Korean J. Ecol., 23(2): 187~192, 2000

Effect of *Phytolacca americana* Extracts on the Activities of AsPOX and GuPOX during Germination Process of *Cassia mimosoides* var. *nomame*

Lee, Ho-Joon and Yong-Ok Kim

Department of Biological Sciences, Division of Natural Science, Konkuk University

ABSTRACTS: After *Cassia mimosoides* var. *nomame* was treated with *Phytolacca americana* extracts during the germination process, its effect on the activities of AsPOX and GuPOX were examined. As the concentration of *P. americana* extract increased, the activity of AsPOX decreased while that of GuPOX increased. During the germination process, the activity of AsPOX was lower than the control, while the activity of GuPOX was over 2 times higher than the control. Activity of total peroxidase by IEF was extremely high at pI 6.4 and pI 6.6 when treatment was made with a 30% concentration of *P. americana* extract. The treatment with phenolic compounds, caffeic acid and benzoic acid did not show much difference from the control although a slight increase was observed at pI 6.6. Activity of GuPOX in *C. mimosoides* was over 4 times higher in roots than in shoots. Namely, because GuPOX activity of *C. mimosoides* was increased by extracts of *P. americana*, defense enzyme, GuPOX, was generated against external stress, and we could certified the activity increase at pI 6.4, especially in root.

Key Words: AsPOX, GuPOX, Germination, Phenolic compound, Peroxidase.

INTRODUCTION

There are many secondary metabolites acting as plant allelochemicals, such as phenolic compounds, volatiles, terpenoids, flavonoids and alkaloids. Among them, phenolic compounds are the most abundant in cells (Einhellig and Rasmussen 1973, Lodhi 1976, Whittaker and Feeny 1971) and are known as inhibitor of seed germination, seedling growth, cell division, protein synthesis and the enzyme activity (Bhowmik and Doll 1984, Horsley 1976, Lodhi and Killingbeck 1981, Rice *et al.* 1981). Allelochemicals inhibit the seed germination and seedling growth, and change the content of protein synthesis and enzyme activities in plants. Also, proteins stored in seeds are hydrolized into amino acids, which are then re-synthesized to new proteins, increasing total protein content (Evans and Bhatt 1977, Gaspak *et al.* 1973, Jones and Armstrong 1971, Khan *et al.* 1973).

According to Alscher and Hess (1995) and Allen *et al.* (1997), plants produce enzymes, such as peroxidase, catalase and superoxide dismutase, for self-protection against outside stress. Peroxidase, in general, exists in the form of isotype enzymes, which have a different molecular structure while activating in the same substrate. During the shoot formation period, the type of isoperoxidase is more important than the total amount of peroxidase (Scandalios 1990) and is involved in plant growth as well as in many plant defense mechanisms (Lagrimini 1991, Knorzer *et al.* 1996). Especially, AsPOX is a major enzyme in removing toxins, caused by external stress, and plays a major role in protecting the plant from active oxygen (Asada 1992, Karpinski *et al.* 1997, Mittler and Zilinskas 1991). GuPOX is also produced when the plant is mechanically damaged or contaminated by disease-organisms (Lagrimini and Rothstein 1987). While caffeic acid and ferulic acid reduced the activity of phosphorylase (Rice 1984), other phenolic compounds were reported to increase the activities of proteinase, peroxidase and catalase (Gasper *et al.* 1985, Loehenstein and Linsey 1961).

Although there is a great need for the study of allelochemicals, not many studies have been made locally. *P. americana*, an invader species which became naturalized in Korea, is widely dispersed, and inhibits the growth of surrounding plants, and thus it was selected to study the effects of its allelochemicals. Water soluble extracts of *P. americana* were used in this study for the purpose of evaluating their effects on the organ differentiation of plants. The study was focused on the physiological mechanisms of AsPOX and GuPOX in plants upon stimulation from allelochemicals through analyzing the activity of isotype enzymes.

MATERIALS AND METHODS

Materials

Seeds from *C. mimosoides* were used for germination and seedling growth, and water soluble

extracts were made from *P. americana*. *C. mimosoides* seeds show a high rate of germination in a short period and are very sensitive to water soluble extract treatment. Water soluble extracts from *P. americana* are known to have a strong inhibiting effect on germination and seedling growth, even at lower concentrations (Kim *et al.* 1997). 200 grams of leaf of *P. americana* was boiled and extracted with distilled water at 80℃ for 48 hours and the extraction was then centrifuged at 15,000 rpm for 30 minutes (Centrikon T-1045, Kontron Co.). The upper parts of the solution were collected and stored at 4℃ before being used.

Water soluble extracts from *P. americana* were filtered with filter paper (diameter of 150 mm, pore sizes of 5 μm and 1 μm), then diluted to 10%, 25%, 50%, 75% and 100% concentrations for the purpose of seed germination study. After being selected for even size, *C. mimosoides* seeds were sterilized for 3 minutes in a solution of 3~5% sodium hypochloride and then washed 3~5 times with distilled water. Thirty seeds were sowed in a petri-dish (diameter of 90 mm) and placed in 28℃ incubator (Hotpack Phila., PA., U.S.A) with light for 12 days of germination. Samples for activity measurement of isozymes were collected at days 3, 6, 9 and 12 and stored in the freezer (−50℃).

Activity measurement of AsPOX and GuPOX

For the activity measurement of AsPOX and GuPOX, 1 ml of extraction buffer, containing ascorbate and EDTA, was added into liquid N-frozen *C. mimosoides* seeds, which were mashed in a grinding bowl then homogenized (Table 1). After being left at 4℃ for 20 minutes, it was centrifuged at 13,000 × g for 20 minutes (Kontron, Centrikon T-126). The amount of soluble protein was measured through protein assay kit (Bio-Rad; USA) using the method of Bradford (1976). Activity of AsPOX and GuPOX was measured using the adopted method from Amako *et al.* (1994) (Table 2) and re-calculated into unit per 1 μg protein using enzyme kinetics software.

Isoelectric focusing (IEF)

The band pattern of peroxidase was examined through a Mini IEF cell kit (Bio-Rad, Model 111) method. The solution was made at 100℃, containing 5% D-sorbitol, 10% glycerol and 1% agarose (EEO<0.02), and was cooled at 55℃. After adding 2% ampholyte, it was placed on gel support film (Bio-Rad) using a casting tray and left at 4℃ for 12 hours. After 3 MM paper (Whatman, 3030 917) with pore size of 7(w)×4(l) nm was placed on gel, 30 μg of protein solution was added and left for 5 minutes to soak completely into the gel. Electrodes were placed in contact with the gel and execution was made for 15 minutes at 100V, 15 minutes at 200V and 1 hour at 450 V.

IEF marker (Sigma, pI 3.6~pI 9.3) staining was made by fixing the gel in fixed solution with 30% methanol, 5% trichloroacetic acid and 3.5% sulfosalicyclic acid. Afterwards the gel was stained again in the solution with 0.2% coomasie brilliant blue R-250, 28% ethanol and 14% acetic acid, and was destained in a solution of 28% ethanol and 14% acetic acid.

Table 1. Composition of AsPOX and GuPOX extraction buffer (pH 7.0) for soluble protein

Contents	Concentration (mM)
KH_2PO_4	25
K_2HPO_4	25
EDTA	1
Ascorbate	1

Table 2. Composition of substrate solution for AsPOX and GuPOX activity assay

	Contents	Concentration (mM)
AsPOX	Potassium phosphate (pH 7.0)	50
	EDTA	0.1
	Ascorbate	0.5
GuPOX	Potassium phosphate (pH 7.0)	50
	EDTA	0.1
	Guaiacol	5
	Hydrogen peroxide	0.3

RESULTS AND DISCUSSION

Activity measurement of AsPOX and GuPOX

Activities of AsPOX and GuPOX were measured during the germination process of *C. mimosoides* seeds treated with different concentrations of *P. americana* extracts. Since the strains of isoperoxidase have been reported to be more important than total peroxidase in the differentiation process of plant (Scandalios 1964), measurements were made of the activities of AsPOX and GuPOX, which are generally known to be sensitive to external stimulation in plants (Figs. 1, 2 and 3).

Activity of AsPOX increased up to the 25% concentration and then dramatically fell thereafter (Fig. 1). While Kim *et al.* (1997) reported that *C. mimosoides* showed improved seed germination and seedling growth in treatments of low concentration, 10~30%, over control, Rice (1984) reported reduced activity of many enzymes by treatment with plant extracts. The reduced activity of AsPOX, observed in this study, is believed

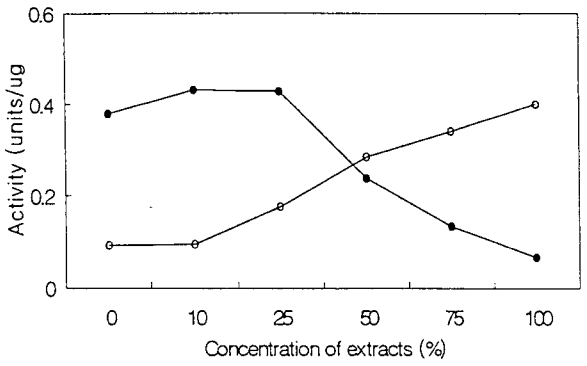

Fig. 1. Effect of *Phytolacca americana* extracts on AsPOX (●—●) and GuPOX (○—○) activity of *Cassia mimosoides* var. *nomame*

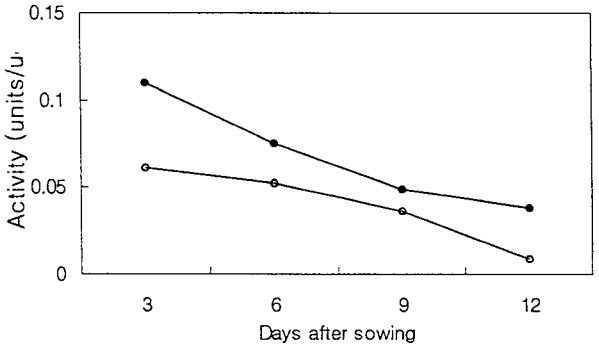

Fig. 2. Comparison of the AsPOX activity of control (●—●) and *Phytolacca americana*. extracts (○—○) during 12 days after sowing *Cassia minosoides* var. *nomame*

to agree with Rice (1984).

Contrary to AsPOX, the activity of GuPOX was dramatically increased as concentration increased (Fig. 1). Generally, plants increase the activity of peroxidase upon external stress as a self-protection mechanism (Gasper *et al.* 1985, Seeni and Gnanam 1981, Wayne *et al.* 1970, Yoo and Kim 1988) and it is considered that GuPOX, rather than AsPOX, is responsible for the increase of peroxidase activity because the activity of GuPOX was increased in the germination process of *C. mimosoides* seeds treated with *P. americana* extracts. Also, the concentration of 30% was deemed to be the critical concentration since the most dramatic changes in the activities of AsPOX and GuPOX were observed between 25% and 50% concentrations (Fig. 1).

During the germination process of *C. mimosoides* seed after treatment of 30% of *P. americana* extracts, samplings of seedling plants were made on days 3, 6, 9 and 12 for the measurement of AsPOX and GuPOX (Figs. 2 and 3). The activity of GuPOX was reduced as germination proceeded in *C. mimosoides* seeds and was lower in the

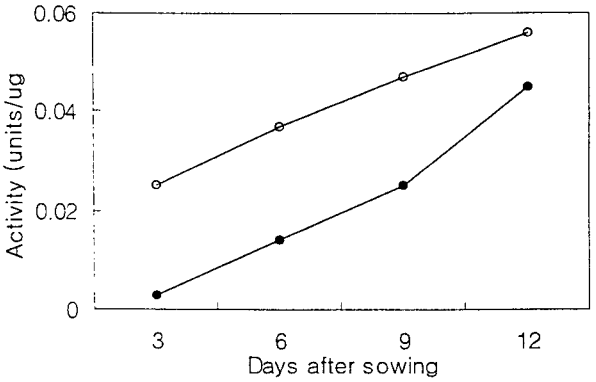

Fig. 3. Comparison of the GuPOX activities of control (●—●) and *Phytolacca americana* extracts (○—○) during 12 days after sowing *Cassia minosoides* var. *nomame*

treated than in the control (Fig. 2). On the other hand, the activity of GuPOX increased as germination proceeded and it was over 2 times higher in the treated than in the control (Fig. 3). It was again confirmed that GuPOX is the isozyme which showed an increase in activity, for the purpose of self-protection, when inhibition occurs on germination and growth in *C. mimosoides* seeds by external stress from *P. americana* extracts.

Comparison of total peroxidase by IEF

During the germination process after *C. mimosoides* seeds were treated with 30% *P. americana* extracts, samplings of seedling plants was made on the 9th day for the comparison of band patterns of total peroxidse by IEF (Fig. 4A). Activity was higher in the treated than in the control, especially at pI 6.6 and pI 6.4, which

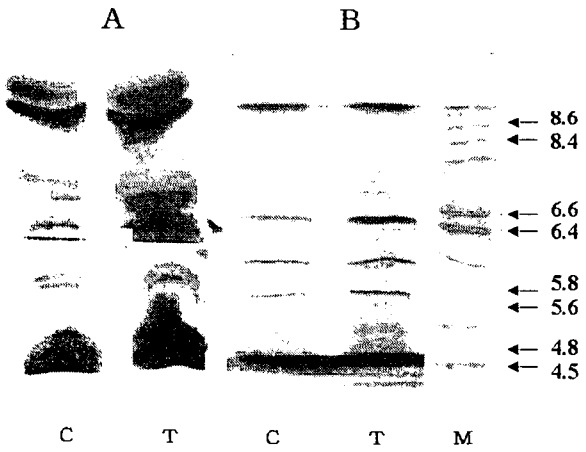

Fig. 4. Comparison of peroxidase isozymes in seedling (A) and root region (B) between control group (C) and treated plant (T) by isoelectric focusing in the range of pH 3~10. M: IEF marker pH 3~10.

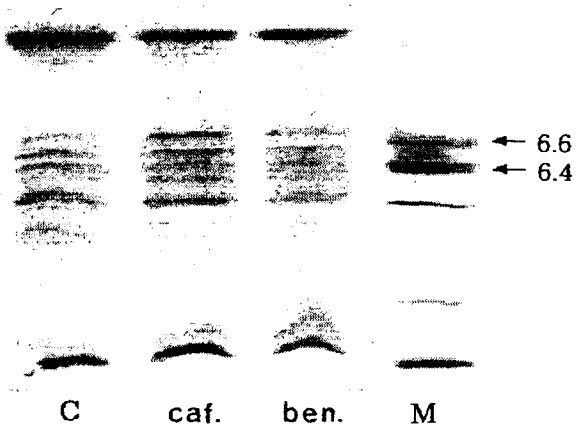

Fig. 5. Comparison of peroxidase isozymes between treated group of caffeic acid (caf.) and benzoic acid (ben.) by isoelectric focusing in the range of pH 3~10. M: IEF marker pH 3~10.

indicates that GuPOX, whose activity was increased by *P. americana* extracts, is distributed at pI 6.6 and pI 6.4. Hogan and Manner (1990) reported that phenolic compounds inhibit cell and tissue differentiation and growth. Kim *et al.* (1987) observed a tendency of negative correlation between cell growth and activity of isozymes, since peroxidase activity becomes lower as cell growth rates increase and vice versa.

Germination and seedling growth of *C. mimosoides* was reduced by *P. americana* extract treatment (Kim *et al.* 1997), and the restricted growth of seedling plants by *P. americana* extract treatment caused the increased activity of total peroxidase in the treated. Especially GuPOX is believed to be the main cause for this increase. From the germination and seedling growth test with the most abundant phenolic compounds in *P. americana* extracts, which were analyzed by HPLC, caffeic acid and benzoic acid showed the highest inhibitory effects (Kim *et al.* 1997). The band pattern of total peroxidase by IEF was therefore examined with caffeic acid and benzoic acid treatment (Fig. 5).

Although there was not much difference between the treated and the control, the benzoic acid treated showed rather slightly lower activity than the control, while the caffeic acid treated showed slightly higher activity at pI 6.6. It is therefore considered that the higher activity observed at pI 6.5 and pI 6.4 in Fig. 4A was not due to the effects from individual phenolic compounds, but due to the aggregate effects of phenolic compounds.

Activity of GuPOX between shoots and roots in *C. mimosoides*

Because the activity of AsPOX was low (Fig. 2) during the germination process of *C. mimosoides* seeds treated with different concentrations of *P. americana* extracts, we therefore measured the activities of GuPOX in the shoots and roots on the 9th day (Fig. 6). The activity of GuPOX increased in proportion to the concentration in shoots and roots: the concentration in roots was 4 times higher than that in the shoots (Fig. 6). Yoo and Kim (1988) reported that activity of the root region was highly sensitive to external stimulation, as a result of total peroxidase activity on the cotyledon, lower cotyledon axis and root. Espelie *et al.* (1986) reported that extracts affected the root region. Activity of total peroxidase in root region measured high because of root differentiation restraint by *P. americana* extracts. The peroxidase activity in root region was higher than control when treatment was made with 30% concentration *P. americana* extract (Fig. 4B). pI 6.6 was GuPOX related to the shoot differentiation and pI 6.4 was isozyme related to the root differentiation of the *C. mimosoides* judging from the high activity of pI 6.4. Enzyme activity of the plants was reported to increase due to stress, injurious substances, aridity and cold-weather damage (Seeni and Gnanam 1981, Wayne *et al.* 1970, Yoo and Kim 1988). Enzyme activity was increased by the treatment of *P. americana* extracts, and it can be due to the increased activity of GuPOX (defense enzyme), especially in roots, as a response of resistance to the stress from the extracts which causes a reduction in growth. The increased

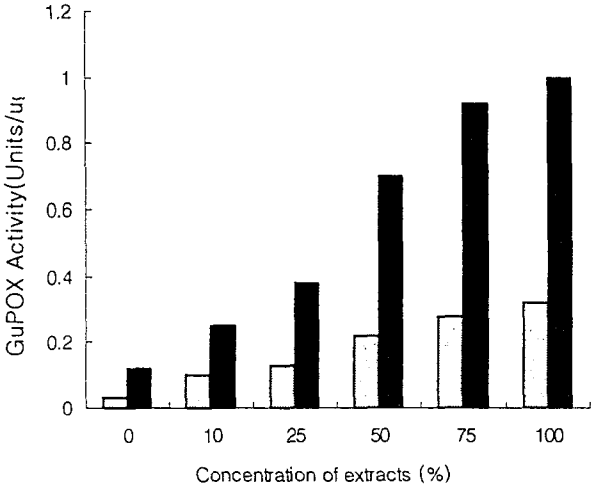

Fig. 6. Comparison of GuPOX activity between root (■) and stem (□) treated with *Phytolacca americana* extract.

activity of peroxidase is therefore considered as a physiological mechanism of plants against allelochemicals.

ACKNOWLEDGEMENTS

This research was supported by Korea Research Foundation (Project No. BSRI-98-4441)

LITERATURE CITED

Allen, R.D., R.P. Webb and S.A. Schake. 1997. Use of transgenic plants to study antioxidation defenses. Free Radical Biol. and Med. 23: 473-479.

Alscher, R.G. and J.L. Hess. 1995. Antioxidants in higher plants. CRC Press, Boca Raton, pp. 1-174.

Amako, K., G.X. Chen and K. Asada. 1994. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol. 35: 497-504.

Asada, K. 1992. Ascorbate peroxidase: A hydrogen peroxide scavenging enzyme in plants. Physiol. Plant. 85: 235-241.

Bhowmik, P.C. and J.D. Doll. 1984. Allelopathic effects of annual weed residues growth and nutrient uptake of corn and soybeans. Agron. J. 76: 383-388.

Bowers, W.S. and R. Nishida. 1980. Juvocimenes: potent juvenile hormone mimics from sweet basil. Science 209: 1030-1032.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Einhellig, F.A. and J.A. Rasmussen. 1973. Allelopathic effects of *Rumex crispus* on *Amaranthus retroflexus*, grain sorghum and field corn. Amer. Mid. Nat. 90: 79-86.

Espelie, K.E., V.R. Franceschi and P.E. Kolattukudy. 1986. Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. Plant Physiol. 81: 487-492.

Evans, L.E. and G.M. Bhatt. 1977. Influence of seed size, protein content and cultivar on early seedling vigor in wheat. Can. J. Plant Sci. 57: 929-935.

Gaspar, T., C. Penel, F.J. Castillo and H. Greppin. 1985. A two-step control of basic and acidic peroxidase and its significance on growth and development. Physiol. Plant. 64: 418-423.

Gaspak, T., A.A. Khan and D. Fries. 1973. Hormonal control of isoperoxidase in lentil embryonic axis. Plant Physiol. 51: 146-149.

Hogan, M.E. and G.D. Manners. 1990. Allelopathy of small everlasting (*Antennaria microphylla*): Phytotoxicity to leaf spurge (*Euphorbia esula*) in tissue culture. J. Chem. Ecol. 16: 931-939.

Horsley, S.B. 1976. Allelopathic interference among plants. II. Physiological models of action. Proc. Fourth North Amer. For. Biol. Workshop, pp. 93-136.

Jones, R.L. and J.E. Armstrong. 1971. Evidence for osmotic regulation of hydrolytic enzyme production in germinating barley seeds. Plant Physiol. 48: 137-142.

Karpinski, S., C. Escobar, B. Karpinski, G. Creissen and P.M. Mullineaux. 1997. Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. Plant Cell 9: 627-640.

Khan, A.A., R. Verbeek, E.C. Waters, Jr. and H.A. van Onckleen. 1973. Embryoless wheat grain: A natural system for the study of gibberellin induced enzyme formation. Plant Physiol. 51: 641-645.

Kim, S.S., S.H. Wender and E.C. Smith. 1987. Isolation and characterization of two isoperoxidases from tobacco tissue culture. Phytochemistry 19: 165-168.

Knorzer, O.C., J. Durner and P. Boger. 1996. Alterations in the antioxidative system of suspension-cultured soybean cells (*Glycine max*) induced by oxidative stress. Physiol. Plant. 97: 388-396.

Lagrimini, L.M. and S. Rothstein. 1987. Tissue specificity of tobacco peroxidase isoenzymes and their induction by wounding and tobacco mosaic virus infection. Plant Physiol. 84: 438-442.

Lagrimini, L.M. 1991. Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. Plant Physiol. 96: 577-583.

Lee, H.J., Y.O. Kim and N.K. Chang. 1997. Allelopathic effects on seed germination and fungus growth from the secreting substances of some plants. Korean J. Ecol. 20: 181-189.

Lodhi, M.A.K. and K.T. Killingbeck. 1981. Effects of pine-produced chemicals on selected understory species in a *Pinus ponderosa* community.

Lodhi, M.A.K. 1976. Role of allelopathy as expressed by dominating trees in a lowland forest in controlling the productivity and pattern of herbaceous growth. Amer. J. Bot. 63: 1-8.

Loebenstein, G. and N. Linsey. 1961. Peroxidase activity in virus-infected sweet potatoes. Phytopathology 55: 533-537.

Mittler, R. and B.A. Zilinskas. 1991. Purification and characterization of pea cytosolic ascorbate peroxidase. Plant Physiol. 97: 962-968.

Rao, M.V., G. Paliyath, D.P. Ormrod, D.P. Murr and C.B. Watkins. 1997. Influence of salicylic acid on H_2O_2 -metabolizing enzymes. Plant Physiol. 115: 137-149.

Rice, E.L. 1984. Allelopathy. 2nd edn. Academic Press, New York and London.

Rice, E.L., C.Y. Lin and C.Y. Huong. 1981. Effects of decomposing rice straw on growth of and nitrogen fixation by *Rhizobium*. J. Chem. Ecol. 7: 333-344.

Scandalios, J.G. 1964. Tissue specific isozyme variation in maize. J. Heredity 5: 281-285.

Scandalios, J. G. 1990. Response of plant antioxidant defense genes to environmental stress. Adv. Genet. 28: 1-41.

Seeni, S. and A. Gnanam. 1981. Isozymes of glucose-6-phosphate dehydrogenase and NAD^+ malate dehydrogenase in shoot forming foliar discs of tobacco. Plant and Cell Physiol. 222: 969-977.

Wayne, A., J.V. Jacobsen, J.G. Scandalios and J.E. Varner. 1970. Deuterium oxide as a density label of perioxidase in germination barley embryos. Plant Physiol. 45: 148-152.

Whittaker, R.H. and P.P. Feeny. 1971. Allelochemics:

Chemical interactions between species. Science 171: 757-770.
Yoo, W.I. and S.S. Kim. 1988. Purification and characterization of an anionic isoperoxidase from Korean radish root. Korea Biochem. J. 21: 207-213.
(*Received March 10, 2000*)