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REDUCTION OF PARAQUAT TOXICITY IN MAIZE LEAVES BY BENZYLADENINE

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The protective effect of a cytokinin benzyladenine (BA), against toxicity of paraquat (PQ), a widely used herbicide and a well-known oxidative stress inducer, was investigated in the leaves of maize. Maize leaves have been pretreated with BA at concentrations of 1, 10 and 100 μM and afterwards treated with PQ. At all concentrations tested, BA retarded PQ-induced decreases in chlorophyll, carotenoid and ascorbic acid contents. Pretreatment with 10 and 100 μM of BA significantly increased superoxide dismutase (SOD) activity after 8 h of PQ treatment but there was no significant change in SOD activity in the leaves pretreated with BA at 12 and 24 h. However, peroxidase activity significantly increased in 100 μM of BA pretreated leaves. Results indicate that pretreatment with BA reduce PQ toxicity and BA-treated plants might become more tolerant against oxidative stress.

Keywords: Benzyladenine – paraquat – oxidative stress – peroxidase – superoxide dismutase – ascorbic acid – chlorophyll – carotenoids – *Zea mays*

INTRODUCTION

The herbicide paraquat has been widely used as a non-selective contact herbicide. It is believed that PQ damages plants by photogeneration of superoxide radical ($O_2^{\cdot -}$) and other toxic oxygen species such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) [10, 11]. PQ accepts an electron from photosystem I, and resulting PQ radicals react rapidly with molecular oxygen forming superoxide radical and, subsequently H_2O_2 and $OH^{\cdot}$ [2]. Thus, toxicity of PQ stems from the generation and activity of oxygen species that lead oxidative stress in biological systems. Many biotic and abiotic stresses (pollutants, herbicides, extremes of temperature and high light, high O_2 pressures, salinity and pathogen invasion) cause increases in toxic, reactive oxygen species (ROS) in plant cells [17, 32]. These oxygen species are potentially highly capable of peroxidizing cellular components such as chlorophyll, proteins and membrane lipids, as shown in PQ treated tissues [23, 28]. Therefore, it is not surprising that also phytohormone content is changed. It is very important because plant

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hormones are main signals in root to shoot communication and *vice versa* [21]. In consequence, the change of hormonal balance might play the key role in the sequence of events induced by stress [15, 29]. Also, the inhibition of growth under stress conditions is the result of inhibition of cell division and/or cell elongation. Growth regulators have been reported to promote cell division and cell elongation [22].

Cytokinins, a large group of plant hormones, promote cell division and, acting both in synergy and antagonism with other plant hormones, influence a wide range of events during plant growth. They effect photosynthetic parameters directly (chlorophyll and photosynthetic protein synthesis and degradation, chloroplast composition and ultrastructure, electron transport, opening of stomata) or indirectly (mediated by changes in growth, sinks for photosynthates, morphology and anatomy) [31, 35]. Also, cytokinins delay senescence [21], while oxidative stress usually accelerates this syndrome [27]. Plant cells can be protected against oxidative stress by various radical-scavenging systems, including low molecular-weight antioxidants such as ascorbic acid, glutathione, α-tocopherol and carotenoids, as well as by antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), glutathione reductase (GR; EC 1.6.4.2) which participate in scavenging ROS [12, 13]. It is probable that antisenescent action mechanism of cytokinins might be in relation to the antioxidants or antioxidant enzymes. Besides, cytokinins are known to protect leaves against heat shock-induced damage [14]. On the other hand, leaves of stressed plants usually exhibit reduced cytokinin content [29]. Therefore, the reduction in plant growth under stress conditions could be an outcome of altered hormonal balance and hence exogenous application of growth regulators under stress conditions could be the possible means of reversing the effects of abiotic stress. The present paper reports the results of exogenous application of BA on the changes in some antioxidants and antioxidant enzymes activities in oxidative stressed maize leaves, and whether exogenously applied BA could protect the plants against PQ toxicity in maize leaves.

MATERIALS AND METHODS

Plant material and treatments

Maize (*Zea mays* L. cv RX 947) seeds obtained from Agriculture Research Center in Trabzon were sown in plastic pots (11 cm high, 23 cm top and 13 cm bottom diameter) filled with soil and sand (5 : 1). They were maintained in a growth chamber under a 16-h light/8-h dark regime with a light intensity of 350 μE m^{-2} s^{-1}, 75% relative humidity, and day/night temperatures of 25/22 °C. Ten-day-old maize plants were sprayed until run off with BA at concentrations of 1, 10 and 100 μM, each containing 0.05% Tween 20 as a surfactant for 4 days. Then, for paraquat treatment, leaves of 14-d-old plants were exposed in a surface application to 10^{-4} M paraquat (methyl viologen) in a 0.05% solution of the Tween 20 for 24 h into the light period.

Control plants were only sprayed with 0.05% Tween 20 in distilled water. Foliar samples were collected for analyses at 8, 12 and 24 h following PQ application, immediately frozen in liquid nitrogen and stored at –20 °C for determination of enzyme activities. All treatments were repeated at least three times on different days.

Determination of chlorophyll and carotenoids

For chlorophyll and carotenoid determinations, the leaves were homogenized in 5 ml of 80% acetone and centrifuged at 3000 rpm for 5 min. The optical density of the supernatant was read at 450, 645 and 663 nm with a spectrophotometer. The amounts of total chlorophyll and carotenoids were estimated according to Arnon [1] and Jaspars [16], respectively.

Determination of ascorbic acid

The determination of ascorbic acid was performed using the procedure of Shieh and Sweet [33] with pure ascorbic acid as the standard. Two g samples were homogenized with 0.01 M phosphate-citric acid buffer, pH 3.0, filtered and centrifuged at 5000 rpm, for 5 min at 25 °C. The supernatant was used to determine the ascorbic acid content. The assay mixture consisted of 0.5 ml of 0.01 M phosphate-citric acid, pH 3.0, 2.4 ml of 2,2'-Cu-biquinoline solution (1.0 mM 2,2'-biquinoline and 0.38 mM $CuCl_2 \cdot 2H_2O$) and 0.1 ml of the plant extract. Ascorbic acid content was determined spectrophotometrically at 540 nm.

Determination of SOD activity

Leaf tissue (0.5 g) was ground to a powder in liquid nitrogen and then homogenized in 5 ml of cold 50 mM phosphate buffer (pH 7.0), containing 1 mM EDTA, 0.05% triton, 2% polyvinylpolypyrrolidone, and 1 mM ascorbic acid. The homogenate was filtered through two layers of cheesecloth and centrifuged at 20,000 *g* for 20 min at 4 °C. The supernatant was used for enzyme analyses.

SOD activity assay was based on the method of Beauchamp and Fridovich [3] as modified by Dhindsa and Matowe [9], which measures the inhibition in the photochemical reduction of nitroblue tetrazolium (NBT). In the spectrophotometric assay the 1 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionin, 75 μM NBT, 2 μM riboflavin, and 50 μl of the plant extract. Riboflavin was added last and the reaction was initiated by placing the tubes under fluorescent white light. The reaction was terminated after 10 min by removal from the light source. Reaction product was measured at 560 nm. The volume of supernatant corresponding to 50% inhibition of the reaction was assigned a value of 1 enzyme unit.

Determination of POD activity

0.5 g leaf tissue was ground to a powder in liquid nitrogen and then homogenized in 5 ml of cold 0.2 M sodium phosphate buffer (pH 7.0). The homogenate was filtered through two layers of cheesecloth and then centrifuged at 20,000 *g* for 20 min at 4 °C [6]. The supernatant was assayed for the enzymatic activity. Peroxidase activity was measured using a modification of the procedure described by Rodriguez and Sanchez [30]. The assay mixture contained 1.4 ml of 0.05 M phosphate citrate buffer (pH 4.6), 1 ml of 40 mM guaiacol and 0.5 ml of 26 mM H_2O_2. The mixture was incubated for 15 min at 25 °C. The assays were initiated by the addition of the enzyme extract and the formation of the oxidized tetraguaiacol polymer was monitored at 420 nm for 3 min. Peroxidase activity was expressed as ΔA_{420}/min/g fresh weight.

Statistical analysis

Analysis of variance of data was evaluated by the Statistical Package for Social Sciences (SPSS for Windows 9.0). DUNCAN's Multiple Range Test was employed to determine the statistical significance of differences among the means.

RESULTS

PQ's main activity is exhibited in the light, where photosystem I is responsible for its reduction. So, in this study the plants were illuminated (350 μE m^{-2} s^{-1}) for 8, 12 and 24 h after they were sprayed with PQ. Physiological injuries included bleached and necrotic spots on the adaxial surface of the leaves were observed. Visible effects were dependent on exposure time. Until 8 h of continuous illumination plants treated with 100 μM PQ still looked healthy, but after that time the leaves began to tilt and, bleached and necrotic spots became evident. Visible leaf injury developed on all leaves within 24 h after treatment with PQ.

Effect of BA on photosynthetic pigment contents

Figure 1 represents the total chlorophyll content in maize leaves exposed to PQ after pretreated with BA solutions of different concentrations. A decrease in chlorophyll level induced by PQ was observed after 12 h of treatment. After 24 h of treatment, total chlorophyll content decreased by 41.6% in PQ-treated leaves compared to the control. At all concentrations tested, BA retarded chlorophyll degradation with the greatest effect at 100 μM. In this treatment, the chlorophyll content only decreased by 22.3%. Also, following of the treatment with 100 μM PQ, carotenoid contents of the leaves were significantly decreased (Fig. 2). After 24 h of treatment, the loss of

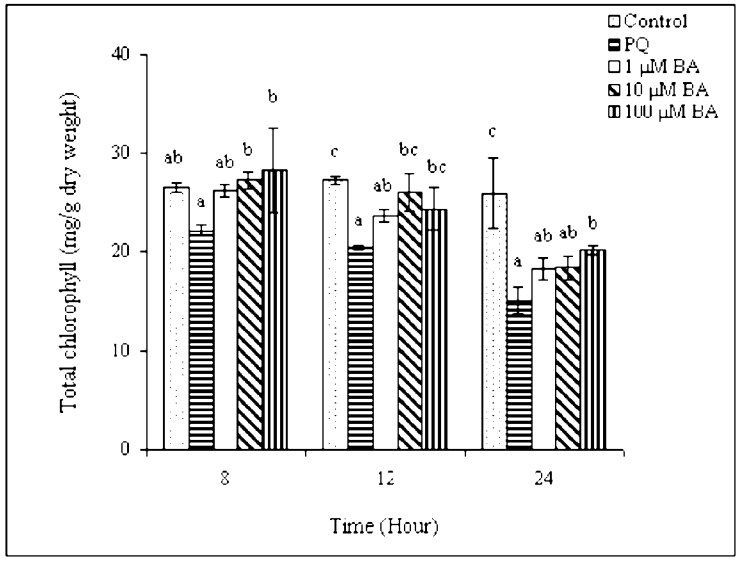

Fig. 1. Changes in total chlorophyll content in maize leaves exposed to PQ after pretreated with BA. Vertical bars represent standard deviation of average of four replications. Within each hour, data followed by different letters are significantly different from each other (P = 0.05) according to Duncan's test

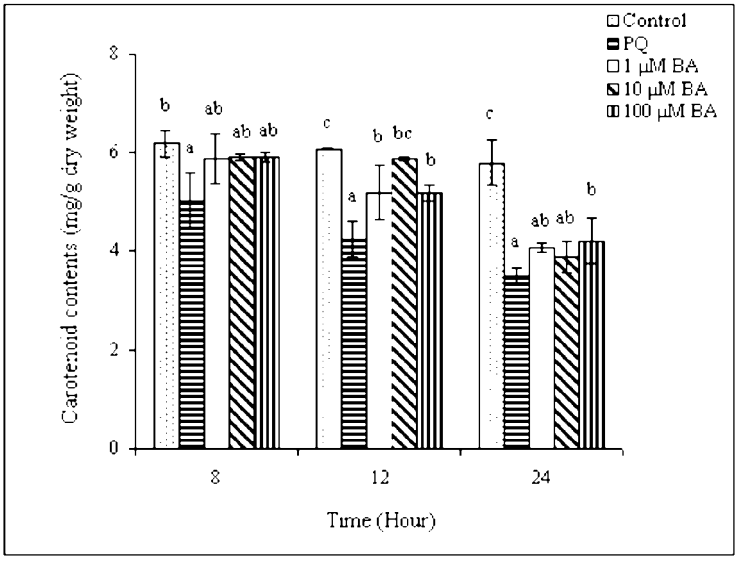

Fig. 2. Changes in carotenoid content in maize leaves exposed to PQ after pretreated with BA. Vertical bars represent standard deviation of average of four replications. Within each hour, data followed by different letters are significantly different from each other (P = 0.05) according to Duncan's test

carotenoid content was 39.7% in the leaves treated with PQ compared to the control. There was no statistically difference in carotenoid contents between the leaves pretreated with BA and those pretreated with water at 8 h after PQ treatment. However, after 12 h of treatment, all concentrations of BA significantly prevented the loss in carotenoid levels induced by PQ. Also, it was found that carotenoid contents of the leaves pretreated with BA were more than those of unpretreated leaves at 24 h and the effect of 100 µM of BA was statistically significant.

Effect of BA on ascorbic acid content

Changes in ascorbic acid level in maize leaves were determined for 8, 12 and 24 h after they were sprayed with PQ. Ascorbic acid content was significantly reduced following of PQ treatment. For example, it was found that ascorbic acid level decreased 69.9% in PQ-treated plants after 24 h of PQ treatment. All concentrations of BA significantly reversed PQ-induced ascorbic acid reduction by inhibiting the decrease in its content with respect to that of PQ-treated leaves. Especially, 1 and 10 µM of BA significantly prevented the decrease in ascorbic acid content. After 24 h of treatment, ascorbic acid content of the leaves pretreated with 1 and 10 µM of BA decreased by 27.5% and 41.3%, respectively (Fig. 3).

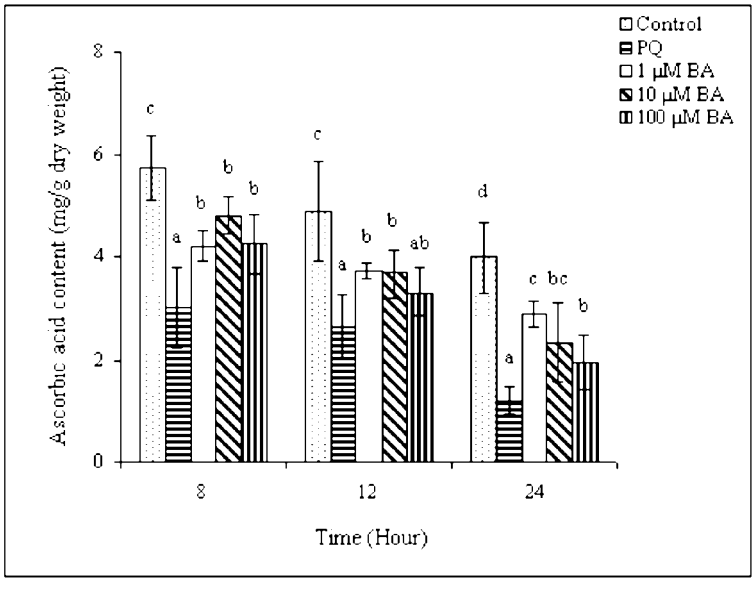

Fig. 3. Changes in ascorbic acid content in maize leaves exposed to PQ after pretreated with BA. Vertical bars represent standard deviation of average of four replications. Within each hour, data followed by different letters are significantly different from each other (P=0.05) according to Duncan's test

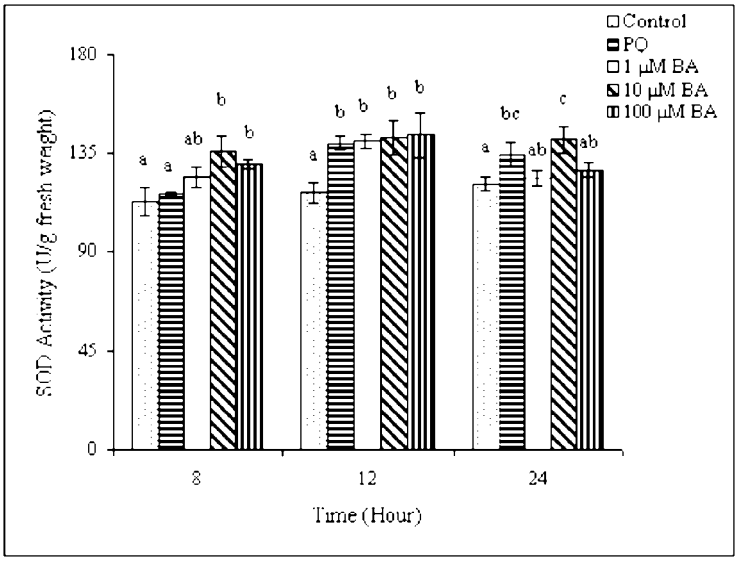

Fig. 4. Changes in SOD activity in maize leaves exposed to PQ after pretreated with BA. Vertical bars represent standard deviation of average of four replications. Within each hour, data followed by different letters are significantly different from each other (P=0.05) according to Duncan's test

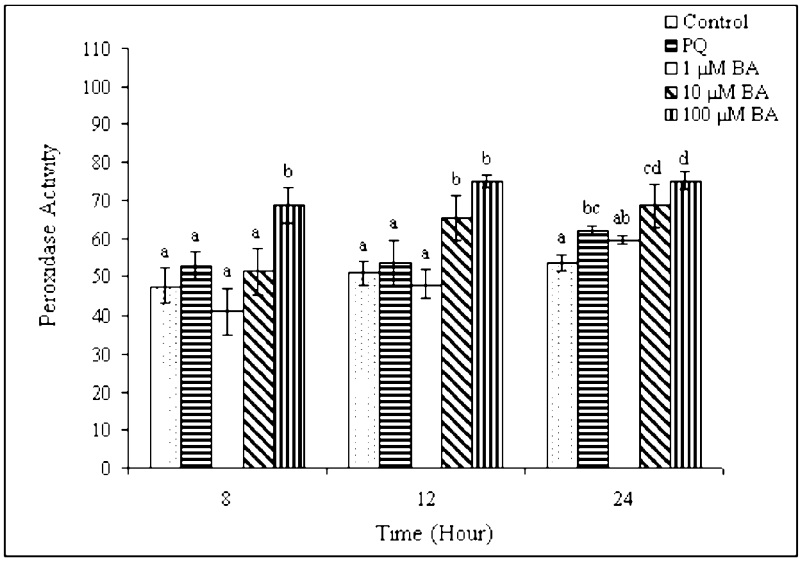

Fig. 5. Changes in POD activity in maize leaves exposed to PQ after pretreated with BA. Vertical bars represent standard deviation of average of four replications. Within each hour, data followed by different letters are significantly different from each other (P=0.05) according to Duncan's test

Effect of BA on SOD activity

The results of investigations into the effect of BA on SOD activity in maize leaves under oxidative stress are shown in Fig. 4. SOD activity gradually increased after the plants were treated with PQ but statistically significant increases were recorded at 12 and 24 h. Pretreatment with BA at concentrations of 10 and 100 μM significantly increased SOD activity after 8 h of PQ treatment. There was no significant change in SOD activity in the leaves pretreated with different concentrations of BA at 12 and 24 h in comparison with the leaves pretreated with water.

Effect of BA on POD activity

After PQ treatment, POD activity gradually increased. It was found that POD activity increased 15.7% after 24 h of PQ treatment. Pretreatment with 100 μM of BA significantly increased POD activity. Also, POD activity in 10 μM of BA pretreated leaves increased at 12 and 24 h but the increase in POD activity at 24 h was not statistically significant. However, pretreatment with 1 μM of BA had no significant effect on POD activity (Fig. 5).

DISCUSSION

In this study, the protective effects of BA against oxidative stress of maize leaves were investigated. Oxidative stress was induced by treating the plants with the herbicide PQ. In our study, 100 μM PQ induced oxidative damage in maize leaves incubated under continuous light. Although the exact role that BA plays during stress remains to be elucidated, the deleterious effect of some stresses can be alleviated by exogenous application of BA [20, 37]. Data presented in our study showed that pretreatment of maize leaves with BA reduced the damage produced by 100 μM PQ to different degrees, according to the studied parameter and the concentrations tested.

Chlorophyll loss, observed as a consequence of PQ treatment, was significantly prevented in maize leaves by the exogenous addition of BA at concentrations of 10 and 100 μM. PQ is known to cause significant losses of chlorophyll via oxygen free radicals [5, 24]. Also, oxidative stress mediated by superoxide and H_2O_2 causes disorganization of cellular and chloroplastic membrane [36] and the breakdown of chlorophyll [19]. Cytokinins have antisenescence properties and prevent chlorophyll breakdown [21, 35]. In addition, BA was recorded to activate chlorophyll synthesis in detached *C. pepo* cotyledons [4].

The results presented also show that all concentrations tested of BA significantly decreased the loss in carotenoid levels induced by PQ after 12 h of treatment. Carotenoids are known to act as efficient quenchers of triplet chlorophyll [25] and singlet oxygen [8], and elevated amounts of these accessory pigments in BA-pretreated leaves should enhance the capacity to limit the damage caused by ROS.

Ascorbic acid is also a key antioxidant in the detoxification of ROS. It was determined that PQ treatment significantly decreased ascorbic acid content in maize leaves. Pretreatment with BA prevented the decrease in ascorbic acid-level induced by PQ. The increment in ascorbic acid content can increase oxidant tolerance in plants. Ascorbic acid has the capacity to directly eliminate several different ROS including singlet oxygen, superoxide and hydroxyl radicals [26]. So, the increment of ascorbic acid content in BA-pretreated maize leaves indicates an enhanced capacity to scavenge ROS. Also, it was reported that both BA and ascorbic acid had similar effects and ameliorate negative effects of water stress in *Cassia angustifolia* [34]. On the other hand, the observed reduction in cytokinins under stress conditions [29] points towards the possibility that BA level might be a limiting factor under stressed conditions and explaining why exogenous application of BA resulted in prevented photosynthetic pigments and ascorbic acid losses.

In addition to non-enzymatic oxygen radical scavengers such as carotenoids and ascorbic acid, antioxidative enzymes could also play an important role in protection against oxidative stress [7, 19a]. So, in this study SOD and POD activities were measured in PQ-treated leaves and it was determined that these enzyme activities gradually increased after PQ treatment. These results are consistent with those obtained by Pastori and Trippi [27] who found increases in SOD and ascorbate peroxidase (AP) activities in maize leaves incubated in PQ. However, Kirtikara and Talbot [18] found that AP activity in PQ-treated plants was similar to the control and SOD level did not significantly change in the PQ-treated tomato plants. Also, we found that pretreatment with 10 and 100 μM of BA significantly increased SOD activity after 8 h of PQ treatment but there was no significant change in SOD activity in the leaves pretreated with BA at 12 and 24 h in comparison with the leaves pretreated with water. However, POD activity was significantly higher in 10 and 100 μM of BA-pretreated leaves than unpretreated leaves. The increases in activities of antioxidant enzymes may contribute to the reduction of PQ toxicity.

Our data show that PQ toxicity in maize leaves can be reduced by BA and there may be a relationship between BA and antioxidant protection against PQ-mediated damage.

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