

Peroxidase Changes as Indicators of Herbicide-Induced Stress in Aquatic Plants

S. L. SPRECHER, A. B. STEWART AND J. M. BRAZIL¹

ABSTRACT

Increase in peroxidase enzyme activity and change in number of isozymes expressed have been associated with

reactions to environmental stress in terrestrial and aquatic plants. Such alterations may be useful in monitoring herbicide efficacy in submersed weeds. Two aquatic species, Eurasian watermilfoil (*Myriophyllum spicatum* L.) and hydrilla (*Hydrilla verticillata* (L.f.) Royle), showed a two- and three-fold increase in peroxidase activity, respectively, when exposed to 12- to 48- μ g/l fluridone for 30 days.

¹ASCI Corporation, 1720 Clay Street, Suite 3, Vicksburg, MS 39180.

Peroxidase levels varied among tissues in untreated watermilfoil, with roots and leaves having higher activity than stems and apical tips. Fluridone-treated hydrilla showed relatively greater increase of this enzyme in tips than in shoots. Extracts from untreated plants of both species were electrophoresed in starch gels, and activity staining revealed peroxidase isozymes.

Key words: enzyme analysis, efficacy, tissue specificity, *Myriophyllum spicatum*, *Hydrilla verticillata*, fluridone.

INTRODUCTION

An early and reliably quantifiable signal of herbicide effect would have value for weed control in the aquatic environment by indicating sufficient contact time, or obviating re-application. Here we present a brief review of peroxidase (PRX) as a general indicator of stress in plants, and summarize some preliminary work on monitoring levels of this enzyme in hydrilla and Eurasian watermilfoil (hereafter called milfoil) following treatment with fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)-phenyl]-4(1*H*)-pyridinone).

The group of enzymes that have peroxidase (PRX) activity (donor: H₂O₂ oxidoreductases, EC 1.11.1.7) occur in almost all plant tissues. These glycosylated heme proteins, which are monomers of approximately 40 to 50 kD, oxidize certain substrates at the expense of hydrogen peroxide, and rid the cell of excess peroxide produced by metabolism under both normal and stress conditions (10).

PRX analysis is frequently encountered in plant studies due to the enzyme's broad involvement in metabolism and ease of measurement. Changes in PRX activity have been associated with a wide array of physiological processes involved with auxin function and cell wall synthesis, and are readily monitored in crude extracts via the colored products formed by the enzyme as it reacts with a variety of substrates *in vitro* (10, 16, 20, 22, 23).

PRX activity in plant tissue tends to be negatively correlated with auxin level and growth rate, and positively correlated to lignin synthesis (19, 20). Consequently, many normal processes of the plant life cycle are marked by significant fluctuations in PRX levels: abscission, aging and senescence, apical dominance, cold tolerance, dormancy, fruit development and ripening, seed development, germination and early growth, sex expression, and tuberization (1, 20, 22, 24). The association with auxin and lignification has made PRX analysis informative in the study of plant response to external stimuli such as light, temperature, irritation and wounding, parasites and pathogens, and variation in ion status (3, 6, 7, 13, 21).

Although poststress elevation in PRX levels may be limited because of its binding to cell wall components, or to

the iron-dependent availability of heme (14), changes in PRX activity have been used to monitor stresses imposed by cold, drought, abscissic acid, gamma irradiation, salt, hypoxia, ion deficiency or toxicity, and pollutants (6, 10). In an evaluation of heavy metal and pesticide contaminants in sediment, Byl and Klaine (5) showed that increasing concentrations from 10 mg/l of the herbicide sulfometuron methyl (methyl 2-[[[(4,6-dimethyl-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl] benzoate) produced a dose-dependent elevation in PRX activity in unrooted apical portions of hydrilla.

Instances of stress-induced response suggested that alterations in PRX activity and isozyme expression following exposure to certain herbicides could be used to monitor stress or indicate lethal dose in submersed aquatic plants. To assess PRX response to fluridone, a slow-acting herbicide which suppresses carotenoid synthesis, we evaluated protocols for activity and isozyme analysis in hydrilla and milfoil, and monitored enzyme levels in untreated and treated tissues.

MATERIALS AND METHODS

Extraction. Crude extracts of plant tissue contain levels of PRX activity that readily support spectrometric and electrophoretic investigation. In the average plant cell the majority of peroxidases are cytoplasmic, with approximately 55% characterized as freely soluble, and 30% as less freely soluble. Of the remaining 15% which are complexed to cell walls and membranes, the ionically bound 10% can be extracted with salt solutions (13). The rest are covalently bound and insoluble, but able to be extracted following cellulase and pectinase treatments (20).

Acetate and phosphate solutions between 4.5 and 7.0 in pH are common extraction buffers for soluble PRX (*e.g.* 3, 15), with salt solutions such as CaCl₂ and KCl being used to release ionically bound enzyme (5, 13, 21). Buffer additives for enzyme stabilization include EDTA, mannitol, insoluble polyvinylpyrrolidone, ascorbate, *etc.* (1, 22). While mercaptoethanol is often used to protect activity of other enzyme systems during extraction for electrophoretic analysis, it interferes with measurement of PRX function.

Substrates. A variety of phenolic or aromatic compounds which are converted to a colored end-product in the presence of H₂O₂ are used as substrates for PRX analysis. Guaiacol, ferulic acid, *o*-dianisidine, pyrogallol, phenol, and benzidine are commonly used in kinetic studies (16, 20, 22). For isozyme development following electrophoresis, an insoluble end-product which will precipitate in the matrix at the location of enzyme activity is required. Suitable substrates include 3-amino-9-ethyl carbazole, 4-chloro-1-naphthol and 3,3'-diamino-benzidine (12).

Some PRX isozymes are capable of using a variety of cellular substrates (11), while others are substrate-specific. When guaiacol is converted to tetraguaiacol in the presence of the plant extract, the analysis is said to test for "guaiacol peroxidase" (6). Use of ascorbate will monitor the activity of those isoforms with a high preference for this compound as a reductant; these are termed "ascorbate peroxidases" (1, 16).

Spectrophotometric assays. Once plant extract is added to a suitable reaction solution containing substrate, H_2O_2 , and buffer, activity is characterized by monitoring change in absorbance (optical density) at a specific wavelength over a suitable linear portion of the kinetic curve, usually during the first 5 min of the reaction. The rate of change is reported for a unit time, either per weight of tissue or per total protein concentration in the crude plant extract, quantified with methods such as the Lowry or Bradford tests, usually with the bovine serum albumin standard (4, 11). Activity is also reported as absorbance units based on defined changes in optical density; or as units of enzyme activity, calculated from a standard curve based on commercially quantified horseradish PRX (5, 18). Other definitions of activity include rate of conversion of substrate, based on the extinction coefficient of the product (24), or rate of H_2O_2 reduction (1). Optimal wavelength for absorbance measurement differs with the color properties of the reaction product. For a guaiacol substrate, readings are usually taken at 470 nm; for pyrogallol, at 430 nm. The reaction with ascorbate produces a compound which decreases absorbance of the mixture, and it is monitored in the UV range at 290 or 265 nm (1, 16).

Electrophoresis. PRX occurs in multiple molecular forms (isozymes) which can represent allelic differences at the level of the gene, or post-translational modifications of the same transcript. Application of crude plant extracts to starch gels allows the separation of differently charged PRX isozymes. The cationic forms tend to be associated with auxin-mediated growth regulation, and the more numerous anionic ones with cell wall formation (9, 14). The number of isozymes resolved and their direction of migration depend partly on the pH of the solutions used; many common buffer systems resolve clear tissue-specific profiles for PRX. Analysis of various plant parts with different reaction substrates can show whether particular isozymes are induced or suppressed in response to herbicide treatment.

Current protocols. Material assayed for PRX in this study was taken from plantings initiated in October 1991 and March 1992. Apical portions of milfoil or hydrilla were placed into sediment and grown in 55-L aquaria in a controlled-environment chamber, following previously described regimes for establishment, radiation, day length, and fertilization (17). Untreated milfoil was analyzed from the

first planting. In the second, established hydrilla and milfoil were exposed to fluridone concentrations of 0, 12, 24 and 48 $\mu\text{g/l}$ for 30 or 60 days. Following treatment, herbicide was flushed from the aquaria and replaced with fresh water. PRX activity was monitored pretreatment and at 8, 30 and 60 days after treatment (DAT) was initiated. Biomass data were collected and reported elsewhere (Netherland, Getsinger and Turner, in press).

For PRX analysis, plant parts were harvested immediately before extraction and placed on ice. Tissue was blotted, and a 1.0-, 0.5-, or 0.25-g sample was macerated with a chilled mortar and pestle using 0.1 M NaPO_4 buffer, pH 6.1 (22) or 0.5 M CaCl_2 (5). The extract was poured into a chilled test tube and centrifuged at 2000 g for 5 min. The supernatant was removed and the pellet washed with buffer in a repeat centrifugation. Supernatants were then pooled and filtered through one layer of Miracloth (Calbiochem). A ratio of either 1:10 or 1:20 g fresh weight of tissue to total milliliter volume of extraction buffer was maintained.

Plants were sorted by tissue before analysis. The uppermost 5 cm of apical or axillary growing points were designated as tips; the portion of plant from 5 to 30 cm below apices was used as shoot (stem plus leaves) or separated into leaves and stem. Roots were cleaned of sediment before use.

A 200- μl aliquot of supernatant was combined with 2.8 ml of a reaction solution, consisting of either 0.1 M NaPO_4 , 4 mM guaiacol, and 3 mM H_2O_2 (22), or 5 mM MES, pH 6.0, 80 mM phenol, 44 mM H_2O_2 , and 2 mM aminoantipyrine (5). Absorbance was monitored at 470 nm (guaiacol) or 510 nm (phenol), with readings taken at the end of the first and third minutes after mixing the plant extract and reaction solution together; the rate of activity was reported as change in absorbance per minute per fresh weight of tissue. Three reactions from each extract were run to produce a mean activity per sample. Analysis of variance was done, and treatment means were separated with a Bayesian LSD at the 95% confidence level.

For the horizontal starch gel electrophoresis, the tank (bridge) buffer used was 0.03 M lithium hydroxide (monohydrate) and 0.19 M boric acid, pH 8.1. The same solution was combined with a buffer of 0.05 M TRIS and 6 mM citric acid, pH 8.4, in a ratio of 1:10, and heated with 10 to 12% hydrolyzed starch to produce the gel matrix. Crude plant extract was applied to the solidified gel using paper wicks. The apparatus was placed inside a refrigerator at 4C, and a constant current was applied to the gel for 4 hr before developing in a 0.1 M Na acetate, pH 5.0, solution containing 4 mM 3-amino-9-ethyl carbazole, 10% N,N-dimethyl formamide, and 15 mM H_2O_2 .

RESULTS AND DISCUSSION

The protocols described were successful in monitoring activity and isozymes of PRX in crude extracts produced from aquarium-grown hydrilla and milfoil following treatment with various aquatic herbicides. They have also been found applicable to egeria (*Egeria densa* Planch.; data not shown).

Tests of the extraction procedure showed that little additional PRX activity (0.9% of the total found) was retrieved by washing the pellet a second time, and the single pellet wash method was used for this work. Many herbicide compounds have ring structures, and compounds to be used for treatment are evaluated to ensure that they do not act as PRX substrates when substituted for phenol or guaiacol in reaction solutions. No herbicide-generated activity has been seen.

The different extraction buffers and reaction substrates used in this preliminary work produced varying levels of PRX activity from the same plant material (data not presented). From this we assume that the protocols monitor soluble (PO_4 -extracted) and ionically bound (CaCl_2 -extracted) isozymes, which can differ in activity and substrate specificity. By extracting both types of isozymes from a range of tissues, and reacting them with more than one substrate, it may be possible to describe activity of the various forms of PRX more fully.

Tissue specificity of PRX isozymes in plants is well-established (10), and initial electrophoretic examination of untreated material showed variation in isozyme profiles between leaves and roots in milfoil and among leaves, roots and tubers in hydrilla (data not presented). Analyzing plant parts separately for enzyme activity provided information about PRX levels in untreated tissues and revealed differential changes following herbicide contact.

The low activity measured in apical tips and stem of untreated milfoil from the first planting, compared to leaves and root (Figure 1), is consistent with relative levels of PRX found in other species and with the negative correlation found between PRX and auxin in plant tissues in general (2, 8, 14). Pretreatment levels of PRX in milfoil and hydrilla leaves from the second planting were approximately twice those of shoots or of apical portions (data not shown). Differential increase in enzyme activity was seen in fluridone-treated hydrilla, where apical tips treated with 12 and 24 $\mu\text{g/l}$ were significantly higher in activity than shoots or reference material at 8 DAT (Figure 2). (Milfoil was not analyzed at this time.)

Pre- and posttreatment tissue differences in PRX levels require that well-defined samples be used when activity is reported relative to weight of plant material. The nonparallel increase in PRX between treated tips and shoots shows the desirability of monitoring enzyme changes in more than one tissue. One plant part may be more indicative of stress than

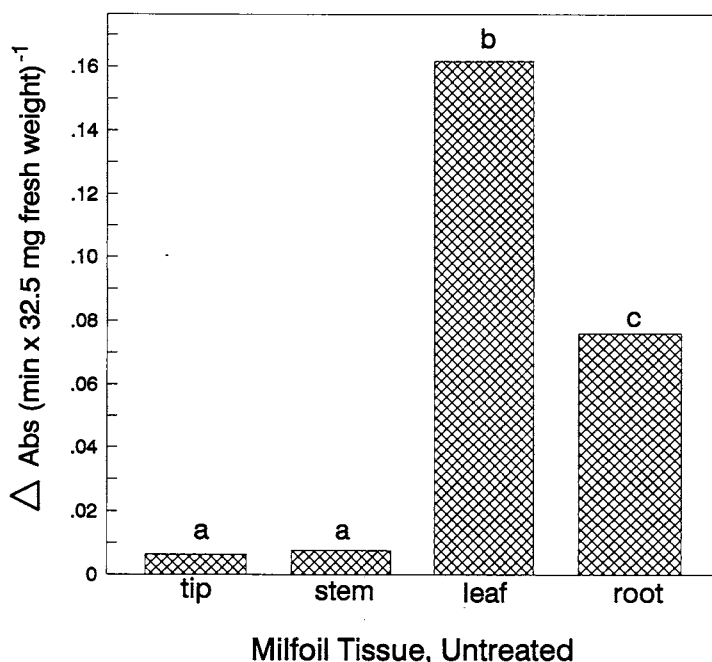


Figure 1. Levels of PRX activity in Eurasian watermilfoil tissue taken from untreated aquarium-grown plants, following reaction with guaiacol substrate. Different letters indicate significant differences between tissue means at the 5% level according to a Bayesian LSD test.

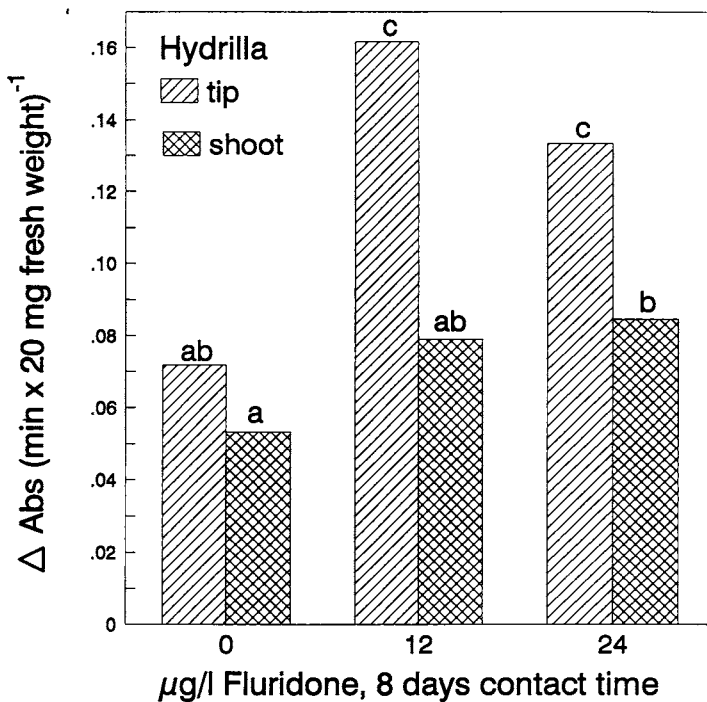


Figure 2. Levels of PRX activity in tips (apical 5 cm) and shoots (stems and leaves below 5 cm) of aquarium-grown hydrilla following 8 days contact time with fluridone at 0, 12 and 24 $\mu\text{g/l}$. Activity reported following reaction with phenol substrate. Different letters indicate significant differences between treatment means at the 5% level according to a Bayesian LSD test.

another, depending on the herbicide's mode of action. With fluridone treatment, stem apices in hydrilla and milfoil become bleached as chlorophyll is reduced. The elevated PRX seen in hydrilla tips suggests a localization of stress response.

Elevated PRX activity with fluridone application was seen in both species. At the end of a 30-day exposure time, milfoil treated with 12, 24 and 48 $\mu\text{g/l}$ was approximately three times higher in activity than reference material, although differences among concentrations were not significant (Figure 3). Shoots from hydrilla treated with 48 $\mu\text{g/l}$ had significantly higher levels than those from plants exposed to lesser concentrations or from untreated material (Figure 3). At 60 DAT, PRX levels in apical tips and shoots of milfoil given 30-day exposure and 30-day recovery did not differ significantly from untreated material, while activity in plants treated continuously for 60 days at 12 and 24 $\mu\text{g/l}$ remained significantly elevated in both tissues (data not shown).

Strong correlations between increase in PRX and herbicide efficacy were not shown here. The 30-day exposure to fluridone resulted in reduced shoot biomass by 90 DAT only in hydrilla treated at 48 $\mu\text{g/l}$ (Netherland, Getsinger and Turner, in press), whereas elevated PRX had been found in both species at 8 and 30 DAT. Milfoil treated for 60 days, which had elevated shoot and tip PRX at that time, did remain reduced in biomass at 90 DAT.

These initial results suggest that PRX monitoring of various tissues may help describe the chronology and severity of herbicide stress in aquaria-grown plants, and be applicable to use in mesocosm systems and the field. By relating PRX activity to other stress indicators, such as phenolic compounds or enzymes such as superoxide dismutase, catalase, and polyphenoloxidase (13, 24), more informative descriptions of metabolic status in both target and nontarget plant species following herbicide treatment may be produced.

ACKNOWLEDGMENTS

This research was conducted under Contract DACW39-90-D-0001, US Army Engineer Waterways Experiment Station. We thank Michael Netherland, Thomas Byl and Kimberly Deevers for a wide range of technical assistance in this study.

LITERATURE CITED

1. Arrigoni, O., L. De Gara, F. Tommasi and L. Rosalia. 1992. Changes in the ascorbate system during seed development of *Vicia faba* L. *Plant Physiol.* 99:235-238.
2. Biles, C. L. and F. B. Abeles. 1991. Xylem sap proteins. *Plant Physiol.* 96:597-601.
3. Borchert, R. 1978. Time course and spatial distribution of phenylalanine ammonia-lyase and peroxidase activity in wounded potato tuber tissue. *Plant Physiol.* 62:789-793.

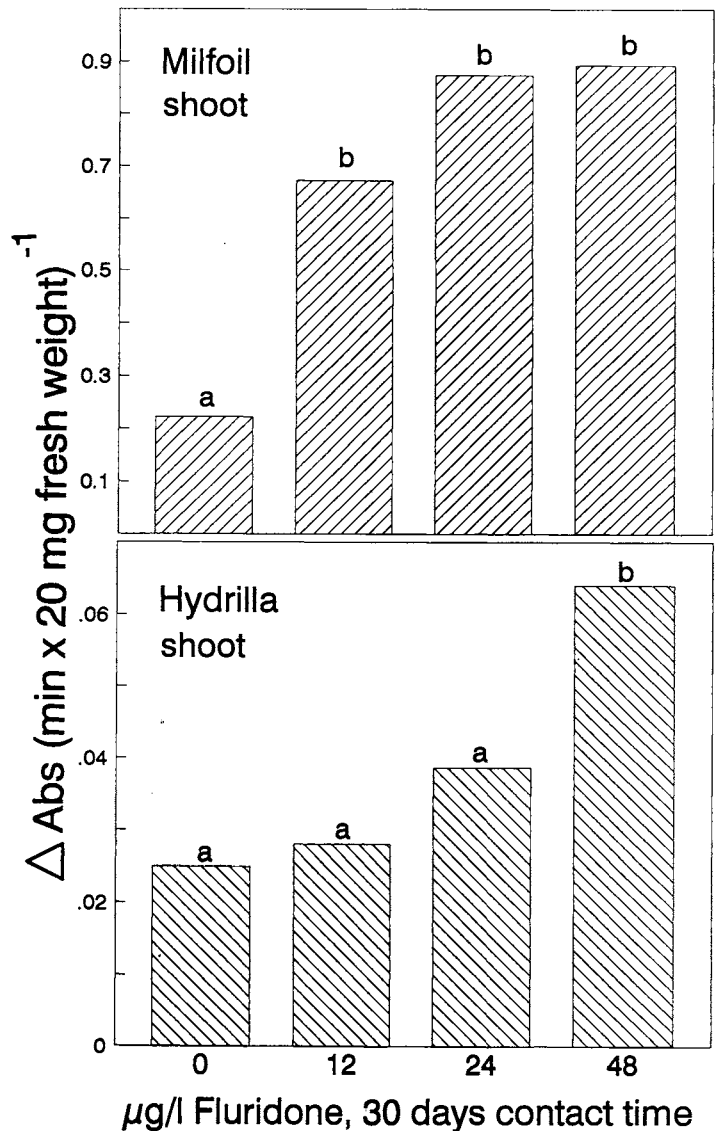


Figure 3. PRX activity in shoots of aquarium-grown Eurasian watermilfoil and hydrilla following 30 days contact time with fluridone at 0, 12, 24 and 48 $\mu\text{g/l}$. Activity reported following reaction with guaiacol (milfoil) or phenol (hydrilla) substrate. Different letters indicate significant differences between treatment means at the 5% level according to a Bayesian LSD test.

4. 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.
5. Byl, T. D. and S. J. Klaine. 1991. Peroxidase activity as an indicator of sublethal stress in the aquatic plant *Hydrilla verticillata* Royle. *In: Plants for Toxicity Assessment: 2nd Volume, ASTM STP 1115*, J. W. Gorsuch, W. R. Lower, W. Wang, and M. A. Lewis (eds.), American Society for Testing and Materials, Philadelphia. pp 101-106.
6. Cakmak, I. and H. Marschner. 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. *Plant Physiol.* 98:1222-1227.
7. 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.
8. Ferrer, M. A., M. A. Pedreno, R. Munoz and A. Ros Barcelo. 1991. Soluble peroxidase gradients in lupin hypocotyls and the control of the level of polarly transported indole-3yl-acetic acid. *J. Plant Growth Regul.* 10:139-146.
 9. Fieldes, M. A. and J. Ross. 1991. Peroxidase activity and relative mobility at anthesis in flax genotrophs and their F₂ progeny: developmental and genetic effects. *Genome* 34:495-504.
 10. Gaspar, T., C. Penel, T. Thorpe and H. Greppin. 1982. Peroxidases 1970-1980. A survey of their biochemical and physiologic roles in higher plants. University of Geneva Press, Geneva, Switzerland.
 11. Gillikin, J. W. and J. S. Graham. 1991. Purification and developmental analysis of the major anionic peroxidase from the seed coat of *Glycine max*. *Plant Physiol.* 96:214-220.
 12. Graham, M. Y. and T. L. Graham. 1991. Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. *Plant Physiol.* 97:1445-1455.
 13. Kahn, V., S. Goldshmidt, J. Amir and R. Granit. 1981. Some biochemical properties of soluble and bound potato tuber peroxidase. *J. of Food Sci.* 46:756-764.
 14. Lagrimini, L. M. 1991. Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. *Plant Physiol* 96:577-583.
 15. Lagrimini, L. M. and S. Rothstein. 1987. Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. *Plant Physiol.* 84:438-442.
 16. Mittler, R. and B. A. Zilinskas. 1991. Purification and characterization of pea cytosolic ascorbate peroxidase. *Plant Physiol.* 97:962-968.
 17. Netherland, M. D., W. R. Green and K. D. Getsinger. 1991. Endothall concentration and exposure time relationships for the control of Eurasian watermilfoil and hydrilla. *J. Aquat. Plant Manage.* 29:61-67.
 18. Pressey, R. 1990. Anions activate the oxidation of indoleacetic acid by peroxidases from tomato and other sources. *Plant Physiol.* 93:798-804.
 19. Pressey, R. 1991. Oxidized oligogalacturonides activate the oxidation of indoleacetic acid by peroxidase. *Plant Physiol.* 96:1167-1170.
 20. Quesada M. A., C. Sanchez-Roldan, A. Heredia, V. Valpuesta and M. J. Bukovac. 1992. Peroxidase and IAA oxidase activities and peroxidase isoenzymes in the pericarp of seeded and seedless "Redhaven" peach fruit. *J. Plant Growth Regul.* 11:1-6.
 21. Shinkle, J. R., S. J. Swoap, P. Simon and R. L. Jones. 1992. Cell wall free space of *Cucumis* hypocotyls contains NAD and a blue light-regulated peroxidase activity. *Plant Physiol.* 98:1336-1341.
 22. Wang, S. Y., H. J. Jiao and M. Faust. 1991. Changes in the activities of catalase, peroxidase, and polyphenol oxidase in apple buds during bud break induced by thidiazuron. *J. Plant Growth Regul.* 10:33-39.
 23. Wareing, P. F. and I. D. J. Phillips. 1981. *Growth and Differentiation in Plants*. 3rd ed. Pergamon Press, Oxford, p 57.
 24. Zheng, R. and Z. Yang. 1991. Lipid peroxidation and antioxidative defense systems in early leaf growth. *J. Plant Growth Regul.* 10:187-189.