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Exogenous 2-aminoethanol can diminish paraquat induced oxidative stress in barley (*Hordeum vulgare* L.)

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Abstract

Effects of unfavourable environmental conditions (stresses) induce stressor specific and unspecific short- and long-term responses in plants. Long-term responses depend on intensity and duration of the stress. Short-term effects comprise the accumulation of reactive oxygen species (ROS), membrane damages by the oxidation of fatty acids, and the release of amino alcohols. They can incite higher stress tolerance in plants. In the present study, shoots of barley (*Hordeum vulgare*) were pre-treated with 2-aminoethanol, and, 2 days later, with the oxidative stress inducing herbicide, paraquat. Pre-treatments with 2-aminoethanol increased the stress tolerance in barley by the stabilization of the cell membranes, the enhanced production of superoxide dismutase and catalase, and the stimulation of glutathione metabolism (GSH, GST). These mechanisms of stress tolerance activation by 2-aminoethanol are discussed.

Introduction

Environmental factors such as water, temperature and nutritional status affect the biochemical responses of plants to stress. Plants have genetically controlled mechanisms that allow them to live and grow under stress (Boyer 1982). Induction of tolerance to stressors (e.g., drought, heavy metals, pathogens, herbicides) is a major criterion for the development of plant growth regulators. The cellular and molecular mechanisms of plants to different stressors have been intensively investigated (e.g., Streb and Feierabend 1999; Bestwick et al. 2001; Mascher et al. 2002; Lascano et al. 2003). Stress conditions are related to an enhanced generation of reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide and hydroxyl radicals (Asada 1999). ROS are destructive to DNA, protein and lipids. Changes in the content antioxidant molecules (e.g., ascorbic acid, glutathione, carotenoids) and antioxidative enzymes (e.g., superoxide dismutase [SOD], peroxidases [POD], catalase [CAT]) have been related to oxidative stress (Gogorcena et al. 1995; Bergmann et al. 1999). ROS detoxification involves dismutation of superoxide radicals by SOD, to hydrogen peroxide, a partially reduced ROS (Bowler et al. 1992; Mascher et al. 2002). The enzymes CAT and POD detoxify the cellular hydrogen peroxide (Fridovich 1983; Bowler et al. 1992). In plants, various types of SOD metalloproteins have been found in different cellular

compartments. Cu/Zn isoforms are located in cytosol and chloroplast, Mn-SOD is mitochondrial and Fe-SOD is chloroplastic (Bowler et al. 1992; Mascher et al. 2002).

Several important biogenic amines have been found in plants that have morphogenetic and stress-physiological significance. 2-Aminoethanol (2-AE), as used in this paper, is a naturally occurring compound in plant cells. It plays an important role in embryogenesis, organ development and the formation of intracellular membranes. It is well known that plant exposure to stress (e.g., drought, heavy metals, paraquat etc.) results in disintegration of biological membranes and the decomposition of their phospholipids, and in the liberation of 2-AE and choline. The elevated level of amines due to the exposure to stress induces an alarm reaction that activates cellular resistance and tolerance mechanisms (Bergmann et al. 1994; Leinhos and Bergmann 1995). It has also been suggested that external 2-AE might function as a signal for initiating stress tolerance and may serve as a membrane stabilizer, too. One hundred fifteen field trails and 140 scale experiments on farms at 17 different sites in East Germany with different climate and soil conditions have been carried out to investigate the influence of 2-AE and its metabolite, choline, on the yield and quality of barley, wheat, rye, and potato. Under these field conditions and treatments, yield accessions of 5% (under low stress conditions) were increased to 20% (under severe stress conditions) (Bergmann et al. 1999). Moreover, for several years, mechanism and reproducibility of these effects have been studied in pot experiments under treatments with abiotic stressors (Bergmann et al. 1983; Eckert et al. 1988; Bergmann et al. 2002; Mascher 2002). Higher plant vitality was the main reason for the yield-improving effect of the native amino alcohol application in unfavourable environments (Bergmann et al. 1999, 2002). Moreover, the stronger physiological activity in tillers increased the transport of assimilates and N-compounds to the root region (Eckert et al. 1988). Root growth has therefore also been stimulated by 2-AE or choline (Lippmann et al. 1995).

The herbicide paraquat (methyl viologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride) exerts its phytotoxic effects by catalyzing the transfer of electrons from Photosystem I to molecular oxygen. Resulting accumulation of superoxide radicals in chloroplasts cause lipid peroxidation and membrane destruction. Paraquat can readily penetrate into leaf tissue through the cuticula. Shortly after spraying, photosynthetic activity decreases, membranes rupture, and the treated parts of the plant wilt due to the loss of turgor within hours (Lehoczki et al. 1992; Chang and Kao 1997). In the final stage, necrotic lesions appear on the leaf surface and the plant dies.

In a previous paper (Mascher et al. 2004), we characterized the effects of 2-AE pre-treatments on barley stressed by drought and paraquat. The higher stress tolerance of 2-AE pre-treated plants was denoted by increased SOD production and the stabilization of membranes in chloroplasts. In the present work, we examined the short-term responses of the antioxidant systems of barley plants pre-treated with 2-AE to the oxidative stress exerted by paraquat.

Materials and methods

Plant material and growth conditions

Barley (*Hordeum vulgare* L. cv. Alexis) plants (13 each) were grown in pots with 0.5 kg quartz sand (particle size 0.1 – 0.63 mm, water capacity 18%). The following nutrients were added (amounts per pot): 1.0 g N as NH_4NO_3, 0.5 g P as KH_2PO_4, 1.0 g K as K_2SO_4, 0.6 g Mg as $MgCl_2$ and 3.0 g Ca as $CaCO_3$ powder, 27.2 mg Fe as $FeCl_3$ and 27.2 mg Fe as Fe–EDTA, 13.6 mg Mn, 6.8 mg Cu, 3.4 mg Zn, 1.36 mg B, and 1.36 mg Mo. The experiment was carried out in a growth chamber with a light/dark regime of 16 h/22 °C and 8 h/16 °C. Light intensity was 100 μE m^{-2} s^{-1}.

2-Aminoethanol and paraquat treatment

At the 3-leaf stage of the 10-day-old seedlings half of the pot cultures were spayed (pre-treated) with aqueous 2-aminoethanol (Merck) solution (0.5 mg 2-AE/plant). Two days after 2-AE pre-treatment, all of the potted plants were sprayed with aqueous solutions containing 0, 0.1, 1.0 and 5.0 mM paraquat (SIGMA) in sublethal concentrations and exposed to continuous light (100 μE m^{-2} s^{-1}). The sublethal doses of paraquat had been determined in previous experiments. Four replicates were used per treatment.

One day after paraquat application, barley plants were harvested.

Harvest procedure

After harvesting, shoots and roots were separated. The plant material was frozen and lyophilized for the determination of the dry weight. The dry material was ground in a stainless steel mill (IKA, A11 basic). The powder was then stored at −20 °C until analysis.

Determination of chlorophyll and carotenoids

Chlorophyll and carotenoids were extracted by homogenizing 100 mg (dry weight) of powdered leaf tissue (powder) in 10 ml acetone solution (80% v/v acetone, 15% water, 5% conc. NH_3 solution [25%]). After centrifugation at $4000 \times g$ for 10 min, chlorophyll and carotenoid content in the supernatant were analyzed spectrophotometrically at 480, 645, 647, 652, 663, 664 and 750 nm, as described by Schopfer (1989).

Determination of malondialdehyde (MDA)

The level of lipid peroxidation was analyzed in terms of MDA content. Shoots were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at $10{,}000 \times g$ for 15 min. To 1 ml aliquot of the supernatant, 4 ml of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at $10{,}000 \times g$ for 5 min, the absorbance was determined at 532 nm. The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} (Sairam and Srivastava 2002).

Preparation of extracts for enzyme assays and protein determination

The lyophilized and ground shoot samples (500 mg) were homogenized on ice with an Ultraturrax (Jahnke and Kunkel, Stauffen, Germany) for 2 min in 3 ml of homogenizing solution containing 100 mM potassium phosphate buffer, 2% (w/v) polyvinylpyrrolidone and 2 mM dithioerythritol (pH 7.2) and extracted for 2 h at 4 °C under gently stirring. The homogenate was filtered through one layer of Miracloth (Calbiochem), centrifuged at 10,000 g and 4 °C for 20 min. The supernatant was dialyzed against 4 l of 5 mM potassium phosphate buffer at 4 °C for 8 h (several changes of the buffer). The dialyzed extracts were cleared by centrifugation ($10{,}000 \times g$, 10 min) and used for further analyses.

The protein concentration was determined according to the method of Lowry et al. (1951) using human serum albumin as a standard. Absorbance was determined using a UV/VIS spectrophotometer.

Superoxide dismutase (SOD) isoenzymes

The non-denaturing PAGE of the crude protein extracts was carried out on 12% polyacrylamide gels ($120 \times 110 \times 1$ mm) using a Biometra electrophoresis system (Biometra, Göttingen) accorsding to the manufacturers specifications. Protein solutions (40 μg) were loaded on to the gel and separated at 4 °C, 120 V and 30 mA for 3 h.

Immediately after electrophoresis, the activity of SOD isoenzymes was visualized using the NBT staining procedure (Beauchamp and Fridovich 1971; Mascher 2002). The gel was incubated in 2.5 mM nitroblue tetrazolium chloride solution (NBT) at 25 °C for 20 min and then soaking in 50 mM potassium phosphate buffer at pH 7.5 containing 0.028 mM riboflavin and 0.3% (v/v) tetramethylethylenediamine (TEMED) for 30 min in the dark. The gel was then illuminated to induce the photoreactive staining-process caused by the SOD activity. The stained gels were scanned and analyzed with a raytest system (Raytest Isotopenmessgeraete, Straubenhardt/Germany; scanning software AIDA 2.0).

SOD-isoenzymes were differentiated by performing the activity stains gel previously incubated at 25 °C for 30 min in 50 mM potassium phosphate buffer at pH 7.5, containing 2 mM KCN or 5 mM H_2O_2. Cu/Zn-SODs are inhibited by KCN and H_2O_2; Fe-SODs are inactivated by H_2O_2 but resistant to KCN and Mn-SODs are resistant to both inhibitors (Fridovich 1986).

Peroxidases (POD) activity

Guaiacol POD activity was based on the determination of guaiacol oxidation (Lagrimini 1991;

Mascher 2002). In the presence of H_2O_2, POD catalyzes the conversion of guaiacol to tetraguaiacol (orange product). This reaction can be recorded at 470 nm. The reaction mixture contained 100 mM citric acid/potassium phosphate buffer (pH 5.0), 33 mM guaiacol and 0.3 mM H_2O_2. Horse-radish POD (SIGMA) was used as standard enzyme.

Catalase (CAT) activity

CAT activity was determined by monitoring the rate of loss of H_2O_2 at 25 °C (Azevedo et al. 1998). The 1 ml reaction mixture contained 50 mM phosphate buffer (pH 7.5) and 2 mM H_2O_2. The reaction was initiated by the addition of 25 μl of desalted leaf extract. Activity was determined by the decrease in A_{240} ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$) due to H_2O_2 consumption. Standard curves were created with purified CAT from *Aspergillus niger* (SIGMA) and non-enzymatic H_2O_2-decomposition was determined using boiled barley leaf extracts.

Glutathione reductase (GR) activity and total glutathione content

GR activity was determined by recording the increase in absorbance in the presence of oxidized glutathione (GSSG, SIGMA) and DTNB (5,5′-dithiobis-2-nitrobenzoic acid) (Sairam et al. 2002). The 1 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 1.5 μmol DTNB, 0.2 μmol NADPH and 25 μl desalted enzyme extract. The reaction was started by adding 0.2 μmol GSSG. The increase in absorbance at 412 nm was recorded spectrophotometrically at 30 °C over a period of 2 min. Standard curves were developed using purified GR from bakers yeast (SIGMA).

Extraction of glutathione was carried out by grinding 100 mg freeze dried plant material (Ultraturrax) in 2 ml of 5% (w/v) sulfosalicylic acid at 4 °C. After centrifugation at 10,000 × *g* for 15 min (4 °C), the supernatants were immediately assayed. The total glutathione content (GSH plus GSSG) was determined spectrophotometrically at 412 nm, using GR from bakers yeast, DTNB and NADPH (Griffith 1980).

Glutathione-S-transferase (GST) activity

GST activity was determined using the method of Sudhakar et al. (2001). The reaction mixture consisted of enzyme extract, and 100 mM potassium phosphate buffer (pH 6.5) containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM reduced glutathione. The enzyme activity was measured at 340 nm. Standard curves were created with purified GST from horse liver (SIGMA).

Statistical analysis

The experiments were repeated several times, at least three times under the same conditions with essentially the same results. All measurements were subjected to analyses of variance (ANOVA) to determine the least significant difference (LSD). The significance in this paper refers to statistical significance at the $p < 0.05$ level.

Results

Stabilization of biomass and the content of chlorophyll and carotenoids of paraquat stressed barley plants by 2-aminoethanol

Paraquat induced oxidative stress (intensities 0.1–5.0 mM) reduced fresh weight (Figure 1) and chlorophyll, but not carotenoid content (Table 1) of not 2-AE pre-treated barley shoots within 24 h. Pre-treatment with 2-AE altered the response to paraquat. Fresh weight was highest in shoots with 2-AE pre-treatment and low paraquat stress (0.1 mM, Table 1). The 2-AE applications stabilized plants in biomass and pigment content, but the protection against higher paraquat concentrations was not complete (Table 1 and Figure 1). We detected the best protection of chlorophyll and carotenoids by 2-AE at high paraquat stress.

Exogenous 2-AE prevented paraquat induced lipid peroxidation

In barley plants not protected by 2-AE pre-treatment the paraquat induced oxidative stress

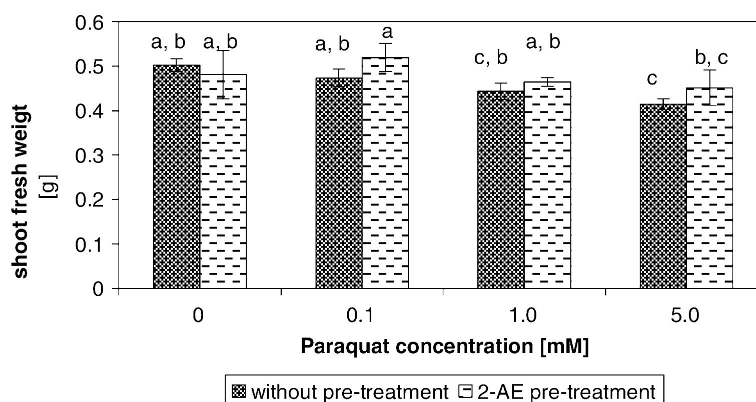

Figure 1. Paraquat caused reduction of the biomass and its stabilization by 2-AE pre-treatment (bars represent ± SD, $n = 12$ replicates; treatments with the same letter are not significantly different at $P < 0.05$ as assessed by LSD test).

Table 1. Changes of the contents of chlorophyll and carotenoids in shoots of 2-AE pre-treated and not pre-treated barley plants at different paraquat concentrations.

	0 mM paraquat	0.1 mM paraquat	1.0 mM paraquat	5.0 mM paraquat
Chlorophyll (mg g^{-1} DW)				
Without pre-treatment	12.18 ± 0.76 (a)	11.08 ± 0.22 (b, c)	11.38 ± 0.33 (b)	10.71 ± 1.08 (c)
2-AE pre-treatment	11.89 ± 0.79 (a)	11.37 ± 0.34 (b)	11.42 ± 0.59 (b)	11.51 ± 0.69 (a, b)
Carotenoids (mg g^{-1} DW)				
Without pre-treatment	0.477 ± 0.020 (a)	0.463 ± 0.016 (a)	0.479 ± 0.014 (a)	0.464 ± 0.005 (a)
2-AE pre-treatment	0.458 ± 0.034 (b)	0.454 ± 0.014 (b)	0.498 ± 0.010 (c)	0.497 ± 0.005 (c)

Values ± SD, $n = 12$ replicates; treatments with the same letter are not significantly different at $p < 0.05$ as assessed by LSD test.

resulted increasingly in the formation of malondialdehyde (MDA) as an indicator of membrane lipid peroxidation (Figure 2). A pre-treatment with 2-AE uncoupled the formation of MDA from the intensity of paraquat stress.

Stimulation of ROS detoxifying enzymes by 2-AE

To test the hypothesis that 2-AE antioxidant capacity may be mediated through their scavenging properties, SOD isoenzymes were analyzed as

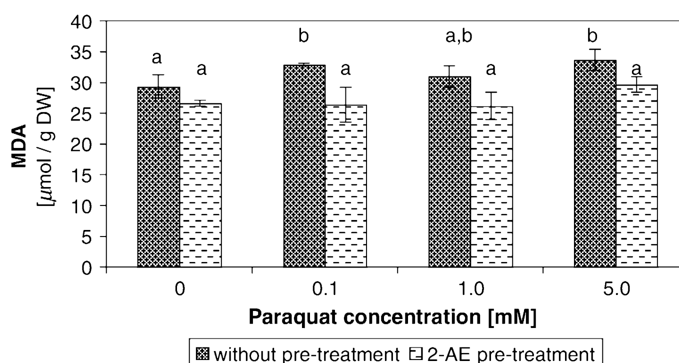

Figure 2. Repression of malondialdehyde formation (lipid peroxidation) in shoots of 2-AE pre-treated barley under paraquat stress (bars represent ± SD, $n = 12$ replicates; treatments with the same letter are not significantly different at $p < 0.05$ as assessed by LSD test).

indicators of a protective effect of 2-AE against oxidative stress. The protein extracts from barley shoots were subjected to non-denaturing PAGE showed three distinct bands of SOD isoenzymes (Figure 5), which were identified as three Cu/Zn-SODs on the basis of their sensitivity to KCN and H_2O_2. Cu/Zn-SODs were designated as Cu/Zn-SOD I, Cu/Zn-SOD II and Cu/Zn-SOD III according to their increasing mobility in polyacrylamide gel. Cu/Zn-SOD II was the major isoenzyme in shoots. The major Cu/Zn-SOD in shoots occurs in chloroplasts (Bowler et al. 1992; Mascher 2002). We could not detect a new SOD isoenzyme in plants treated with paraquat, but the SOD activity changes in barley shoots to the superoxide radical generating effect of paraquat in chloroplasts remained moderate. Slight increases in SOD activity were recorded for plants, not pre-treated with 2-AE, at low paraquat concentrations (Figure 3). Comparatively higher were the increases in SOD activity when plants had been pre-treated with 2-AE, were subsequently exposed to 0 and 0.1 mM paraquat.

The major differences between 2-AE pre-treated and not-pre-treated barley plants under paraquat stress were found in the activities of hydrogen peroxide detoxifying enzymes. Paraquat significantly inhibited catalase and guaiacol peroxidases in the absence of a 2-AE pre-treatment. When 2-AE pre-treated plants were exposed to paraquat, the activity of CAT was significantly stimulated (Figure 4) and reached nearly to the 2-fold that in plants without pre-treatment at the level of 1.0 mM paraquat. The inactivation of CAT by paraquat was prevented by 2-AE.

The multiple molecular forms/isoenzymes of peroxidases were inhibited by all paraquat treatments. The pre-treatment with amino alcohol could not prevent this inhibition (Figure 4).

Effects of 2-AE on glutathione, GR and GST under paraquat stress

Paraquat exposure of untreated barley shoots resulted in a significantly enhanced content of glutathione (Figure 5). This enhancement of glutathione concentration was stimulated by a 2-AE pre-treatment. Usually, the total content of this antioxidant increased about 2-fold after the application of the amino alcohol relative to untreated plants. The ratio between reduced and oxidized form of glutathione decreased with increasing paraquat concentration (Table 2). This oxidative effect of paraquat stress could be prevented by a leaf application of the amino alcohol.

The activity of the enzyme GR was not influenced in the shoots of all paraquat treated plants. In opposite to this findings, the pre-treatment with 2-AE resulted in significantly higher activity of GR in response to the lowest paraquat dose (Figure 5).

GST activity was significantly increased in the shoots of untreated barley plants treated with the highest paraquat concentration (5.0 mM) (Figure 5). The application of 2-AE (at two days prior to paraquat treatment) elevated the activity

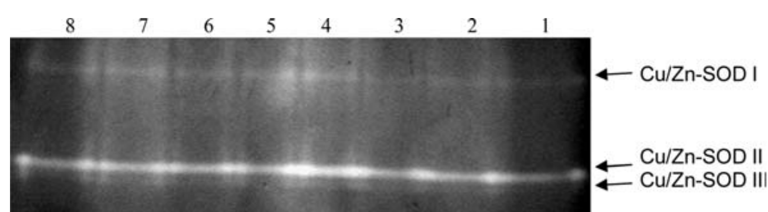

Figure 3. Protective effect of a pre-treatment with 2-AE to shoots of paraquat stressed barley plants expressed by the increase of SOD activity within 24 h. Zymograms in native PAGE refer to SOD from shoots sprayed with increasing paraquat concentrations. The gel was loaded with 40 μg of protein. lane 1 – 0 mM paraquat; lane 2 – 2-AE +0 mM paraquat; lane 3 – 0.1 mM paraquat; lane 4 – 2-AE +0.1 mM paraquat; lane 5 – 1.0 mM paraquat; lane 6 – 2-AE +1.0 mM paraquat; lane 7 – 5.0 mM paraquat; lane 8 – 2-AE +5.0 mM paraquat.

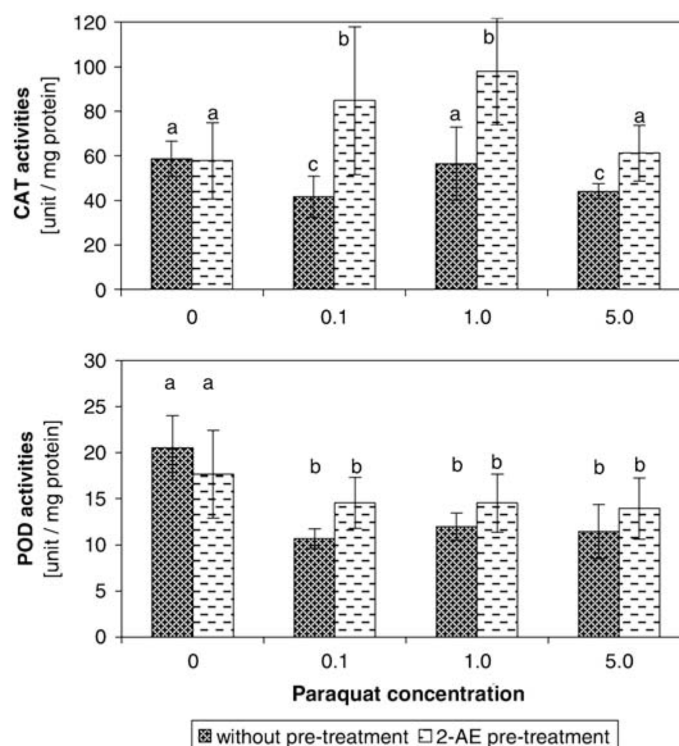

Figure 4. Comparison of the activities of catalase (CAT) and peroxidases (POD) in paraquat stressed barley plants and protective effects of the 2-AE pre-treatment (bars represent ± SD, $n = 12$ replicates; treatments with the same letter are not significantly different at $p < 0.05$ as assessed by LSD test).

of GST at low and medium paraquat concentrations to the 1.5-fold relative to plants without pre-treatment. GST activities were indentical for untreated plants at 5.0 mM paraquat and for 2-AE pre-treated plants at 0.1–5.0 mM paraquat.

Discussion

Barley shoots exposed to the oxidative stress of paraquat for 24 h showed short term responses whose nature and extent could be influenced by a pre-treatment with 2-AE. The herbicidal effects of paraquat reduced fresh weight and chlorophyll, but not the carotenoid content of the wilting plants (Table 1; Figure 1), whose membranes were slightly damaged by lipid peroxidations (Figure 2). All of these detrimental effects were relieved by a pre-treatment of the plants with 2-AE. Their pre-treatment resulted generally in a higher glutathione concentration and in a stabilized level of reduced glutathione by activation of GR than in plants without 2-AE application (Figure 5). Glutathione and glutathione dependent processes play a key role in plant stress response (Foyer et al. 1997). Plants with an increased capacity of glutathione biosynthesis respond faster to environmental challenges (Foyer et al. 1994, 1997; Noctor and Foyer 1998). In 2-AE treated plants, the higher glutathione content was accompanied by an enhanced activity of GST after paraquat treatment. Levine et al. (1994) postulated also increased activities of GST in response to an elicitor stimulated oxidative burst. We suppose, that the higher glutathione content, the stimulation of GR activity, and the increased GST activity in response to 2-AE pre-treatment and paraquat stress allow a better detoxification of the products of lipid peroxidation and membrane deterioration, which generated as a result of oxidative stress. However, a similar role of GST in counteracting abiotic stress imposed by herbicides has received far less attention due to the protective role of GST in catalysing the conjugation and detoxification of many types of herbicides including thiocarbamates, chloroacetanilides, diphenyl ethers

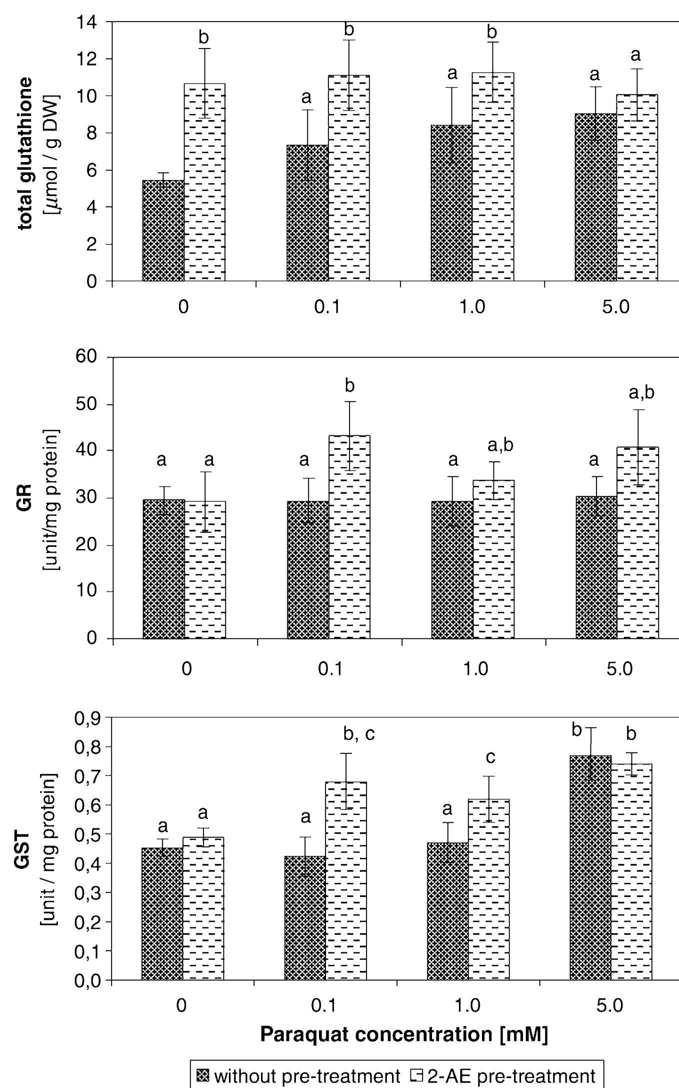

Figure 5. Comparison of the content of total glutathione, the activities of glutathione reductase (GR) and glutathione-S-transferase (GST) in paraquat stressed barley plants and protective effects of the 2-AE pre-treatment (bars represent ± SD, $n = 12$ replicates; treatments with the same letter are not significantly different at $P < 0.05$ as assessed by LSD test).

Table 2. Changes in ratio of reduced and oxidized form of glutathione in shoots of 2-AE pre-treated and not pre-treated barley plants at different paraquat concentrations.

	0 mM paraquat	0.1 mM paraquat	1.0 mM paraquat	5.0 mM paraquat
Ratio GSH/GSSG				
Without pre-treatment	0.48	0.44	0.47	0.42
2-AE pre-treatment	0.49	0.49	0.47	0.49

and paraquat (Cole 1994; Cummins et al. 1999) Knörzer et al. (1996) postulated the potential roles of GST in herbicide tolerance by prevention of oxidative stress. In agreement with this hypothesis, Mascher et al. (2004) showed by electron micrograph studies that paraquat induces membrane

damages in chloroplasts of barley plants, which could be limited by pre-treatment with 2-AE.

As mentioned above, we previously demonstrated that 2-AE pre-treatment of barley plants can cause tolerance to drought and paraquat stress (Bergmann et al. 2002; Mascher 2002; Mascher et al. 2004). However, our results indicate that 2-AE functions like a low stressor and a protector against stress. The application of 2-AE leads to unusual high concentrations of this membrane component in the extracellular space, might be an 'alarm signal', because the outside of the plasmalemma is in general almost devoid of those free components. In response to this exogenous 2-AE, we measured higher activities of SOD (Figure 3) and a higher concentration of total glutathione (Figure 5). This metabolic process to higher stress tolerance consumes assimilates and energy, and results in lower contents of proteins (data not shown) and photosynthetic pigments in 2-AE pre-treated reference plants (Table 1).

Our previous (Mascher et al. 2004) and present results indicate that the main difference in the antioxidant system responses and the associated tolerance/susceptibility of barley plants with and without 2-AE pre-treatment to drought stress or paraquat application modulates the glutathione cycle (Figure 5) and the capacity of ROS detoxification by stimulation/protection of SOD (Figure 3) and CAT (Figure 4) under stress conditions. Many studies have analyzed the phenomenon of cross-tolerance (Bowler et al. 1992; Smirnoff 1993; Iturbe-Ormaetxe et al. 1998). Lascano et al. (2003) reported that drought resistant cultivars of wheat did not exhibit cross-tolerance to paraquat stress. On the contrary, they showed higher sensitivity to paraquat than the drought sensitive cultivar, as indicated by the response of the antioxidant system. These results are consistent with the idea that amino alcohol pre-treated plants are better adapted than untreated ones to respond to different stressors, and suggest that this difference could be related to a higher ROS detoxifying capacity. Plants with high levels of antioxidants, either constitutive or induced by stress tolerance activating substances, are more tolerant to oxidative damages. Consequently, strengthening of the antioxidant mechanisms by plant pre-treatment with amino alcohol could be helpful in the development of a broad tolerance to adverse stress conditions.

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