in all cultivars except Onda. There is directly a correlation between the germination percentage and α-amylase activity (Table 1).

In all cultivars seedling shoot length, seedling fresh and dry weight have decreased by increasing concentrations of VA. Moreover, Onda's shoot length was reduced in higher concentrations of VA rather than other cultivars (Table 2). Also seedling root length, seedling fresh and dry weight were decreased by increasing concentrations of VA in all cultivars (Table 3).

In adult plants, shoot length and shoot dry weight were reduced by increasing concentrations of VA in all cultivars. Shoot fresh weight was decreased by increasing concentrations of VA in Shafagh but we did not observe the decreasing effects of VA in the other cultivars (Table 4). The root length and the fresh weight were reduced by increasing concentrations of VA in all cultivars but there was no significant difference in dry weight (Table 5). Also, chlorophyll content had been reduced in the presence of increasing concentrations of VA. Peroxidase activity in all cultivars was increased when rice adult plants were subjected to increasing concentrations of VA (Table 6). It seems that growth factors in adult plant are more resistant than seedling period.

Table 1: Effects of vanillic acid at different concentrations on germination percentage, germination rate and α-amylase activity after 7 days of treatment

	Germination percent (%)				Germination rate (day)				α-amylase activity (U mL^{-1})			
Cultivars	Control	10	20	25	Control	10	20	25	Control	10	20	25
Taroom mahalli	92.5a	85a	15b	2.5b	3.96a	3.95a	3.87a	0.75b	2.24a	1.86a	1.32a	0.68b
Taroom deilamani	95.5a	87.5a	65a	5b	3.6a	4.03a	3.85a	1.75b	1.62a	1.67a	1.6a	0.41b
Shafagh	82.5a	50b	5c	0c	3.11a	3.86a	0.75b	0b	3.83a	3.37a	2.02b	2.13b
Onda	87.5a	92.5a	95a	67.5a	3a	3.15a	3.21a	3.19a	0.28a	0.39a	0.38a	0.32a

Values (n = 4) within a row were not followed by the same letter are significantly different (p<0.05)

Table 2: Effects of vanillic acid at different concentrations on shoot seedling growth factors after 14 days of treatment

	Shoot length (cm)				Shoot fresh weight (g)				Shoot dry weight (g)			
Cultivars	Control	10	20	25	Control	10	20	25	Control	10	20	25
Taroom mahalli	5.98a	3.77b	0.37c	0.04c	0.22a	0.13b	0.01c	0.001c	0.02a	0.01ab	0.005b	0c
Taroom deilamani	5.39a	3.66b	2.14b	0.04c	0.2a	0.14ab	0.08b	0.0005c	0.02a	0.01ab	0.007b	0c
Shafagh	5.46a	1.7b	0.15c	0c	0.28a	0.09b	0.01c	0c	0.02a	0.009b	0.002bc	0c
Onda	7.03a	6.21a	3.4b	1.59b	0.31a	0.25ab	0.15bc	0.07c	0.03a	0.02ab	0.007b	0.003c

Values (n = 4) within a row were not followed by the same letter are significantly different (p<0.05)

Table 3: Effects of vanillic acid at different concentrations on root seedling growth factors after 14 days of treatment

	Root length (cm)				Root fresh weight (g)				Root dry weight (g)			
Cultivars	Control	10	20	25	Control	10	20	25	Control	10	20	25
Taroom mahalli	6.38a	2.19b	1.04c	1.01c	0.84a	0.31b	0.03c	0. 01c	0.24a	0.01b	0.007c	0c
Taroom deilamani	6.97a	2.89b	1.23bc	1.01c	0.91a	0.51ab	0.02b	0.015b	0.22a	0.18a	0.006b	0b
Shafagh	5.61a	2.16b	2.38b	0c	0.76a	0.3b	0.3b	0c	0.2a	0.006b	0.007b	0b
Onda	10.23a	4.47b	1.52c	1.04c	1.84a	0.77b	0.05c	0.04c	0.6a	0.4a	0.009b	0.002b

Values (n = 4) within a row were not followed by the same letter are significantly different (p<0.05)

Table 4: Effects of vanillic acid at different concentrations on Shoot growth factors in adult plant after 30 days of treatment

	Shoot length (cm)				Shoot fresh weight (g)				Shoot dry weight (g)			
Cultivars	Control	25	50	100	Control	25	50	100	Control	25	50	100
Taroom mahalli	16.15a	16.45a	11.44b	12.77b	0.6a	0.56a	0.45a	0.43a	0.07a	0.06a	0.03b	0.02b
deilamani	13.44a	12.85a	13.58a	10.86b	0.33a	0.27a	0.29a	0.25a	0.05a	0.05a	0.06a	0.03b
Shafagh	16.4a	16.25a	14.47ab	12.75b	0.62a	0.34b	0.3b	0.28b	0.08a	0.07a	0.05a	0.02b
Onda	15.35a	15.42a	15.17a	11.43b	0.68a	0.59a	0.66a	0.57a	0.06a	0.05a	0.05a	0.02b

Values (n = 4) within a row were not followed by the same letter are significantly different (p<0.05)

Table 5: Effects of vanillic acid at different concentrations on root growth factors in adult plant after 30 days of treatment

	Root length (cm)				Root fresh weight (g)				Root dry weight (g)			
Cultivars	Control	25	50	100	Control	25	50	100	Control	10	20	25
Taroom mahalli	3.97a	2.65b	2.9b	2.89b	0.04a	0.04a	0.02b	0.02b	0.018a	0.015a	0.015a	0.015a
Taroom deilamani	3.53a	3.55a	2.16b	2.09b	0.05a	0.04ab	0.03b	0.03b	0.027a	0.021a	0.01a	0.01a
Shafagh	8.57a	9.42a	8.43a	6.18b	0.06a	0.03b	0.03b	0.03b	0.036a	0.035a	0.029a	0.031a
Onda	4.58a	4.22a	4.97a	2.18b	0.44a	0.23b	0.21b	0.2b	0.023a	0.018a	0.011a	0.01a

Values (n = 4) within a row were not followed by the same letter are significantly different (p<0.05)

Table 6: Effects of vanillic acid at different concentrations on peroxidase and chlorophyll content after 30 days of treatment

	Chl (mg g^{-1} fresh weight)				POD (μ mol min^{-1} mg^{-1} protein)			
Cultivars	Control	25	50	100	control	25	50	100
Taroom mahalli	2.66a	2.1a	0.85b	0.43b	0.5b	0.77a	0.74a	0.98a
Taroom deilamani	3.17a	2.84a	1.14b	0.69b	0.28b	0.34b	0.82a	0.93a
Shafagh	3.15a	3.22a	1.05b	0.52b	0.67b	0.78b	0.7b	2.1a
Onda	3.74a	2.69a	2.15a	0.71b	0.62b	0.73b	0.62b	0.9a

Values (n= 4) within a row were not followed by the same letter are significantly different (p<0.05)

DISCUSSION

In this study, we observed that germination percentage was positively correlated with the activity of α-amylase. α-amylase is considered to play a major role in degradation of reserve carbohydrate to soluble sugars during germination. Also, α-amylase is induced by gibberellic acid in seeds. Kato-Naguchi and Macias (2005) studied effects of 6-methoxy-2-benzoxazolinone (MBOA) on the germination and α-amylase activity in lettuce seeds and found that MBOA inhibited the germination and induction of α-amylase. So, MBOA may inhibit α-amylase induction in antagonism with gibberellin-induced events in α-amylase translation process. Also, Komai (1981) and Watanabe *et al.* (1982) found antigibberellin activity by sesqiterpen faenesol and sesqiterpen lactone argrophylline A and b-selinene. Thus, it is possible that VA affects on gibberellic acid like other allelochemical compounds (MBOA, sesqiterpen faenesol, sesqiterpen lactone, argrophylline A and b-selinene). Furthermore, we observed decreasing effects of rice aqueous extract (including phenolic compounds such as VA) on α-amylase activity and germination percentage (Aghajanzadeh *et al*., 2007).

Einhellig (1995) proposed primary effect of phenolic acids on the plasma membrane. Multiple physiological effects have commonly been observed from treatments with many allelochemical phenolics. These effects lead to reduce in physiological factors including decreases in plant growth, absorption of water and mineral nutrients, ion uptake, leaf water potential, shoot turgor pressure, osmotic potential, dry matter production, leaf area expansion, stomatal aperture size, stomatal diffusive conductance and photosynthesis (Booker *et al*., 1992; Chou and Lin, 1976; Einhellig and Kuan, 1971; Einhellig and Rasmussen, 1979; Gerald *et al*., 1992; Patterson, 1981). Blum *et al*. (1999) suggested that derivatives of benzoic and cinnamic acids inhibit plant growth by affecting many physiological processes such as ion uptake and hydraulic conductivity (i.e., water uptake). Benzoic and cinnamic acids also increased ion leakage from cucumber roots (Yu and Matsui, 1997) and enhanced the accumulation of abscisic acid (Holapp and Blum, 1999), a key substance in the regulation of stomatal behavior (Jia and Zhang, 1999). With regard to VA is a phenolic compound we suggest that decreasing in rice growth factors may be as a result of VA effect on mentioned physiological factors. Suppressing effect of ferulic and vanillic acids on plant growth have been shown at concentration of up to 10 mM (Kuiters, 1990). Present results in this investigation indicated that VA had decreasing effect on growth factors at 10 mM and more than it. Yang *et al* (2002) indicated that accumulation of chlorophyll and porphyin contents were more inhibited as the phenolic concentrations increased; also they suggested Mg-chelatase may be major target of the three phenolic compounds (ferulic, p-coumaric and o-hydroxyphenyl acetic acid). Some phenolic compounds, such as o-hydroxyphenylacetic, ferulic and p-coumaric acid significantly induce the activities of chlorophyllase a and b and Mg-dechelatase a and b in rice seedlings (Yang *et al*., 2004). Rice (1984) has suggested that the synthesis of porphyrin precursors of chlorophyll biosynthesis may be impeded by some allelopathic compounds. Allelopathic agents might inhibit photosynthesis by inducing peroxidation (Yu and Matsui, 1997). VA severely inhibit suppress the photosynthesis of soybean(Patterson, 1981) as well as chlorophyll reduction in soybean and sorghum (Einhellig and Rasmussen, 1979). In present research this decreasing effect of VA on chlorophyll has been observed in rice cultivars, too. Also, reducing in chlorophyll content, in turn diminishes total plant growth.

Increased peroxidase (POD) activity have been observed in soybean seedling after treatment with benzoic and cinnamic acids (Baziramakenga *et al*., 1995). A stimulation of the activities of POD by exposure to environmental stress has been documented in *Ramalina farinacea*, *Lycopersicon pennellii* Correll and *Chlorella rulgaris Beij* (Malanga and Puntarulo, 1995; Shalata and Tal, 1998; Deltoro *et al*., 1999).

Allelopathic agents such as phenolic compounds also increased the activities POD in cucumber. It should be noted, however, that many phenolic acids could act as peroxidase substrates. The great increase in POD activity by these acids was, therefore, partly attributed to allelopathic effect (Yu *et al*., 2003). In this study, we observed that the increasing concentrations of VA lead to increase in POD which it may be corresponded with study of Yu *et al*. (2003).

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