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Responses to chilling stress in French bean seedlings: antioxidant compounds

H. M. EL-SAHT

Department of Botany, Faculty of Science, University of Mansoura, Mansoura, Egypt

Abstract

French bean (*Phaseolus vulgaris* L. cv. Contender) plants at five developmental stages (4, 8, 12, 16 and 20 d after sowing) were exposed to one of three treatments: *1* - 25 °C (control), *2* - exposure to chilling at 10 °C only for 2 d prior to sampling, and *3* - long-term exposure to chilling at 10 °C. Short- and long-term chilling decreased plant growth. Higher concentrations of ascorbate and glutathione were found in the chilling-treated plants throughout the different period of growth in comparison with those in the control plants. The activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase increased in the chilling-treated seedlings while activities of catalase and peroxidase and of β-carotene content decreased in young chilling-treated plants and slightly increased in older ones.

Additional key words: ascorbate, ascorbate peroxidase, β-carotene, catalase, glutathione, glutathione reductase, peroxidase, *Phaseolus vulgaris*, superoxide dismutase.

Introduction

Chilling injury is a physiological disorder commonly found in crops indigenous to tropical and subtropical regions. Exposure of chilling-sensitive seedlings to non-freezing temperatures below 10 °C decreased root conductivity and caused seedling dehydration (Rikin and Richmond 1976), decreased root growth (Jennings and Saltveit 1994) accompanied by the development of lateral roots (Greencia and Bramlag 1971). These symptoms of chilling injury increase with the duration of exposure and with the reduction in temperature, and they are often evident only after the tissue has been transferred to non-chilling temperatures.

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Abbreviations: APO - ascorbate peroxidase; ASA - reduced ascorbate; BSA - bovine serum albumine; CAT - catalase; DASA - dehydroascorbate; DTT - dithiothreitol; GR - glutathione reductase; GSH - glutathione reduced; GSSG - oxidized glutathione; NBT - nitrobluetetrazolium; POD - peroxidase; SOD - superoxide dismutase.

Chilling temperatures increase the level of potentially lethal toxic oxygen compounds in chilling-sensitive plants, *e.g.*, maize (*Zea mays* L.) mainly because of chilling-induced photoinhibition (Hodgson and Raison 1991, Havaux and Davaud, 1994, Sonokie and Terashima 1994, Hodges *et al.* 1996). In this process, a reduction in the rate of CO_2-fixation due to low temperature stress (Foyer *et al.* 1994a) leads to an inadequate supply of the natural electron acceptor, NADP, resulting in an over-reduction of the photosystem reaction centers (Öquist and Huner 1993). Molecular oxygen may then act as an electron acceptor in place of $NADP^+$, producing O_2^- (Robinson 1988).

Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APO), can eliminate these toxic oxygen by-products. In conjunction with these enzymes, antioxidant compounds such as ascorbate, glutathione and β-carotene can also play important roles in the removal of toxic oxygen compounds.

Ascorbate can react directly with O_2^-, OH^- and singlet oxygen ($^1O_2$), and can remove H_2O_2 through the glutathione-ascorbate cycle (Halliwell 1987). Reduced ascorbate can be regenerated by the monohydroascorbate reductase and dehydroascorbate reductase, the oxidation of another antioxidant compound, glutathione (Foyer *et al.* 1994b). Glutathione can also be oxidized by direct reaction with $^1O_2$ and OH^- (Foyer *et al.* 1994a). The resulting oxidized glutathione (GSSG) is then converted back to the reduced form (GHS) by glutathione reductase (Foyer and Halliwell 1976).

β-carotene, may quench both $^1O_2$ and the excess chlorophyll excitation energy not readily passed on through the photosystem (Knox and Dodge 1985). In this work, it is though of interest, to study further the effects of chilling on growth and synthesis of antioxidant compounds in French bean seedlings.

Materials and methods

Plants: French bean (*Phaseolus vulgaris* L. cv. Contender) exhibited differential sensitivity to chilling during germination, emergence and early growth in both the laboratory and the field (Hasaneen and El-Saht 1993). Seeds of similar size were germinated for 4 d in the dark at 25 °C until the coleoptile length was 2 cm. The seedlings were then pinned into rectangular styrofoam rafts and cultured on trays containing Hoagland solution (1/4 - strength) in a growth chamber (16-h photoperiod with a photon flux density of 450 - 500 μmol m^{-2} s^{-1}. Plants were grown for 4, 8, 12, 16 and 20 d under temperature: *1*) 25 °C (control), *2*) 25 °C with a 2-d period of 10 °C prior to harvesting (short-term chilling), and *3*) 10 °C (long-term chilling).

Total glutathione (GSSG + GSH) was determined by the method of Gossett *et al.* (1994). Over ice, 1 g of frozen tissue was grounded with sterilized sand and 5 cm^3 of ice-cold 6 % (v/v) phosphoric acid (pH 2.8) containing 1 mM EDTA in a mortar and pestle. The homogenate was centrifuged at 22 000 *g* for 15 min and the supernatant was removed and then filtered through ultrafilter. Total glutathione was measured in

a reaction mixture consisting of 0.4 cm^3 of solution A [1 mM Na_2HPO_4, 4 mM NaH_2PO_4, 0.15 mM EDTA, 0.14 mM 5,5-dithiobis-(2-nitrobenzoic acid) and 0.05 cm^3 dm^{-3} of bovine-serum albumin (BSA)], 0.32 cm^3 of solution B [0.05 mM EDTA, 12.5 mM imidazole, 0.05 cm^3 dm^{-3} BSA, 5 U mg^{-1}(protein) glutathione reductase (*Sigma Diagnostics*, Canada), 0.4 cm^3 of the plant extract diluted 1:50 in 5 % (m/v) Na_2HPO_4 (pH 7.5), and 0.8 mM NADPH]. The reaction was measured spectrophotometrically (*Milton Roy Spectronic 1001 plus*, *Fisher Scientific*, Ottawa, Canada) following the change in absorbance at 412 nm for 10 min.

Total ascorbate (ASA + DASA) was determined by a modified method of Gossett *et al.* (1994). Over ice, 1 g of frozen tissue was ground with sterilized sand and 10 cm^3 of 5 % (v/v) phosphoric acid with a motar and pestle. The homogenate was centrifuged at 22 000 *g* for 15 min. Total ascorbate was determined in a reaction mixture consisting of 0.2 cm^3 of supernatant, 7.5 mM KH_2PO_4 buffer (pH 7.4) containing 5 mM EDTA and 0.1 mM dithiotheritol (DTT) to reduce DASA to ASA. After 10 min at room temperature, 0.05 % (m/v) N-ethylmaleimide was added to remove excess DTT. Colour was developed in the reaction mixture with the addition of 0.4 cm^3 of 10 % (m/v) TCA, 0.4 cm^3 of 4.4 % (v/v) *o*-phosphoric acid, 0.4 cm^3 of dipyridyl in 70 % (v/v) ethanol and 0.2 cm^3 of 3 % $FeCl_3$. The reaction mixture was incubated at 40 °C for 1 h and quantified spectrophotometrically at 525 nm. Ascorbate standards were between 1 and 50 μmol ascorbate in 5 % (v/v) phosphoric acid.

β-carotene was extracted from seedlings according to Lichtenthaler (1987). Under dim-light and over ice, 1 g of tissue was ground up with 5 cm^3 aceton and sterilized sand in a mortar and pestle. The mortar was washed with 5 % (m/v) ascorbic acid in deionized H_2O and the wash mixed with the reaction. A subsequent 2.5 cm^3 hexane addition followed by centrifugation (1000 *g*) was performed and the top layer removed. This hexane extraction was repeated twice. The hexane extract was then dried in darkness and the residue taken up in 0.5 cm^3 chloroform. Extracts were stored under nitrogen at -75 °C untill assayed. β-carotene concentration was determined by HPLC (Miller and Yang 1985) using a *LIXB 2150* binary pump, *LKB 2151* variable wavelength monitor, and a *LKB 2152* controller. The column was a *CSC-Sil 80 A /ODS2* (4.6 × 25 mm). The column was eluted with a methanol + actinitrile + chloroform (25:60:15 v/v/v) at a flow rate of 1.5 cm^3 min^{-1}. Standards were prepared using a stock solutions of 0.125 mg cm^{-3} β-carotene in chloroform.

Enzyme extraction and assay: Seedlings (5 g) were homogenized to a fine powder in a mortar under temperature -5 °C. Subsequently soluble proteins were extracted by grinding the powder with a small amount of sterilized sand on ice, in 5 cm^3 of 50 mM Tris-HCl, pH 7.0, containing 20 % (v/v) glycerol, 1 mM ascorbate, 1 mM EDTA, 1 mM GSH, 5 mM $MgCl_2$, 1 mM DTT. After two centrifugation steps (6 min at 12 000 *g* and 16 min at 22 000 *g*), the supernatant was stored in liquid nitrogen for determination of the activities of SOD, CAT, POD, APO and GR.

Superoxide dismutase activity was determined according to the method of Giannopolitis and Ries (1977). One enzyme unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the rate of nitrobluetetrazolium (NBT) reduction measured at 560 nm. The reaction mixture contained 1.3 μM riboflavin, 13 mM methionine, 63 μM nitroblutetrazolium (NBT) in 0.1 M phosphate buffer (pH 7.8), and 0.05 cm^3 of enzyme extract in final volume of 3 cm^3. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT.

Catalase activity was determined according to Aebi (1983) by measuring changes in absorbance at 240 nm corresponding to the decomposition of H_2O_2 in reaction mixture containing 50 mM KH_2PO_4, pH 7.0, 10 mM H_2O_2 and enzyme extract in a final volume of 1 cm^3 at 25 °C.

Ascorbate peroxidase was assayed as the decrease in absorbance at 290 nm due to ascorbate oxidation, by the method of Nakano and Asada (1981). The reaction mixture contained 50 mM KH_2PO_4, pH 7.0, 1 mM sodium ascorbate, 2.5 mM H_2O_2 and enzyme extract in a final volume of 1 cm^3 at 25 °C .

Glutathione reductase activity was assayed as the increase of absorbance at 340 nm due to the connection of GSSG to 1-chloro-2,4-dinitrobenzen (CDNB) as described by Drotar *et al.* (1985), in 100 mM KH_2PO_4, pH 7.0, containing 2 mM CDNB, 2 mM GSSG and enzyme extract in a final volume of 2 cm^3.

Peroxidase activity was assayed by the method of Chance and Maehly (1955) using pyrogallol as the reductant. The POD assay mixture contained 20 mM pyrogallol, 1 mM H_2O_2 and enzyme extract in a final volume of 2 cm^3. Purpurogallin formation was measured at 430 nm.

The results from triplicate samples and triplicate determinations were statistically analysed using the least significant difference (LSD) at 5 and 1 % levels (Snedecor and Cochran 1980).

Results and discussion

During the whole experimental period all growth parameters (root length, shoot length and fresh mass) measured increased with increasing age of French bean seedlings. Low temperature treatment induced significant reduction in growth parameters in comparison with controls at all growth stages, the decrease was more pronounced at long-term than short-term chilling (Table 1).

Concentrations of total ascorbate and glutathione in chilling-treated seedlings were significantly increased in comparison with control seedlings. The content of total ascorbate was more increased due to short-term chilling than due to long-term chilling and a reverse situation was observed for total glutathione content (Table 2). Oxidative stress enhances ascorbate and glutathione levels (Smith 1985, May and Leaver 1993, Foyer *et al.* 1994a, Hodges *et al.* 1996). This fact support the hypothesis that the chilling-treated bean plants accumulated greater amount of toxic oxygen compounds than the control.

Table 1. Effect of short- and long-term chilling treatments on growth parameters of French bean seedlings (4 to 20-d-old). Means significantly different from control at * - $P \leq 0.05$, ** - $P \leq 0.01$.

Plant age [d]	Length of root [cm]			Length of shoot [cm]			Fresh mass [g seedling^{-1}]		
	control	short	long	control	short	long	control	short	long
4	2.4	2.0**	1.4**	6.5	5.7**	2.4**	1.2	0.9**	0.4**
8	6.5	5.7**	3.2**	10.4	8.3**	6.5**	2.4	1.7**	0.7*
12	8.5	6.2**	4.5**	19.2	17.0**	8.7**	3.4	2.1**	1.2**
16	9.8	8.1**	6.5**	22.2	20.1**	9.6**	4.6	3.8**	2.2**
20	11.3	9.8**	7.1**	28.6	21.1**	11.0**	5.2	4.3**	3.1**
LSD at 5 %	0.12	0.10	0.07	0.32	0.28	0.12	0.06	0.04	0.02
LSD at 1 %	0.18	0.15	0.10	0.48	0.42	0.18	0.09	0.06	0.03

Table 2. Effect of short- and long-term chilling treatments on ascorbate, glutathione, and β-carotene contents [mmol g^{-1}(f.m.)] in French bean seedlings (4 to 20-d-old). Means significantly different from control at * - $P \leq 0.05$, ** - $P \leq 0.01$.

Plant age [d]	Ascorbate			Glutathione			β-carotene		
	control	short	long	control	short	long	control	short	long
4	7.2	9.4**	8.6**	1.46	3.2**	4.2**	54.8	18.1**	14.0**
8	13.1	14.6**	13.2**	0.74	6.6**	8.2**	49.2	34.1**	26.8**
12	9.4	24.0**	19.2**	0.90	7.6**	10.0**	26.3	46.6**	34.0**
16	7.5	30.4**	26.0**	0.98	8.2**	13.2**	20.4	50.2**	39.1**
20	6.7	36.6**	32.0**	0.99	9.9**	16.6**	18.0	56.4**	49.2**
LSD at 5 %	0.36	0.47	0.43	0.07	0.16	0.21	2.7	0.9	0.7
LSD at 1 %	0.54	0.71	0.64	0.10	0.24	0.31	4.1	1.3	1.0

Concentration of β-carotene was significantly lower in the chilling-treated seedlings relative to that in control seedlings only in those seedling treated for 4 and 8 d. Decrease in β-carotene content have been observed under short-term chilling in tomato (Walker and McKersie 1993) and during long-term chilling in spinach (Schoner and Krause 1990). However, after 12, 16 and 20 d the concentrations of β-carotene was significantly higher in chilling treated seedlings than that of controls. The magnitude of increase was more pronounced at short-term than at long-term chilling treatment (Table 2). Oxidative stress enhances β-carotene content (Foyer *et al.* 1994a) and chilling-treated plants have greater levels of toxic oxygen compounds than the controls (Hodges *et al.* 1996).

The short-term or long-term chilling treatment affected the activities of enzymes participating in the H_2O_2-scavenging ascorbate-glutathione cycle (Tables 3 and 4). A short-term as well as a long-term chilling treatments led to gradual increase in specific GR activity and specific APO activity (Table 3). SOD activity firstly (4 d) decreased but at 8, 12, 16 and 20 d treatments progressive increase in SOD activity due to chilling was apparent. Seedlings under short-term and long-term chilling treatment for 4, 8, 12 d showed significant reductions in CAT activity, while at

16 and 20 d, a significant increase in CAT activity was apparent as compared with control seedlings. A decline in CAT activity was reported in chilling-treated maize (Zhang *et al.* 1990) and *Vigna* (Mukherjee and Choudhuri 1983) plants. The decrease

Table 3. Effect of short- and long-term chilling treatments on activity of glutathione reductase [mmol(red. GSH) mg^{-1}(protein) s^{-1}] and ascorbate peroxidase [mmol(oxid. asc.) mg^{-1}(protein s^{-1}] in French bean seedlings (4 to 20-d-old). Means significantly different from control at * - $P \leq 0.05$, ** - $P \leq 0.01$.

Plant age [d]	Glutathione reductase control	short	long	Ascorbate peroxidase control	short	long
4	0.30	0.33**	0.42**	0.29	0.30	0.36**
8	0.31	0.41**	0.44**	0.30	0.35**	0.43**
12	0.32	0.43**	0.46**	0.31	0.37**	0.48**
16	0.34	0.47**	0.49**	0.33	0.48**	0.53**
20	0.35	0.53**	0.57**	0.34	0.51**	0.65**
LSD at 5 %	0.01	0.01	0.02	0.01	0.01	0.02
LSD at 1 %	0.02	0.02	0.03	0.02	0.02	0.03

Table 4. Effect of short- and long-term chilling treatments on activity of superoxide dismutase [U mg^{-1}(protein) s^{-1}], catalase [mmol(red. H_2O_2) mg^{-1}(protein) s^{-1}] and peroxidase [mmol(oxid. pyrogallol) mg^{-1}(protein) s^{-1}] in French bean seedlings (4 to 20-d-old). Means significantly different from control at * - $P \leq 0.05$, ** - $P \leq 0.01$.

Plant age [d]	Superoxide dismutase control	short	long	Catalase control	short	long	Peroxidase control	short	long
4	11.3	11.0	10.8**	24.5	18.3**	11.7**	13.4	11.6**	9.5**
8	10.4	11.8*	13.8**	22.0	19.5**	14.3**	13.8	10.8**	8.8**
12	8.8	14.7**	15.4**	21.6	20.1**	16.5**	14.4	15.9**	16.0**
16	8.3	15.3**	15.8**	17.3	24.4**	18.8**	15.3	17.8**	17.7**
20	8.0	15.7**	16.0**	15.2	24.8**	20.3**	16.5	19.9**	19.7**
LSD at 5 %	0.56	0.58	0.63	1.2	0.9	0.6	0.68	0.58	0.47
LSD at 1 %	0.84	0.87	0.94	1.8	1.3	0.9	1.00	0.87	0.71

in CAT activity, in the present study, could be explained partly by photoinactivation of the enzyme. During further development a re-synthesis of CAT is supposed. POD activity was reduced under short-term and long-term chilling treatment for 4 and 8 d, while it was increased at 12, 16 and 20 d due to chilling (Table 4). An increase of POD activity also was observed in other chilling-treated plants (Badiani *et al.* 1990, Siegel 1993, Zhang and Kirkham 1994). POD could be synthesized *de novo* (Siegel and Galston 1966) or released from membranes. Chilling stress also increase the accumulation of POD substrates, *e.g.* ascorbate (Table 2).

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