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Effect of Foliar Application of Pyridoxine on Antioxidant Enzyme Activity, Proline Accumulation and Lipid Peroxidation of Maize (*Zea mays L.*) under Water Deficit

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Abstract

The effects of pyridoxine foliar application on protein content, lipid peroxidation, proline accumulation, and superoxide dismutase and catalase activities were studied in water stressed leaves of Zea mays L. S.C 704. The experiment was conducted in factorial with a randomized complete block design with three replications. The experiment was done with combination of water deficit stress at two stages of growth, and with three concentrations of pyridoxine (vitamin B6) (0, 75, 150 ppm). The activity of catalase increased, activity of superoxide dismutase reduced, protein content decreased but proline and malondialdehyde content increased under water stress. Our results also showed that vitamin B6 can not act as an antioxidant in plant at least through the foliar application method.

Keywords: Antioxidant enzymes, maize, malondialdehyde, pyridoxine, water deficit stress

Introduction

One of the most crucial functions of plant cells is their ability to respond to fluctuations in their environment. Understanding the connections between a plant's initial response and the downstream events that constitute successful adjustment to its altered environment is one of the next grand challenges of plant biology. Adverse environmental conditions such as drought and high soil salinity are among the major factors limiting the growth and productivity of land plants. These abiotic stresses can result in the accumulation of reactive oxygen species (ROS) and other toxic compounds (Xiong and Zhu, 2002). Production of ROS during environmental stress is one of the main causes for decreases in productivity, injury, and death that accompany these stresses in plants. ROS are produced in both unstressed and stressed cells, and in various locations (Upadhyaya and Panda, 2004). In plant cells chloroplasts, mitochondria and peroxisomes are important intracellular generators of ROS (Elstner, 1991). Photosynthetic plants have a strong demand for combating oxidative stress and other abiotic stresses (Xiong and Zhu, 2002). An initial oxyradical product, the superoxide radical ($O_2^{-\cdot}$), upon further reaction within the cell, can form more ROS such as hydroxyl radicals and singlet oxygen. ROS play an important role in endonuclease activation and consequent DNA damage (Hagar *et al*., 1996). Plant cells respond defensively to oxidative stress by removing the ROS and maintaining antioxidant defense compounds at levels that reflect ambient environmental conditions (Scandalios, 1997). The mechanisms that act to adjust antioxidant levels to afford protection include changes in antioxidant gene expression (Cushman and Bohnert, 2000). Some well-known antioxidants in plants include glutathione, vitamin C, vitamin E, antioxidant enzymes and carotenoids. Catalases, Superoxide Dismutase, Peroxides, are few antioxidant enzymes. This raises an interesting question as to whether plants also employ pyridoxine as an antioxidant. Pyridoxine, pyridoxal, and pyridoxamine are collectively called vitamin B6. As a cofactor for many enzymatic reactions, especially those involved in amino acid metabolism, vitamin B6 is required by all organisms (Trotel-Aziz *et al*., 2003). However, the biosynthesis pathway of vitamin B6 and its roles in plant growth and development are not well elucidated compared with microbial systems. This vitamin is required for plant development and tolerance to oxidative radicals generated by abiotic stresses (Chen and Xiong, 2005).

The objective of this work was to investigate the effect of water deficit stress and pyridoxine spraying on protein content, activities of antioxidant enzymes, lipid peroxidation, and lignin in leaves of maize.

Materials and methods

Seeds of maize (*Zea mays* L. S.C 704) were sown in loam silt-soil in pots and the plants were grown in greenhouse (with 14hr photoperiod and supplementary light, provided by fluorescent lamps with 250 µmol m^{-2} s^{-1} intensity). The experiment was conducted using three replications in a factorial design. The experiment was done with combination of water deficit stress at two stages of growth, and three concentrations of pyridoxine (0, 75, 150 ppm). The plants were treated with water deficit or pyridoxine of combination of both in two different stages as follows: Vegetative growth phase in which the plants had 10 leaves (V10) and the flowering stage in which the first tassels emerged (R1). The plants were treated for one weak and pyridoxine was sprayed on leaves in the third day of this period. Water potential (Ψ (was -0.5 and -7.5 bar at the initiation of stress (24h after irrigation) and the end of the treatment (end of the weak), respectively. The water potential was measured by pressure plates system.

Sampling

Three days after the foliar application of Pyridoxine, middle leaves of each plant were harvested and frozen in liquid N_2 and kept at −80°C until used for biochemical analysis.

Preparation of extracts

Leaf samples (0.2 g) were homogenized in a mortar and pestle with 3 ml ice-cold extraction buffer (25 mM sodium phosphate, pH 7.8). The homogenate was centrifuged at 18,000 g for 30 min at 4°C, and then the supernatant was filtered through filter paper. The supernatant fraction was used as a crude extract for the assay of enzyme activity and protein content. All experiments were carried out at 4°C.

Catalase activity assay

The reaction mixture contained 100 µl crude enzyme extract, 500 µl 10 mM H_2O_2 and 2400 µl 25 mM sodium phosphate buffer. The decrease in the absorbance at 240 nm was recorded for 1 min by spectrophotometer (Cintra 6, GBC, Australia). Catalase activity was expressed as ΔA/mg protein/min (Cakmak and Horst, 1991).

Superoxide dismutase activity assay

The reaction mixture contained 100 µl riboflavin 1 µM, 100 µl L-methionine 12 mM, 100 µl EDTA 0.1 mM (pH 7.8), 100 µl Na_2Co_3 50 mM (pH 10.2), and 100 µl nitroblue tetrazolium, 75 µM in 2300 µl sodium phosphate buffer 25 mM (pH 6.8), with 200 µl crude enzyme extract in a final volume of 3 ml. Superoxide dismutase activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were illuminated with a fluorescent lamp (120 W); for 15 minutes and then the absorbance at 560 nm was measured. Identical tubes that were not illuminated served as blanks. One unit of superoxide dismutase was defined as the amount of enzyme activity that was able to inhibit by 50% the photo-reduction of NBT to blue formazan. The superoxide dismutase activity of the extract was expressed as ΔA/mg protein/min (Giannopolitis and Ries 1977).

Protein assay

Total protein content was determined by Bradford method (1976), using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Lipid peroxidation Rate

The level of membrane damage was determined by measuring malondialdehyde as the end product of peroxidation of membrane lipids (De Vos *et al*., 1991). In brief, samples were homogenized in an aqueous solution of trichloroacetic acid (10% w/v), and aliquots of the filtrates were heated in 0.25% thiobarbituric acid. The amount of malondialdehyde was determined from the absorbance at 532 nm, followed by correction for the non-specific absorbance at 600 nm. The concentration of malondialdehyde was determined using the extinction coefficient of malondialdehyde (ε=155 mM cm^{-1}).

Proline assay

Leaf Samples (0.2 g) were homogenized in a mortar and pestle with 3 ml sulphosalicylic acid (3% w/v), and then the homogenate was centrifuged at 18,000 g for 15 min. Two milliliters of the supernatant were then put into a test tube into which 2 ml of glacial acetic acid and 2 ml of freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) were added. Tubes were incubated in a water bath for 1 h at 100°C, and then allowed to cool to room temperature. Four milliliters of toluene were added and mixed on a vortex mixer for 20 seconds. The test tubes were allowed to stand for at least 10 min to allow the separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube, and its absorbance was measured at 520 nm by spectrophotometer (GBC, Cintra 6, Australia). The concentration of proline was calculated from a proline standard curve and was expressed as mmol per gram of fresh weight (Bates *et al*., 1973).

Determination of lignin content

Cell wall preparations were obtained by homogenization of frozen samples in water with a mortar and pestle followed by centrifugation at 1000 g and sequential washing of the pellet with EtOH, $CHCL_3$-MEOH (2:1) and acetone and then drying in air. Lignin content of wall preparations was measured via a modified acetyl bromide procedure. The lignin content was determined by measuring of absorbance at 280 nm using specific absorption coefficient value of 20.0 g^{-1} L cm (Iiyama and Wallis, 1990).

Statistical analysis

All data were analyzed using SAS software (SAS Institute Inc, 1997). Each treatment was analyzed in three replications. When analysis of variance (ANOVA) showed significant treatment effects, Duncan's multiple range test was applied to compare the means at $P<0.05$.

Results and discussion

Water stress at V10 and R1 stages reduced protein content of leaf samples, compared to those control plants. Protein content of pyridoxine sprayed-plants was 0.073 mg/g fresh weight at V10 and 0.078 mg/g fresh weight at R1 stage (Tab. 1). However, there was not significant differences between protein content of water stressed plants treated with different concentrations of pyridoxine neither at V10 nor R1 stages (Tab. 2).

The catalase activity was observed in water deficit stress at V10 stage was higher than that at R1 stage. Catalase activity was increased by 7% over control values in water deficit stress at V10 stage (Tab. 1). Also catalase activity on water deficit stress and spraying at V10 stage in 75 and 150 ppm were more than other treatments (Tab. 2) and mentioned enzyme activity had negative correlation with protein content.

Superoxide dismutase enzyme under water deficit stress at V10 stage and at R1 stage 4 and 6 percent has decreased in comparison without stress. Also its activity on spraying pyridoxine at R1 stage had 5.5 percent more activity than at V10 stage. Mentioned enzyme activity on 75 and 150 ppm 10 percent decreased in front of 0 ppm (Tab. 1).

Proline content in leaves of corn on water deficit stress at R1 stage and at V10 stage and without water deficit stress were 0.025, 0.018 and 0.015 mg/g fresh weight respectively. Proline content under water deficit stress at R1 stage and at V10 stage 66 and 20 percent increased in comparison without stress (Tab. 1). Also proline content in various concentration and both times of spraying, water deficit stress at R1 stage showed more praline than other water deficit stress (Tab. 1). Accumulation of praline under water deficit stress showed the most correlation with malondialdehyde and lignin, it had high negative correlation with chlorophyll a too.

Malondialdehyde content of leaves in water deficit stress at R1 stage and at V10 stage and without water deficit stress were 2.15, 1.91 and 1.68 $\mu M\ cm^{-1}$ respectively. It can show that water deficit stress at R1 stage and at V10 stage increased malondialdehyde level 27 and 13 percent in front of without water deficit stress (Tab. 1). Also there was not difference between various treatments of concentrations and spraying times (Tab. 2). It had high correlation with proline content.

Lignin percent in both water deficit stress time (at R1 stage and at V10 stage) and without water deficit stress was 0.033 and 0.030 respectively. It showed that water deficit stress condition increased lignin about 10 percent (Tab. 1). Also there was not difference between various treatments of concentrations and spraying times (Tab. 2). Of course the mentioned trait has high correlation with malondialdehyde and proline content.

In this study, the most activity of catalase was observed on water deficit stress at V10 stage and the lowest of protein was observed this time too, this fact can showed they have contrary relation. Because at V10 stage plant is producing new organs and it has high metabolism, thereupon ROS generation will be increased under water deficit stress condition. It seems that, enhance in produce ROS can increase substrate for catalase reaction. Increased superoxide dismutase and catalase activities in response to water deficit stress have been reported (Halliwell and Gutteridge, 1989). However, in the present study, decrease in superoxide dismutase activities in response to water deficit stress was observed similar this result has been reported (Bai *et al.*, 2005).

Our results showed that drought caused changes in antioxidant enzymes activity in leaves of maize. These results are, in part, similar to those obtained by other researcher (Ghorbanli *et al.*, 2004). Shalata and Tal also reported that in salt-tolerant tomato superoxide dismutase and catalase were effective antioxidant enzymes (Shalata and Tal, 1998).

Reduction of protein content in both of at V10 stage and at R1 stage under water deficit stress plants suggests that water deficit stress may cause generation of ROS. ROS can have effect on protein synthesis which may be related to a decrease in the number of polysomes (Creelman *et al.*, 1990), or causes protein denaturation (Schwanz *et al.*, 1996). However, protein content of leaves of control water deficit was higher than that of water deficit stress. Also, maybe decomposing of protein is essential for produce amino acids that they have role in adaptation to drought stress. Spraying at R1 stage had better effect. It can be depended to more adaptation of plants to water deficit stress and good effect of pyridoxine. Changes in proteins results from a variety of environmental stresses such as water deficit stress reported by Yordanova *et al.,* 2004. Accumulation of proline was reported in many plant species under diverse abiotic stress conditions (Delauney and Verma, 1993), and in this study proline content in leaves increased under water deficit stress condition and it was more at R1 stage, because in this stage nitrogen compound is not used for produced new organs, and they can used for proline producing. Stress condition maybe has effect on produce of proline process. Osmotic stress-induced ABA gene expression in response to dehydration (salt, drought). It has been proposed that ABA is the hormone responsible for inducing proline production in stressed plants (Makela *et al.*, 2003).

Role of pyridoxine was not clear on mentioned trait just in 150 ppm concentration it was decreased. It maybe depended to its effect on produce of proline and other process in proline synthesize. Proline synthesis in this sys-

Tab. 1. Means comparison of water deficit stress , time of spraying and pyridoxine concentration effects on mentioned traits

Treatments		Protein (mg/gF.W)	Catalase (ΔA/ mgprotein/min)	Superoxide dismutase (ΔA/mg protein/min)	Proline (mg/g F.W.)	Malondialdehyde (µM cm-1)	Lignin percent
Water deficit stress	R1 stage	0.076 b	342 b	497 b	0.0251 a	2.15 a	0.033 a
	V10 stage	0.072 c	359 a	486 b	0.0182 b	1.91 b	0.033 a
	Without water stress	0.079 a	335 b	518 a	0.0151 c	1.68 c	0.030 b
Time of spraying	R1 stage	0.078 a	330 b	513 a	0.0201 a	1.94 a	0.032 a
	V10 stage	0.073 b	360 a	487 b	0.0198 a	1.89 a	0.033 a
Pyridoxine concentration	0 ppm	0.077 a	323 c	529 a	0.0197 ab	1.96 a	0.032 a
	75 ppm	0.076 a	367 a	486 b	0.020 a	1.90 a	0.032 a
	ppm 150	0.075 a	346 b	492 b	0.0192 b	1.89 a	0.033 a

V10: Vegetative growth phase in which the plants had 10 leaves. R1: The flowering stage in which the first tassels emerged. All the values followed by the same letter are not statistically different at the $p<0.05$ probability level.

Tab. 2. Means interaction of water deficit stress , time of spraying and pyridoxine concentration effects on mentioned traits

Treatments			Traits					
Water stress time	Time of spraying	Pyridoxine (ppm)	Protein (mg/g F.W.)	Catalase (ΔA/ mgprotein/min)	Superoxide dismutase (ΔA/mg protein/min)	Proline (mg/g F.W.)	Malondialdehyde (µM cm-1)	Lignin percent
V10 stage	V10 stage	0	0.072 de	366 d	529 bcde	0.018 c	2.03 abc	
		75	0.073 cd	446 a	514 cdef	0.019 c	1.90 bcde	0.031 bcde
		150	0.070 de	418 abc	469 efg	0.018 c	1.88 bcde	0.033 abc
	R1 stage	0	0.072 de	368 d	529 bcde	0.018 c	2.01 abc	0.033 abc
		75	0.071 de	383 dc	483 defg	0.018 c	1.94 bcde	0.031 bcde
		150	0.072 de	330 e	495 defg	0.019 c	1.74 cdef	0.034 abc
R1 stage	V10 stage	0	0.073 cd	364 d	562 abc	0.026 a	2.27 a	0.033 abcd
		75	0.070 ef	396 bcd	498 cde	0.025 ab	1.96 abcd	0.033 abc
		150	0.069 e	428 ab	584 a	0.025 ab	2.04 abc	0.034 a
	R1 stage	0	0.073 cd	364 d	562 abc	0.026 a	2.27 a	0.034 a
		75	0.075 cde	382 dc	570 ab	0.025 ab	2.19 ab	0.033 abcd
		150	0.072 de	386 bcd	544 abcd	0.024 b	2.19 ab	0.033 abc
Without stress	V10 stage	0	0.085 ab	271 e	495 defg	0.015 d	1.62 ef	0.030 de
		75	0.085 ab	284 e	413 fhg	0.014 de	1.63 def	0.031 bcde
		150	0.081 bc	295 e	413 fgh	0.015 d	1.74 cdef	0.031 bcde
	R1 stage	0	0.086 ab	320 de	495 defg	0.014 e	1.58 ef	0.025 e
		75	0.090 a	294 e	448 fgh	0.016 d	1.77 cdef	0.031 bcde
		150	0.085 ab	322 de	495 defg	0.15 d	1.74 cdef	0.031 bcde

V10: Vegetative growth phase in which the plants had 10 leaves. R1: The flowering stage in which the first tassels emerged. All the values followed by the same letter are not statistically different at the $p<0.05$ probability level.n This Tab. for nay biochemical analysis although not strange, but confusing. This can be deleted.

tem relies on increased transcription of the D1-pyrroline-5-carboxylate synthesize and prevention of its degradation requires inactivation of the proline dehydrogenase enzyme (Trotel-Aziz *et al*., 2003). The beneficial role of proline in plant stress tolerance as suggested by early correlative studies was recently confirmed by genetic as well as transgenic studies, which demonstrated that proline can increase the tolerance of plants to abiotic stress (Hong *et al*., 2000). This is probably due to, among others, the ability of osmolytes to scavenge reactive oxygen species (Hong *et al*., 2000), although the underlying mechanism is presently unclear.

Malondialdehyde has been known as the end product of peroxidation of membrane lipids. Water deficit stress by increase of generation ROS is responsible for stress-dependent peroxidation of membrane lipids (Upadhyaya and Panda, 2004). H_2O_2 is a signaling intermediate in programmed cell death (Alvarez and Lamb, 1997). It is mostly consumed in peroxidation of membrane lipids and increased mechanical strength and lowers the extensibility of plant cell walls (Schopfer, 1996). Lipid peroxidation is often used as an indicator of increased oxidative damage (Jagtap and Bhargava, 1995). Water deficit stress by increase of ROS can play important role in production of lignin. Hydrogen peroxide as an electron acceptor for wall bound peroxidases, play a major role in polymerization of phenolic monomers in the synthesis of lignin and establishment of covalent bonds between lignin and carbohydrate in cell walls (Fry, 1986). Increase of lignin in cell wall can use for indicate of produce ROS like hydrogen peroxide in plant cell under abiotic stress.

Conclusions

In conclusion, this study has shown that foliar application of pyridoxine can not improved the growth conditions for plants under water deficit stress. The water deficit stress increased lipid peroxidation whereas, activity of antioxidant enzymes and proline increased.

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