Comparison of resistance to drought of three bean cultivars

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Abstract

The aim of the present work was to evaluate oxidative stress and plant antioxidant system of three contrasting bean (*Phaseolus vulgaris* L.) genotypes in the response to drought. Drought was imposed 14 d after emergence, by withholding water, until leaf relative water content reached 65 %. Water stress increased lipid peroxidation (LPO), membrane injury index, H_2O_2 and $OH^{\bullet}$ production in leaves of stressed plants. Activities of the antioxidative enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APOX) increased significantly under water stress in all the studied cultivars, while catalase (CAT) increased in cvs. Plovdiv 10 and Prelom, but decreased in cv. Dobrudjanski ran. Furthermore cv. Plovdiv 10 which had the highest APOX and CAT activities also showed the lowest increase in H_2O_2 and $OH^{\bullet}$ production and LPO while cv. Dobrudjanski ran showed the lowest increases (and often the lowest values) in the antioxidant enzyme activities and the highest increases of H_2O_2 and $OH^{\bullet}$ production, and LPO. On the basis of the data obtained we could specify cv. Plovdiv 10 and cv. Prelom as drought tolerant and cv. Dobrudjanski ran as a drought sensitive.

Additional key words: lipid peroxidation, oxidative stress, *Phaseolus vulgaris* L., reactive oxygen species.

Introduction

Drought affects not only water relations, but also induces stomatal closure and decreases the photosynthetic rate and growth. Closure of stomata decreases CO_2 concentration in leaf mesophyll tissue and results in an accumulation of NADPH. Under such conditions, where NADP is a limiting factor, oxygen acts as an alternate acceptor of electrons from the thylakoid electron transport chain, resulting in the formation of superoxide radical ($O_2^{\bullet}$) (Cadenas 1989).

Superoxide radical and its reduction product H_2O_2 are potentially toxic compounds, and can also combine by the Haber-Weiss reaction to form the highly toxic hydroxyl radical ($OH^{\bullet}$) (Sairam *et al.* 1998). A large number of reports deal with the deleterious effects of reactive oxygen species (ROS), which production is stimulated under water stress conditions (Malenčić *et al.* 2000, Blokhina *et al.* 2003). ROS cause lipid peroxidation and consequently membrane injuries, protein degradation, enzyme inactivation (Sairam *et al*. 2005), thus induce oxidative stress. Tolerant genotypes, therefore, should not only be able to retain sufficient water under drought, but should also have a highly active system to protect against oxidative injury. Plants possess several tissue antioxidant enzymes for protection against ROS, such as superoxide dismutase (SOD), ascorbate peroxidase (APOX) and catalase (CAT). During drought conditions high activities of antioxidant enzymes are associated with lower levels of lipid peroxidation, being connected to drought

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Abbreviations: APOX - ascorbate peroxidase; CAT - catalase; I % - membrane injury index; LPO - lipid peroxidation; MDA - malondialdehyde; PPFD - photosynthetic photon flux density; ROS - reactive oxygen species; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloroacetic acid.
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tolerance (Bowler *et al.* 1992). In fact, an increased metabolic capacity of these enzymes may be part of a general antioxidative system in plants involving regulation of protein synthesis or gene expression (Foyer *et al.* 1994, Scandalios *et al.* 1997).

The aim of the present investigation, conducted with three bean genotypes, was to evaluate the membrane tolerance to water deficit and to characterize the effect of water stress on some of plant antioxidant systems.

Materials and methods

Three contrasting cultivars of common bean (*Phaseolus vulgaris* L.) were used: cv. Plovdiv 10, cv. Dobrudjanski ran and cv. Prelom. Seeds were washed in distilled water, surface sterilized and germinated on moist filter paper in Petri dishes, maintained at 28 ºC in the dark, for 3 d. After germination, seedlings were cultivated in pots filled with 3.5 kg of dry sand in a greenhouse (3 seedlings per pot). Pots were watered to full capacity each second day, and irrigated with the Hoagland nutrient solution twice a week. Plants were placed in a semi-controlled greenhouse, under natural light (maximum PPFD up to 1200 μmol m^{-2} s^{-1}), day/night temperature 27 - 32/ 15 - 18 ºC, and relative humidity between 75 % (morning) and 50 % (late afternoon). Water stress was progressively induced in 14-d-old plants by withholding irrigation until leaf relative water content reached 65 %. The measurements were carried out on the first trifoliate leaf at the end of the stress period.

Hydrogen peroxide content was determined according to Alexieva *et al.* (2001). Leaf tissue (500 mg) was homogenized in ice bath with 5 cm^3 of a cold 0.1 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged (12 000 *g*, 15 min, 4 ºC) and 0.5 cm^3 of the supernatant was added to 0.5 cm^3 of 100 mM potassium phosphate buffer (pH 7.0) and 1 cm^3 of 1 M KI. The absorbance was read at 390 nm on spectrophotometer *UV-160A* (*Shimadzu*, Tokyo, Japan). The production of hydroxyl radicals was measured in a trapping reaction with dimethyl sulfoxide. As a primary product of this reaction, the concentration of methanesulfuric acid was measured colorimetricaly with Fast Blue BB salt (Babbs and Gale 1987).

For the measurement of lipid peroxidation of leaf cell membranes, the thiobarbituric acid (TBA) test, which determines malondialdehyde (MDA) as a final product of lipid peroxidation, was used (Heath and Packer 1968). Leaf material (500 mg) was homogenized in 5 cm^3 of 0.1 % (m/v) TCA solution. The homogenate was centrifuged (10 000 g, 20 min, 4 ºC) and 0.5 cm^3 of supernatant was added to 1 cm^3 0.5 % (m/v) TBA in 20 % TCA. The mixture was incubated in boiling water for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. The samples were then centrifuged (10 000 *g*, 5 min, 4 ºC) and the absorbance of the supernatant read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex (red pigment) was calculated using a coefficient of absorbance 155 mM^{-1} cm^{-1}.

For the electrolyte leakage test, fifteen leaf discs (0.5 cm^2) per sample were cut and rinsed three times with distilled water to remove the solutes from damaged cells and veins. The discs were floated for 24 h on distilled water. The conductivity was monitored after that period with a conductometer *GLP 31* (*Crison*, Barcelona, Spain). The percentage of relative damages of droughted leaves was expressed as an injury index (I %) calculated according to the formula: $I\ \% = [1-(T-D/T-C)] \times 100$ (Scherbakova and Kacperska-Palacz 1980), where D and C represent the conductivity of the electrolytes released by drought treated and control samples, respectively. T was considered as being the total electrolyte conductivity, measured after heating the samples, at temperature of 90 ºC, for 2 h.

The activity of chloroplastic SOD (EC 1.15.1.1) was measured following the method of McCord and Fridovich (1969). For the assay, 5 g of leaves were homogenized for 2 min at 4 °C in 20 cm^3 of cold 0.1 M Tris-HCl buffer, pH 8, containing 0.1 mM EDTA and 0.3 % Triton X-100. Homogenates were filtered through eight layers of cheese cloth and centrifuged (2 000 *g*, 4 min, 4 °C) to remove cell debris. The supernatants were centrifuged (10 000 *g*, 10 min, 4 °C) and the pellets containing chloroplasts were resuspended in the same buffer. Enzyme activity was assayed at 25°C in a 3 cm^3 reaction mixture containing 50 mM phosphate buffer (pH 7.8), 10^{-5} M ferricytochrome *c*, 50 μM xanthine, and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome *c* of 0.025 absorbance unit per min, at 550 nm. The enzyme activity is expressed as units per mg of chlorophyll, one unit being defined as the amount of enzyme required to inhibit the reduction rate of cytochrome *c* by 50 % under the assay conditions. Chloroplastic ascorbate peroxidase (EC 1.11.1.11) activity was measured following the method of Nakano and Asada (1981), using 5 g of fresh leaves that were cut and homogenized in 25 cm^3 of a cold 25 mM HEPES buffer, pH 7.6, containing 0.35 M sorbitol, 1 mM ascorbate and 1 mM EDTA. The crude homogenate was filtered through eight layers of cheese cloth and then centrifuged at 200 *g* for 2 min, 4 °C, to remove cell debris. The supernatant was then centrifuged at 1 000 *g* for 5 min. The chloroplasts remaining in the supernatant were discarded. Those in the pellet were suspended, washed twice, and then resuspended in 50 mM HEPES buffer (pH 7.6) and 350 mM sorbitol. Ascorbate peroxidase was assayed using the prepared extract with an amount of 10 μg Chl and 0.5 mM of ascorbate in a final volume of 2 cm^3, and following the decrease in

absorbance at 290 nm. The enzyme activity is expressed as μmol of ascorbate consumed per mg of chlorophyll per min and for quantification a coefficient of absorbance 2.8 mM^{-1} cm^{-1} was used. The activity of catalase (EC 1.11.1.6) was measured following the method of Patra *et al.* (1978). 5 g of fresh leaves were cut and homogenized in 25 cm^3 of a cold 0.1 M phosphate buffer, pH 7.8. The suspension was filtered through eight layer of cheese cloth, and centrifuged (5 000 *g*, 45 min, 4 °C). 0.2 cm^3 of the supernatant was added to 10 cm^3 0.035 % H_2O_2 and 3 cm^3 extraction buffer at 25°C. The reaction was stopped by adding 10 cm^3 2 % H_2SO_4 after 5 min. The residual H_2O_2 was titrated with 0.01 M $KMnO_4$. The blank consisted of the reaction mixture in which the extract was added to the acidified solution. Catalase activity in the pellet was also determined and was found to be negligible. The enzyme activity is expressed as μmol of H_2O_2 consumed per mg of chlorophyll per min. The data were analyzed statistically using a two-way *ANOVA*, applied for the various measured and calculated parameters, followed by the *F*-ratio test for mean comparison between genotypes or degree of dehydration (for 95 % confidence level).

Results and discussion

Under control conditions there were no significant differences among cultivars in lipid peroxidation (LPO), measured as MDA content (Table 1). Under drought MDA content was higher in Dobrudjanski ran than in Plovdiv 10 and Prelom. Injury index (I %) was highest in Dobrudjanski ran and the lowest in Plovdiv 10. Prelom showed intermediate values.

There was an increase in both H_2O_2 and $OH^{\bullet}$ production under water stress in all genotypes. The lowest H_2O_2 content were observed in Prelom and highest in Dobrudjanski ran both under control and drought conditions, but there was no significant difference between Prelom and Plovdiv 10 under drought, with the later exhibiting the smallest increase (*ca*. 10 %) between control and stress conditions. $OH^{\bullet}$ content showed the lowest value and increase under drought in Plovdiv 10 (with 54 % rise) and the highest value and increase in Dobrudjanski ran (with 238 % rise). Genotype Prelom

Table 1. Content of H_2O_2 [μmol g^{-1} (f.m.)], $OH^{\bullet}$ [mmol g^{-1} (f.m.)] and changes in lipid peroxidation [nmol (MDA) g^{-1}(d.m.)] and electrolyte leakage, expressed as injury index I [%], in the leaves of three bean (*Phaseolus vulgaris* L.) cultivars (Plovdiv 10, Dobrudjanski ran and Prelom) submitted to drought. Means ± SE, n = 5. Different letters express significantly different results between control and drought stressed plants in the same genotype (a, b) or between cultivars within each treatment (r, s, t).

Cultivar	Treatment	H_2O_2	$OH^{\bullet}$	MDA	I
Plovdiv 10	control	4.23 ± 0.21 a/r	0.135 ± 0.011 b/r	114 ± 8.5 b/r	
	drought	4.65 ± 0.24 a/s	0.208 ± 0.013 a/t	169 ± 9.4 a/s	28 ± 1.8 s
Dobrudjanski ran	control	4.46 ± 0.19 b/r	0.143 ± 0.009 b/r	147 ± 9.6 b/r	
	drought	5.91 ± 0.27 a/r	0.483 ± 0.022 a/r	284 ± 12.7 a/r	48 ± 3.1 r
Prelom	control	3.41 ± 0.17 b/s	0.158 ± 0.012 b/r	124 ± 8.6 b/r	
	drought	4.53 ± 0.19 a/s	0.301 ± 0.017 a/s	189 ± 10.4 a/s	35 ± 2.3 s

Table 2. Changes in the antioxidant enzyme activities, in the leaves of three bean (*Phaseolus vulgaris* L.) cultivars (Plovdiv 10, Dobrudjanski ran and Prelom) submitted to drought. APOX - ascorbate peroxidase [μmol(Asc) mg^{-1}(Chl) min^{-1}]; SOD - superoxide dismutase [U mg^{-1}(Chl) min^{-1}]; CAT - catalase [μmol(H_2O_2) mg^{-1}(Chl) min^{-1}]. Means ± SE, n = 5. Different letters express significantly different results between control and drought stressed plants in the same genotype (a, b) or between cultivars within each treatment (r, s, t).

Cultivar	Treatment	APOX	SOD	CAT
Plovdiv 10	control	917 ± 56 a/r	442.6 ± 24.9 b/r	241.2 ± 19.8 b/r
	drought	1037 ± 79 a/r	593.1 ± 29.5 a/r	784.9 ± 25.9 a/r
Dobrudjanski ran	control	254 ± 16 b/s	341.7 ± 22.7 b/s	114.7 ± 8.7 a/s
	drought	350 ± 21 a/t	689.8 ± 29.4 a/r	83.6 ± 4.2 b/t
Prelom	control	296 ± 11 b/s	438.6 ± 21.8 b/r	138.4 ± 10.2 b/s
	drought	635 ± 30 a/s	620.5 ± 24.1 a/r	504.6 ± 14.1 a/s

exhibited an intermediate behavior. There were no significant differences between cultivars under control conditions.

Cultivar Plovdiv 10 exhibited significantly higher values APOX activity under both control and water stress conditions, followed by Prelom and Dobrudjanski ran (Table 2). APOX activity increased significantly in cvs. Dobrudjanski ran and Prelom (38 % and 115 %, respectively). There were no significant changes in APOX activity in cv. Plovdiv 10, but under drought conditions this cultivar revealed a 196 % and a 63 % increase comparatively to cvs. Dobrudjanski ran and Prelom, respectively. Chloroplastic SOD activity increased significantly under water stress in all genotypes. Plovdiv 10 exhibited the highest SOD activity in control plants, followed by the Prelom and Dobrudjanski ran. In the cv. Dobrudjanski ran the SOD activity increased more than two folds after the exposure to drought. There were no significant differences in SOD activity between cultivars under drought conditions. The activity of catalase (CAT) varied significantly among the cultivars and drought treatment. CAT activity increased 225 % in cv. Plovdiv 10 and 265 % in cv. Prelom (Table 2) under stress, while a significant decrease (*ca.* 27 %) was observed in cv. Dobrudjanski ran. Under both control and stress conditions Plovdiv 10 maintained a highest CAT activity, followed by cvs. Prelom and Dobrudjanski ran.

The results obtained from the present study show that, at the end of drought period, an increased I %, MDA and increased H_2O_2, and $OH^{\bullet}$ production were observed in all genotypes, therefore revealing a state of oxidative stress related to membrane damage. H_2O_2 is a strong oxidant produced mainly as a result of scavenging of superoxide radical, and its higher concentration is injurious to cells, resulting in a localized oxidative damage, lipid peroxidation, and disruption of metabolic function and losses of cellular integrity (Foyer *et al.* 1997, Velikova *et al.* 2000). It is well known that H_2O_2 has multifunctional roles in the early stages of plant stress response. H_2O_2 can diffuse to relatively long distances, causing changes in the redox status of surrounding cells and tissues where, at relatively low concentrations, it may trigger an antioxidative response (Foyer *et al.* 1997). Rather than just the scavenging capacity, a fine-tuning of H_2O_2 levels is essential for an efficient control. The rationale of this assumption is that H_2O_2, whilst deleterious to some cellular components, is essential to plants in various biosynthetic reactions and, as suggested by some authors, possibly also in signal transduction pathways, which could contribute to plant defense (Schreck and Baeuerle 1991). In fact, the drought induced production of H_2O_2 in the mesophyll cells may be associated with changes in the cell wall structure (Scandalios *et al.* 1997). Furthermore, H_2O_2 is necessary for the peroxidase-mediated oxidative polymerization of cynnamil alcohols to form lignin, and several enzymatic systems have been proposed as responsible for hydrogen peroxide production, on the surface of plant cells (Lütje *et al.* 2000). It may be therefore suggested that the increased level of H_2O_2 observed by us in the drought treated plants (Table 1) is due to oxidative damages, but eventually may also have a signal function.

H_2O_2, $OH^{\bullet}$ and other ROS can be expected to be responsible for the lipid peroxidation (Sairam *et al.* 2005). The increase of MDA content indicates that the bulk oxidative lipid metabolism in leaves was enhanced by drought, suggesting a relationship between drought and oxidative stress (Munné-Bosch *et al.* 2001). A decrease in membrane stability reflects the extent of lipid peroxidation caused by reactive oxygen species. Premachandra *et al.* (1990) has reported that cell membrane stability is an indicator of drought tolerance. Lower LPO and higher membrane stability (lower electrolyte leakage) has also been reported in drought tolerant genotypes of maize (Pastori and Trippi 1992) and wheat (Sairam *et al.* 1998). Under drought conditions, cultivar Plovdiv 10, which had comparatively lower I % and LPO, also showed higher APOX and CAT activity, as compared to cvs. Prelom and Dobrudjanski ran. APOX and CAT are involved in the scavenging of H_2O_2 (Jagtap and Bhargava 1995), and thus help in ameliorating the adverse effects of oxidative stress. In fact, the higher activities of APOX and CAT would have contributed to the small (*ca*. 10 %) increase of H_2O_2 under the imposed drought conditions. On the other hand, Dobrudjanski ran showed a significant decrease in CAT activity under water stress conditions (Table 2), what may have contributed to the highest H_2O_2 accumulation observed in this cultivar. Du and Klessig (1997) proposed that CAT may be inactivated by binding to salicylic acid or to other cellular components, but the relevancy of these data towards physiological conditions is difficult to assess. Increased APOX and CAT activity in drought tolerant genotypes of pea (Gillham and Dodge 1987), tomato (Walker and Mc Kersie 1993), *Sorghum* (Jagtap and Bhargava 1995) and wheat (Sairam *et al.* 1998) have also been reported. The results are in accordance with other authors reporting similar patterns of APOX and CAT activities in different stress situations, such as iron (Hendry and Brocklebank 1985), arsenic (Stoeva *et al.* 2003) toxicity, and acid rain stress (Velikova *et al.* 2000). As reported by Sgherri and Navari-Izzo (1995), the increase in the activity of scavenging enzymes could be due either to an adaptive change in catalytic properties or to the transcription of the corresponding silent genes. Our results showed that SOD, APOX and CAT activities are increased during the drought period in cv. Plovdiv 10 and cv. Prelom. This could be related to enhanced levels of free radicals or other ROS in plant cells and correlate with a temporal coordination of the production of H_2O_2 *via* SOD and destruction of this peroxide by APOX and CAT. Such coordinated responses are believed to promote plant tolerance to oxidative stress (Foyer *et al.* 1994). It is also possible that increased SOD activity could alter the expression of other metabolic processes associated with water stress. Thus, Gupta *et al.* (1993) have demonstrated that enhanced activity of Cu,Zn SOD

in transgenic plants was associated with increased activity of APOX. Some other authors also reported an increase in SOD activity in plants under oxidative stress (Gupta *et al.* 1993, Kang and Saltveit 2002).

The results presented in this paper clearly indicate that drought treatment induced oxidative stress related to membrane damage. It appears that relative tolerance of genotypes, as reflected by its lower lipid peroxidation and higher membrane stability, is related with the levels of its antioxidant enzymatic activity. APOX, SOD and CAT are involved in overcoming of oxidative stress. The increased activities of antioxidant enzymes act as a damage control system and, thus, provide protection from oxidative stress, resulting in lower LPO and higher membrane stability in tolerant genotypes. On the basis of the data obtained about parameters for stress tolerance evaluation of plants such as electrolyte leakage, lipid peroxidation and activity of antioxidant enzymes cvs. Plovdiv 10 and Prelom can be considered as drought tolerant and cv. Dobrudjanski ran as sensitive to water shortage stress.

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