http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (1): 34-38, 2006
ISSN 1028-8880

An Investigation of Vanillin Imposed Oxidative Stress in Corn (*Zea mays* L.) and the Activities of Antioxidative Enzymes

A.M. Nojavan and M. Khorshidi
Department of Biology, Urmia University, Urmia 57135-165, Islamic Republic of Iran

Abstract: This research studied the physiological effects of vanillin on corn and the activity of oxidative enzymes under vanillin imposed oxidative stress. Vanillin is a secondary metabolite which is produced in many plant species. It leaks out of living roots and shoots of donor plants and decaying material of dead plants into the neighboring environment. The 0.0, 0.5, 1, 2, 4 and 8 mM concentrations of vanillin were used as treatments. Seed germination was not affected seriously after 72 h of incubation at 25°C. Surprisingly, the longitudinal root and shoot growth was significantly reduced. This finding shows its phytotoxic effects against neighboring plants in natural and cultivated ecosystems. Besides its inhibitory effects, by an unknown mechanism of action, vanillin imposed an oxidative stress on corn plants. This was demonstrated by elevated malondialdehyde levels which were positively correlated with increased vanillin concentrations. This finding suggests an oxidative role for vanillin in corn which brings about the elevated activities of antioxidative enzymes. A possible mechanism of vanillin action is proposed.

Key words: Oxidative stress, antioxidative enzymes, SOD, APX, CAT, *Zea mays*

INTRODUCTION

Plants produce an array of organic compounds which apart from playing an important physiological role for the self, result in an interaction between them and neighboring environment[1]. These interactions are diverse and provide the donor plant a number of selective advantages[2]. These compounds are known as secondary metabolites. For many years, the adaptive significance of most plant secondary metabolites were unknown. In recent years, it has been suggested that, these compounds have important ecological functions in plants and may be antiherbivory, antiparasitic, antifungal and antibacterial phytotoxins[3,4]. They may serve as attractants for pollinators and seed dispersing insects and animals. Finally, they may act as agents of plant-plant competition[5].

Plant phenolics, which are a class of secondary metabolites, are biosynthesized via shikimic acid pathway[6]. Plants release these compounds into the environment from their leaves, roots, and decaying residues, which have harmful effects on neighboring plants, a process, that is defined as allelopathy[7]. Most phenolic compounds significantly affect weed growth in the field, while they influence nutrient uptake[8], nutrient cycling[9], enzyme activity[10], water relations[11] and photosynthesis and respiration[12].

Vanillin and vanillic acid are two closely related compounds that are synthesized in many plant species such as *Avena fatua*, *Camelina allysum*, *Cirsium arvense*, *Cyperus esculentus*, *Erica austrlis*, *Imperata cyclindrica*, *Proboscidea louisianica* and *Sasa cernua*[13]. Vanillin is converted to vanillic acid[14]. On the other hand ferulic acid is also converted to vanillic acid[15,16]. It has been reported that, these compounds are released into the soil by decomposing plant material and act as a growth inhibitor[17].

Recently, Shimazo *et al.*[18] reported that vanillin is taken up by fungal plasma membrane. They showed that vanillin is oxidized to vanillic acid by a membrane bound catalyzing system. They suggested that the fungus seems to posses a transporter protein for vanillin and vanillic acid uptake. Recently, the metabolic pathway from ferulic acid to vanillin has also been elucidated at the enzymic and molecular genetic levels[19].

The mechanism of action of vanillin has not been studied in more detail in higher plants. Employing metabolic engineering strategies for alteration the plants ability to produce enough bioactive secondary products or allelochemicals in order to manipulate the growth of unwanted neighboring plants is a prime goal of modern agriculture.

The aims of this research were to study the effects of vanillin on seed germination and seedling growth in corn and to investigate its effects on antioxidative enzymes.

Corresponding Author: M. Khorshidi, Department of Biology, Urmia University, Urmia 57135-165, Islamic Republic of Iran

MATERIALS AND METHODS

Corn seeds (*Zea mays* L. single cross 704) were obtained form Urmia Agricultural Organization (2004). For each experiment, a necessary amount of seeds were sterilized in 1% sodium hypochlorite solution for 10 min. Then they were washed in tap water and finally rinsed with distilled water. The germination percentage of the seeds was 96% after 72 h.

During experiments, 10 sterilized seeds were placed on two sheets of filter papers in 9 cm petri dishes. Then 10 mL of distilled water (untreated controls) or of a vanillin solution (0.5, 1, 2, 4, 8 mM) were added to appropriate petri dishes according to experimental design. Then, the petries were incubated at 25°C. After 5 days, root and shoot length were measured. Finally, the necessary samples of roots and shoots were prepared for biochemical determinations.

A Completely Randomized Design with three replicates for each treatment was used. The mean of treatments were compared with standard error bars.

Measurement of Malondialdehyde: Malondialdehyde contents of treated samples and untreated controls were measured using a thiobarbitoric acid reaction[20].

Preparation of enzyme extract: The 0.5 g FW was homogenized at 4°C in 1 mL of extraction buffer (0.05 M Tris-HCl buffer, PH 7.5, 3 mM $MgCl_2$, 1 mM EDTA and 1.5% W/V PVPP) with mortar and pestle. The extraction buffer used for the APX assay contained 0.2 mM ascorbate. The homogenate was then centrifuged at 25000 g for 20 min and the supernatant was used as the crude extract for the assay of antioxidative enzyme activity[21].

Enzyme assay: Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Dhindsa *et al.*[22].

Catalase (CAT) activity was assayed by measuring the rate of disappearance of hydrogen peroxide using the method of Maehly and Chance[23].

Ascorbate peroxidase (APX) activity was determinated according to the method of Chen and Asada[24] with minor modification.

RESULTS

Table 1 shows that vanillin did not inhibit corn seed germination significantly except at 8 mM that it decreased only about 5%. This suggests that, vanillin absorption takes place slowly and before it reaches to embryo and its

Table 1: The effects of vanillin concentrations on corn seed germination. The data are the means of three replicates±SE

Vanillin concentrations (mM)	0.0	0.5	1.0	2.0	4.0	8.0
Germination (%)	100±0.0	100±0.0	97±3.3	97±3.3	100±0.0	94±3.3

Table 2: The effects of different concentrations of vanillin on shoot and root length. The data are the means of three replicates ±SE

Vanillin concentration (mM)	Stem (cm)	Root (cm)
0.0	4.7±0.12	21±0.58
0.5	4.2±0.15	17±0.58
1.0	3.5±0.12	13.5±0.58
2.0	3.3±0.12	12.5±0.41
4.0	3.0±0.17	11±0.58
8.0	2.5±0.15	9±0.44

Table 3: The effects of different concentrations of vanillin on malondialdehyde content in shoot and root. The data are the means of three replicates ±SE

Vanillin concentration (mM)	Malondialdehyde of stem (μMol g^{-1} fw)	Malondialdehyde of root (μMol g^{-1} fw)
0.0	9.21±0.50	5.1±0.45
0.5	9.85±0.48	6.61±0.54
1.0	10.27±0.56	8.73±0.58
2.0	10.87±0.54	11.62±0.53
4.0	11.45±0.58	14.69±0.61
8.0	12.85±0.55	18.89±0.52

Table 4: The effects of varying vanillin concentrations on the relative activity of superoxide dismutase (percent of control). The data are the means of three replicates ±SE

Vanillin concentration (mM)	Stem (cm)	Root (cm)
0.0	100±5.41	100±3.61
0.5	118.75±4.78	102.86±3.30
1.0	156.25±5.41	105.71±3.06
2.0	193.75±5.41	107.62±3.60
4.0	184.38±4.78	100.95±3.06
8.0	168.75±4.78	143.81±3.61

Table 5: The effects of varying vanillin concentrations on the relative activity of ascorbate peroxidase (percent of control). The data are the means of three replicates ±SE

Vanillin concentration (mM)	Stem (cm)	Root (cm)
0.0	100±3.07	100±3.13
0.5	101.23±2.84	101.67±3.31
1.0	108.61±3.75	103.60±3.64
2.0	117.05±3.01	113.24±3.72
4.0	115.98±3.58	126.32±3.52
8.0	128.69±3.69	204.21±3.74

Table 6: The effects of varying vanillin concentrations on the relative activity of catalase (percent of control). The data are the means of three replicates ±SE

Vanillin concentration (mM)	Stem (cm)	Root (cm)
0.0	100±3.07	100±3.38
0.5	103.96±3.47	102.04±3.60
1.0	109.02±3.58	108.74±3.97
2.0	81.65±3.43	118.22±3.65
4.0	94.30±3.71	139.41±3.76
8.0	119.94±3.62	182.53±4.03

primary site of action the seed starts germination. But, studying the effect of vanillin on root and shoot growth revealed that it inhibits their longitudinal growth meaning fully (Table 2). Interestingly, it inhibited shoot growth more than root growth. This happens because shoot axis starts growthing later than root growth and by this time, vanillin has reached its primary site of action in root tissues.

Increased synthesis of malondialdehyde is an indication of lipid peroxidation in response to oxidative stress imposed by vanillin. Table 3 clearly shows that, by increasing the concentration of vanillin, the amount of malondialdehyde is significantly increased both in roots and shoots. Mean while, the addition of malondialdehyde in roots is greater than in shoots. In other words, its addition in roots is four folds in comparison to shoots which is only about 1.4 folds. This is probably due to the later shoot growth initiation and slower translocation of vanillin to shoots.

The relative activity of superoxide dismutase was measured in comparison to untreated controls both in roots and shoots (Table 4). The activity of SOD increased with increasing the concentration of vanillin. The addition of activity in roots was positively correlated with vanillin concentration, while in shoots, it decreased slightly at 4 and 8 mM vanillin levels.

The activity of Ascorbate peroxidase (APX) was also increased in roots and shoots (Table 5). The addition of relative activity both in roots and shoots were correlated positively with vanillin concentration.

The relative activity of catalse (CAT) was also increased with the addition of vanillin concentration (Table 6). In roots, the addition of activity was always additive with respect to increased vanillin concentration while in shoots, it slightly decreased at 2 and 4 mM and increased again at 8 mM vanillin concentrations.

DISCUSSION

Present studies revealed that, vanillin could not inhibit corn seed germination after 3 days (Table 1). This could be resulted from slower uptake by seed coat and slower translocation towards the prime site of action in embryonic tissues. On the other hand, vanillin inhibited longitudinal shoot and root growth significantly (Table 2). This finding suggests that, vanillin is absorbed by roots and translocated to shoots very effectively. Whether, vanillin is converted to vanillic acid by a plasma membrane enzyme system in corn, like what happens in some microorganisms[18], is unknown. It has not been clarified, if vanillin is converted to vanillic acid inside the cytoplasm or not?

Increased malondialdehyde production both in roots and shoots of corn, clearly suggests that an oxidative stress has been imposed upon corn plants by vanillin (Table 3). In oxidative stress, superoxide radical reacts with unsaturated fatty acids, causing lipid peroxidation and thereby damages membranes. Thus, accumulation of malondialdehyde indicates the damaging of lipids by oxidative stress[25]. Lipid peroxidation of biological membrane leads to structural alterations[26], denaturation of proteins and mutation of DNA[27,28].

The electron transport chains of mitochondria and chloroplasts are the main site of reactive oxygen species (ROS) production. During photorespiration, H_2O_2 is producted in peroxisomes and degradation of fatty acids in glyoxysomes also generates H_2O_2[29].

Fortunately plants have the capacity to cope with oxidative stress agents by eliminating them with an ROS scavenging system. In present study, we investigated the activities of SOD, APX and CAT which are key antioxidative enzymes in Halliwell-asada cycle.

The activity of SOD increased both in roots and shoots. The increased activity of SOD was correlated with the addition of ±vanillin concentration. Increased SOD activity is an indication of vanillin imposed oxidative stress. This is resulted from increased superoxide production which is a generic feature of biotic and adaphic stresses in plants[30,31]. It is a key enzyme in an oxidative defense mechanism[32]. SOD_s are metaloenzymes that convert superoxide radicals to H_2O_2 in chloroplasts and mitochondria ($2H^+ + 2O_2^{\bullet -} \xrightarrow{SOD} H_2O_2 + O_2$). Other isozymes of SOD are also found in cytosol and peroxisomes.

There are two enzymes that scavenge H_2O_2 produced in plant cells. The first one is APX which catalyze the H_2O_2 to monodehydroascorbate (MDHA) (2 Ascorbate + H_2O_2 $\xrightarrow{APX}$ 2 MDHA + $2H_2O$). Different isozymes of this enzyme exists in cytosol[33], chloroplasts[34-36]. Ascorbate is regenerated from MDHA in the chloroplast membrane by ferredoxin[37], or in the stroma by monodehydroascorbate reductase to the expense of NADPH[38]. The second enzyme which converts the H_2O_2 to H_2O and O_2 is catalase[39].

In present study, the relative activities of both APX and CAT increased in positive correlation with vanillin concentration (Table 5 and 6). Increased activities of these enzymes are an indication of increased H_2O_2 production which is resulted from vanillin imposed oxidative stress. The relative activities of SOD, APX and CAT were higher in vanillin treated corn plants than in the untreated controls. The mechanism for this vanillin imposed stress tolerance is not clear but it is related with increased antioxidative activity.

The chloroplasts and mitochondria are not the only source of ROS in stress conditions. The plasmalemma bound NADPH oxidase system also produce $O_2^{\bullet -}$ and H_2O_2 in response to many forms of stress[40]. Finally endoplasmic reticulum, peroxisomes and glyoxysomes are the possible sites of ROS and H_2O_2 production[41,42].

We suggest that, vanillin inhibits electron transport in photosynthetic and respiratory electron transport chains in chloroplasts and mitochondria, respectively. Therefore, electron leaks from some sites of these chains and reaches to molecular oxygen and produces superoxide. In photosynthesis, when NADP+ is not available to accept electrons from the Fd-$NADP^+$ oxidoreductase, electrons are transferred to O_2 and superoxide is produced. Probably, vanillin interferes with ATP or NADPH consuming enzymes in calvin cycle. On the other hand, vanillin may block the electron donor side of photosystem I. In that case the photosystem I may pass electrons to O_2 directly[25].

In respiration, different sites of electron leakage and release of superoxide and hydrogen peroxide have been proposed. One of the sites that is specific to plant mitochondria, is cyanide-insensitive alternative oxidase[43]. Therefore, vanillin may block the oxidative electron transport chain and electrons are switched to be transferred through alternative pathway. The electron leakage from this pathway could be the source of superoxide production.

At last, we propose, detailed studies on: (1) absorption and translocation of vanillin in different plant species, (2) is vanillin converted to vanillic acid? and if so, (3) to find the possible site of conversion and (4) precise determination of vanillin action and site of superoxide production.

REFERENCES

1. Taiz, L. and E. Ziger, 2002. Plant Physiology. 3rd Edn., Sinauer Associates, Inc. Publishers, pp: 690.
2. Reigose, M.J., A. Sanchez-Moreiras and L. Gonzalez, 1999. Ecophysiological approach in allelopathy. Cri. Rev. Plant Sci., 18: 577-608.
3. Horsley, S.B., 1986. Evaluation of hayscented fern interference with block cherry. Am. J. Bot., 73: 668-669.
4. Lawrey, J.D., 1993. Chemical ecology of *Hobsonis christiansenii* a lichencolous hypomycetes. Am. J. Bot., 80: 1109-1113.
5. Salisburay, F.B. and C.W. Ross, 1991. Plant Physiology. 4th Edn., Wadsworth Publishing Company. Belmont California, pp: 628.
6. Herrmann, K.M. and L.M. Weaver, 1999. The shikimate pathway. Ann. Rev. Plant Physiol. Plant Mol. Biol., 50: 473-503.
7. Indergit, K.M.M. and F.A. Einhellig, 1995. Allelopathy: Organisms, processes and adaptations. ACS Symposium Series, American Chemical society. Washington DC,
8. Booker, F.L., U. Blum and F.L. Fiscus, 1992. Short-term effects of ferulic acid on ion uptake and water relations in cucumber seedling. J. Exp. Bot., 93: 649-655.
9. Kalburtji, K.L., J.A. Mosjidis and A.P. Mamolos, 1999. Litter dynamics of low and high tannin sericea lespedeza plants under field conditions. Plant and Soil, 208: 271-281.
10. Devi, S.R. and M.N.V. Prasad, 1992. Effect of ferulic acid on growth and hydrolytic enzyme activities of germinating maize seeds. J. Chem. Ecol., 18: 1981-1990.
11. Barkosky, R.R. and F.A. Einhellig, 1993. Effect of salicylic acid on plant-water relationship. J. Chem. Ecol., 19: 237-247.
12. Hejl, A.M., F.A. Einhellig and J.A. Rasmussen, 1993. J. Chem. Ecol., 19: 559-568.
13. Kohli, R.K., H.P. Singh and D.R. Batish, 2002. Allelopathy in Agroecosystems. Haworth Press, New York, pp: 447.
14. Anthony, L. and L. Don Crawford, 1983. Whole cell bio conversion of vanillin to vanillic acid by *Streptomyces* viridosporus. Applied Environ. Microbiol., 45: 1582-1585.
15. Zhixian, H., L. Dostal and J.P.O. Rossazza, 1993. Mechanisms of ferulic acid conversions to vanillic acid and guaiacol by *Rhodotorula rubra*. J. Biol. Chem., 268: 23954-23958.
16. Narbad, A. and M.J. Gasson, 1998. Metabolism of ferulic acid to vanillin using novel COA-dependent pathway. Microbiology, 144: 1397-1405.
17. Eussen, J.H.H. and G.J. Neimann, 1981. Zeitschrift fur pflanzenphysiol., 102: 263.
18. Shimazo, M., Y. Kobayashi, H. Tanaka and H. Warrishi, 2005. Transportation mechanism for vanillin uptake through fungal plasma membrane. Applied Microbiol. Biotechnol., (In Press).
19. Nicholas, J.W., A. Narbad, C. Faulds and G. Williamson, 2000. Novel approaches to the biosynthesis of vanillin. Curr. Opin. Biotechnol., 11: 490-496.
20. Health, R.L. and L. Packer, 1968. Photoperoxidation in isolated chloroplasts. Arch. Biochem. Biophysiol., 125: 189-198.

21. Kang, H. and M.E. Saltveit, 2002. Chilling tolerance of maize, cucumber and rice seedling leaves and roots are differentially affected by salicylic acid. Physiol. Plant., 115: 571-576.
22. Dhindsa, R.S., P. Plump-dhindsa and T.A. Thorpe, 1981. Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J. Exp. Bot., 32: 93-101.
23. Maehly, A.C. and B. Chance, 1959. The Assay of Catalase and Peroxidase. In: Glick, (D., Ed.). Methods of Biological Analysis. Vol. 1 Interscience Publishers. New York, pp: 357-425.
24. Chen, C.X. and K. Asada, 1989. Ascorbate peroxidase in tea leaves. Occurrence of two isozyme and the differences in their enzymatic and molecular properties. Plant Cell Physiol., 30: 987-998
25. Lawlor, D.W., 2001. Photosynthesis. Springer-Verlag. New York, pp: 386.
26. Bhaumik, G., K.K. Srivastava and W. Selvamurthy, 1995. The role of free radicals in cold injuries. Intl. J. Biomet., 38: 171-175.
27. Breen, A.P. and J.A. Morphy, 1995. Reactions of oxyl radicals with DNA. Free Rad. Biol. Med., 18: 1033-1077.
28. Desimone, M., A. Henk and E. Wagner, 1996. Oxidative stress induce partial degradation of the large subunite of ribulose-1,5- bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. Plant Physiol., 111: 789-796.
29. Inze, D. and V. Montago, 1995. Oxidative stress in plants. Curr. Opin. Biotechnol., 6: 153-158.
30. Foyer, C.H. and J. Harbinson, 1997. The Photosynthetic Electron Transport System: Efficiency and Control. Taylor and Fransis, London, UK, pp: 3-39.
31. Stallings, W.C., K.A. Pattridge, R.K. Strong and M.L. Ludwig, 1984. Manganese and iron superoxide dismutases are structural homologs. J. Biol. Chem., 259: 10695-10699.
32. Van Camp, W., M. Montago and D. Inze, 1994a. Superoxide Dismutases. Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press, Boca Raton, pp: 317-341.
33. Jespersen, H.M. and K.J. Welinder, 1997. Seguence analysis of three novel ascorbate peroxidases from *Arabidopsis thaliana*. Biochem. J., 326: 305-310.
34. Jimnez, A., J.A. Hemmandez and F. Sevilla, 1997. Evidence for the presence of the ascorbate-glutathion cycle in mitochondria and peroxisomes of pea leaves. Plant Physiol., 114: 275-284.
35. Yamaguchi, K., H. Mori and M. Nishimura, 1997. A novel isozyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. Plant Cell Physiol., 36: 1157-1162.
36. Ishikawa, T., K. Yoshimura, K. Sakai, M. Tamoi and M. Takeda, 1998. Molecular characterizeation and physiological role of the glyoxisome-bound ascorbate peroxidase from spinach. Plant Cell Physiol., 39: 23-34.
37. Miyake, C. and K. Asada, 1994. Ferredoxin dependent photoreduction of the monodehydroascorbate radical in spinach thylakoids. Plant Cell Physiol., 35: 535-549.
38. Lopez, F., G. Vansuyt, F. Casse and P. Fourcroy, 1996. Ascorbate peroxidase activity is inhanced in salt-stressed *Raphanus sativus* plants. Physiol. Plant., 97: 13-20.
39. Guan, L.Q. and J.G. Scandalios, 1996. Molecular evolution of maize catalases and their relationship to other eukaryotic and prokaryotic catalases. J. M. Evol., 42: 570-579.
40. Doke, N., Y. Miura, M. S. Leandro and K. Kawakita, 1994. Involvement of Superoxide in Signal Transduction. CRC. Press, Boca Raton, pp: 177-179.
41. Elstner, E. F., 1991. Mechanismes of Oxygen Activation in Different Compartments of Plant Cells. American Society of Plant Physiologists, Rockville, pp: 13-25.
42. Mckersie, B.D. and Y.Y. Leshem, 1994. Stress and Stress Coping in Cultivated Plants. Kluwer Academic Publishers, Dordrecht, pp: 343.
43 Rich, P.R. and W.D. Bonner, 1978. The sites of super oxide anion generation in higher plant mitochondria. Arch. Biochem. Biophysiol., 188: 206-213.