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REVIEW PAPER

Effects of $Ca(NO_3)_2$ stress on oxidative damage, antioxidant enzymes activities and polyamine contents in roots of grafted and non-grafted tomato plants

Gu-Wen Zhang · Zheng-Lu Liu · Jun-Guo Zhou · Yue-Lin Zhu

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Abstract The effects of $Ca(NO_3)_2$ stress on biomass production, oxidative damage, antioxidant enzymes activities and polyamine contents in roots of grafted and non-grafted tomato plants were investigated. Results showed that when exposed to 80 mM $Ca(NO_3)_2$ stress, the biomass production reduction in non-grafted plants was more significant than that of grafted plants. Under $Ca(NO_3)_2$ stress, superoxide anion radical ($O_2\bullet^-$) producing rate, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents of non-grafted plants roots were significantly higher than those of grafted plants, however, nitrate (NO_3^-), ammonium (NH_4^+) and proline contents, superoxide dismutase (SOD, EC1.15.1.1), peroxidase (POD, EC1.11.1.7), catalase (CAT, EC1.11.1.6) and arginine decarboxylase (ADC, EC 4.1.1.19) activities of grafted plants roots were significantly higher than those of non-grafted plants. Regardless of stress, free, conjugated and bound polyamine contents in roots of grafted plants were significantly higher than those of non-grafted plants. The possible roles of antioxidant enzymes, prolines and polyamines in adaptive mechanism of tomato roots to $Ca(NO_3)_2$ stress were discussed.

Keywords Antioxidant enzymes · $Ca(NO_3)_2$ stress · Grafted · Polyamines · Tomato

Abbreviations

ADC	Arginine decarboxylase
CAT	Catalase
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
MDA	Malondialdehyde
NH_4^+	Ammonium
NO_3^-	Nitrate
$O_2\bullet^-$	Superoxide anion radical
PCA	Perchloric acid
POD	Peroxidase
Put	Putrescine
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Spd	Spermidine
Spm	Spermine

Introduction

Nitrogen fertilizer plays an important role in crop yield and quality. Plants depend on an adequate nitrogen supply to synthesize amino acids, proteins, nucleic acids and other cell constituents necessary for

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development (Ruiz and Romero 1999). However, conventional practices of cultivation bring excessive nitrogen fertilization into soil (Quilleré et al. 1994). In northern China, where greenhouse vegetable production plays an important role in people' life, it is very common that too many nitrogen fertilizers (>1,000 Kg ha^{-1}) are applied to greenhouse vegetable for maximal yield (He et al. 2007). Generally, plant growth decreases under a nitrogen supply exceeding 10 mM, a value considered on the threshold of toxicity for some species (Cao and Tibbtts 1998; Jones 1997; Sánchez et al. 2004). Excessive nitrogen fertilization not only degrades surface and ground-water quality, inhibits biological nitrogen fixation, modifies microbial soil biodiversity, but also causes osmotic stress, in which reactive oxygen species (ROS), such as superoxide anion radical ($O_2\bullet^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) are produced. ROS are highly toxic and can highly disrupt normal metabolism of lipids, proteins and nucleic acids and then inhibit plant growth and reduce yield (Sainju et al. 2001). However, plants have a number of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) to protect themselves against the deleterious effects of ROS. In addition, when exposed to stresses, plants can improve their tolerance by accumulation of low molecular mass osmolytes, such as proline, sugars, organic acids and polyamines (Sánchez et al. 2002; Claussen et al. 2006).

Polyamines are small, cationic molecules present ubiquitously in prokaryotic and eukaryotic organisms and involve in the regulation of many basic cellular processes such as DNA replication and transcription, cell proliferation, modulation of enzyme activities, membrane rigidity and stabilization (Liu et al. 2004). The common polyamines found in plants include the diamine putrescine (Put), polyamines spermidine (Spd) and spermine (Spm). Evidence gathered in recent years indicated that the changes in free polyamine levels were closely associated with tolerance of plants to stresses. A stress-induced change in endogenous free polyamine levels has been reported in various species. For example, in pine, free polyamine levels increased significantly during high nitrogen stress (Minocha et al. 2000), similar results were reported in spruce (Erisson et al. 1993) and tomato (Corey and Barker 1989). Also, an increase of free polyamine levels was reported in maize (Jiménez-Bremont et al. 2007) and rice (Roy and Wu 2002) under salt stress. It has been suggested that, due to their polycationic nature, free polyamines might be involved in ROS removing and cellular ionic balance (Willadino et al. 1996).

At physiological pH, polyamines are highly protonated, which can bind to negative charges easily. Polyamines bound to low molecular mass compounds, such as hydroxycinnamic acids are designated as conjugated polyamines, which are soluble in perchloric acid (PCA). Polyamines bound to high molecular mass compounds, such as DNA, proteins, nucleic acids, and membrane phospholipids are designated as bound polyamines, which are insoluble in PCA (Neves et al. 2002). Also, conjugated and bound polyamines are associated with plant tolerance to stresses. When submitted to osmotic stress, conjugated and bound polyamine levels in rape leaf discs increased significantly (Aziz and Larher 1995). When exposed to heat stress, the more heat tolerant tobacco suspension cells exhibited relatively high levels of conjugated polyamine and exogenous application of Spd could improve tomato heat tolerance in both heat tolerant and heat sensitive cultivars (Königshofer and Lechner 2002; Murkowski 2001). Piqueras et al. (2002) reported that high concentration of conjugated Put was correlated with the establishment of normal growth rate in hyperhydric carnation plants. Under chilling stress, the conversion of free polyamines to conjugated polyamines could enhance potato (Mauricio et al. 1999), pepino (Martínez-Romero et al. 2003) and mangosteen fruit (Kondo et al. 2003) chilling tolerance.

In addition, polyamines are associated with plant responses to biotic stresses. Legaz et al. (1998) reported that in sugarcane infected with smut fungus, concentrations of free and conjugated polyamines increased greatly. The study of Waie and Rajam (2003) indicated that transgenic tobacco lines with higher conjugated polyamine levels were more tolerant to fungal wilts. However, very little is known about the significance of polyamines in response to high nitrogen stress in plants.

In horticultural production, grafting is a useful technique to increase yield, avoid disease, alter growth habit and improve stress tolerance (Shaterian et al. 2005). Also, grafting can affect polyamines metabolism and antioxidant enzymes activities of

plants. Miklos et al. (2006) reported that grafting affected the Put level of grapevine leaves and the effect depends on the rootstock genotypes. Konigshofer (1990) reported that grafting changed polyamine levels of needles, shoot-axes, buds and xylem exudates of spruce trees. The study of López-Gómez et al. (2007) indicated that salt stress did not produce significant effect on SOD activity in loquat plants grafted on anger rootstock (salt-tolerant), whereas a significant fall was observed in plants grafted on their own loquat rootstock (salt-sensitive), suggesting that grafting could increase salt tolerance of loquat plants.

The objectives of this experiment were to investigate the effects of $Ca(NO_3)_2$ stress on biomass production, oxidative stress, antioxidant enzymes activities and polyamine contents in roots of grafted and non-grafted tomato plants, and the possible roles of antioxidant enzymes, prolines and polyamines in adaptive mechanism of tomato roots to high nitrogen stress.

Materials and methods

Materials

The commercial tomato hybrid *Lycopersicon esculentum* Mill. cv. 'Kagemusya'(Japanese Takii company, Japan) which is nitrogen tolerant was used as rootstock, and 'Baoda 903'(Chinese Shanghai Academy of Agricultural Science, China) which is nitrogen sensitive was used as scion. Rootstock seeds were sterilized with sodium hypochlorite containing 5% active chloride for 5 min, soaked for 6 h in distilled water after being washed five times, then geminated at 25 ± 1°C for 2 d in moist filter paper in glass culture dishes, scion seeds were sterilized and geminated 3 d later after rootstock. Uniformly germinated seeds were selected, and sown in plastic pots (10×10 cm^2) filled with a 1:1 mixture of peat and vermiculite, one plant per pot. Seedlings were grown under greenhouse conditions of a 65–70% relative aerial humidity. All plants were irrigated with half-strength Hoagland nutrient solution every 2 d. When seedlings had developed four or five true leaves, grafting was made as previously described (Santa-Cruz et al. 2002).

Treatments

Fifteen days later, both grafted and non-grafted plants with the same size were transplanted to 12 l plastic pots containing half-strength Hoagland nutrient solution (one plant per pot), nutrient solution was renewed every 3 d and aerated 24 h each day. When plants had developed seven or eight true leaves, $Ca(NO_3)_2$ treatment was applied, $Ca(NO_3)_2$ was dissoluted into the nutrient solution directly. The experiment consisted of four treatments: (1) grafted plants control (G1), (2) grafted plants + $Ca(NO_3)_2$ (G2), (3) non-grafted plants control (N1), (4) non-grafted plants + $Ca(NO_3)_2$ (N2).

Biomass determination

After 10 d of stress, 40 plants were harvested and divided into shoots and roots, all materials were rinsed three times in distilled water after disinfecting with non-ionic detergent, then blotted on filter paper and weighed. The materials were then dried in a forced-draft oven at 75°C for 48 h and re-weighed.

Determination of $O_2\bullet^-$ producing rate, NO_3^-, NH_4^+, H_2O_2, MDA and proline contents

Roots with the same position were sampled each 2 d after stress, after rinsing in distilled water and blotting on filter paper, they were cut into 5-mm segment for use. $O_2\bullet^-$ producing rate was measured according to Elstner and Heupel (1976). NO_3^- content was measured by nitration of salicylic acid (Cataldo et al. 1975). NH_4^+ content was measured according to the reaction of Berthelot modified by Weatherburn (1967). H_2O_2 content was measured by the titanium method (Patterson et al. 1984). MDA content was measured by the thiobarbituric acid reaction method (Heath and Packer 1968). Proline content was determined according to Bates et al. (1973).

Assay of SOD, POD and CAT activity

For the assay of antioxidant enzymes, samples of root segment (0.5 g) were homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C and the supernatant was used as source of enzyme rude extract solution.

SOD activity was assayed according to Beauchamp and Fridovich (1971). One unit of activity was defined as the amount of enzyme required to cause a 50% inhibition of the reduction of nitroblue tetrazolium (NBT) at 560 nm. POD activity was assayed according to Kochba et al. (1977). One unit of activity was defined as the amount of enzyme required to increase 1 A value in the optical density at 470 nm per min. CAT activity was assayed according to Aebi (1984). One unit of activity was defined as the amount of enzyme required to decrease 0.1 A value in the optical density at 240 nm per min. Total soluble protein contents were determined according to Lowry et al. (1951) utilizing bovine serum albumin (BSA) as a standard.

Assay of ADC activity

For the assay of ADC activity, samples of root segment (0.5 g) were homogenized in 50 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM phenylmethylsulfonyl fluoride, 2 mM pyridoxal phosphate (PLP), 10 mM dithiothreitol (DTT), 5 mM EDTA, 25 mM ascorbic acid and 0.1% PVP. The homogenate was centrifuged at 15,000 *g* for 20 min at 4°C and then supernatant was dialyzed at 4°C in 3 ml of 50 mM potassium phosphate buffer (pH 8.0) containing 0.05 mM PLP, 1 mM DTT, 0.1 mM EDTA for 24 h in darkness. The dialyzed extract was used for enzyme assay.

ADC activity was assayed according to Matsuda (1984). Enzyme activity was expressed in μl CO_2 g^{-1} FW min^{-1}.

Polyamine determination

Polyamine was extracted according to Sharma and Rajam (1995). Samples of root segment as previously (0.5 g) were homogenized in 3 ml of 5% (v/v) PCA and incubated at 0°C for 1 h. Then the homogenate was centrifuged at 20,000 *g* for 30 min. The supernatant was used to determine free and conjugated polyamines and the deposit was used to determine bound polyamines. For conjugated polyamines, 1 ml of the supernatant was mixed with 1 ml of 12 N HCl and hydrolyzed at 110°C for 24 h in flame-sealed glass ampules. After acid hydrolysis, HCl was evaporated by heating at 75°C and the residue was resuspended in 1 ml of 5% (v/v) PCA. This solution served as the conjugated polyamine fractions containing free polyamines and those liberated from polyamine conjugates. For bound polyamines, the deposit was rinsed four times with 5% PCA to remove any trace of conjugated polyamines and then suspended in 2 ml of 1 N NaOH. The mixture was centrifuged at 20,000 *g* for 30 min and hydrolyzed under the same conditions like conjugated polyamines.

Polyamines recovered from the hydrolyzed supernatant, non-hydrolyzed supernatant and the deposit were benzoylated according to Santa-Cruz et al. (1997). About 0.5 ml of the supernatant was mixed with 1 ml of 2 N NaOH and 10 μl of benzoyl chloride. The mixture was vortexed vigorously and incubated for 30 min at 37°C. The reaction was terminated by adding 2 ml of saturated NaCl solution and the benzoyl-polyamines were extracted with 2 ml cold diethyl ether for 10 min after vortexing vigorously. Finally, 1 ml of the ether phase was evaporated to dryness and redissolved in 100 μl of methanol.

Polyamine contents were analyzed by HPLC (Shimadzu LC-10AT, supplemented with a UV-VIS detector (Shimadzu SPD 10AV) with a C-18 reverse phase column (250 × 4.6 mm; 5 μm). Mobile phase consist of water:methanol at 36:64 (v:v), at a flow rate of 0.7 ml $min^{-1}$and monitored at 254 nm. The peaks were identified with reference to the retention time of polyamine standards (Sigma) prepared as described above. Quantitative determination was based on external standards. The calibration curves were $y = 50372$ x + 2253.3, $r^2 = 0.9975$ (for Put); $y = 67151$ x + 3430.6, $r^2 = 0.9958$ (for Spd) and $y = 76690$ x + 2367.3, $r^2 = 0.9966$ (for Spm). Results were expressed as nmol g^{-1} fresh weight.

Statistical analysis

Significances were tested by one-way ANOVA followed by Duncan's test at $P < 0.05$ by SPSS (11.5) software, and the results are expressed as the mean values ± SE of three independents. Each experiment was repeated three times.

Results

Effect of $Ca(NO_3)_2$ stress on biomass production between grafted and non-grafted tomato plants

In the absence of stress, fresh and dry weight of shoots and roots of grafted plants (G1) was significantly

Table 1 Effect of $Ca(NO_3)_2$ stress on biomass production between grafted and non-grafted tomato plants

Treatment	Shoot (g plant^{-1})		Root (g plant^{-1})		DW percentage (%)	
	Fresh weight	Dry weight	Fresh weight	Dry weight	Shoot	Root
G1	113.29 ± 5.17^{a}	11.27 ± 1.56^{a}	38.03 ± 1.96^{a}	2.56 ± 0.18^{a}	9.95^{b}	6.73^{b}
G2	67.67 ± 3.05^{c}	7.20 ± 0.92^{c}	26.25 ± 1.07^{b}	1.80 ± 0.06^{b}	10.64^{a}	6.86^{a}
N1	90.33 ± 2.79^{b}	8.49 ± 1.81^{b}	24.14 ± 2.90^{b}	1.59 ± 0.45^{c}	9.40^{d}	6.59^{c}
N2	39.90 ± 1.98^{d}	3.83 ± 0.92^{d}	13.13 ± 1.21^{c}	0.88 ± 0.04^{d}	9.60^{c}	6.70^{b}

Data are mean ± SE (n = 3). Values with different superscript letters are significantly different at $P < 0.05$

higher than those of non-grafted plants (N1) (Table 1). $Ca(NO_3)_2$ stress significantly inhibited the biomass production of both grafted (G2) and non-grafted plants (N2), which was more marked in N2 than G2. Under stress, fresh weight of shoots and roots of G2 reduced 40.27% and 36.11%, dry weight of shoots and roots of G2 reduced 30.98 and 29.69%. The decrease in N2 was 55.83 and 54.89% in fresh weight and 45.61 and 44.64% in dry weight, respectively. Otherwise, DW percentage of both G2 and N2 increased significantly after stress and the DW percentage of G2 was significantly higher than that of N2 regardless of stress.

Effects of $Ca(NO_3)_2$ stress on NO_3^- and NH_4^+ contents in roots of grafted and non-grafted tomato plants

In the absence of stress (0 day), NO_3^- content of G1 was significantly higher than that of N1 (Fig. 1). Under stress, NO_3^- contents of both G2 and N2 increased significantly during the first 4 days and then stayed stable in G2, however, NO_3^- content of N2 decreased from day 6, throughout the whole stress period, NO_3^- contents of G2 was significantly higher than that of N2. In the absence of stress, there was no significant difference in NH_4^+ content between G1 and N1 (Fig. 1). Under stress, NH_4^+ contents of both G2 and N2 increased significantly and reached the highest values on day 4 and then decreased significantly, from day 2, NH_4^+ content of G2 was significantly higher than that of N2.

Effects of $Ca(NO_3)_2$ stress on $O_2\bullet^-$ producing rate, H_2O_2, MDA and proline contents in roots of grafted and non-grafted tomato plants

In the absence of stress (0 day), there were no significant differences in $O_2\bullet^-$ producing rate and

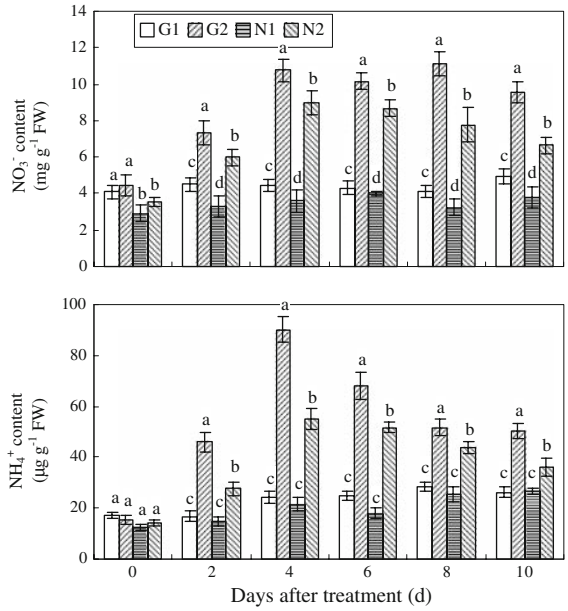

Fig. 1 Effects of $Ca(NO_3)_2$ stress on NO_3^- and NH_4^+ contents in roots of grafted and non-grafted tomato plants. Each histogram represents a mean value of three independent experiments, and the vertical bars indicate SE ($n = 3$). Significances were tested within the same day by one-way ANOVA. Different letters in each bar indicate significant differences ($P < 0.05$)

H_2O_2 content between G1 and N1 (Fig. 2). Under stress, $O_2\bullet^-$ producing rate and H_2O_2 content in both G2 and N2 increased significantly during the first 8 days, then decreased significantly on day 10. On day 10, $O_2\bullet^-$ producing rate and H_2O_2 content of G2 was 50.99 and 95.02% higher than those of G1, N2 was 98.11 and 179.17% higher than those of N1, from day 2, $O_2\bullet^-$ producing rate and H_2O_2 content of N2 was significantly higher than those of G2.

In the absence of stress, MDA content of G1 was significantly higher than that of N1 (Fig. 2). Under stress, MDA content of G1 increased significantly in the first 8 days of stress, then remained unchanged on the day 10,

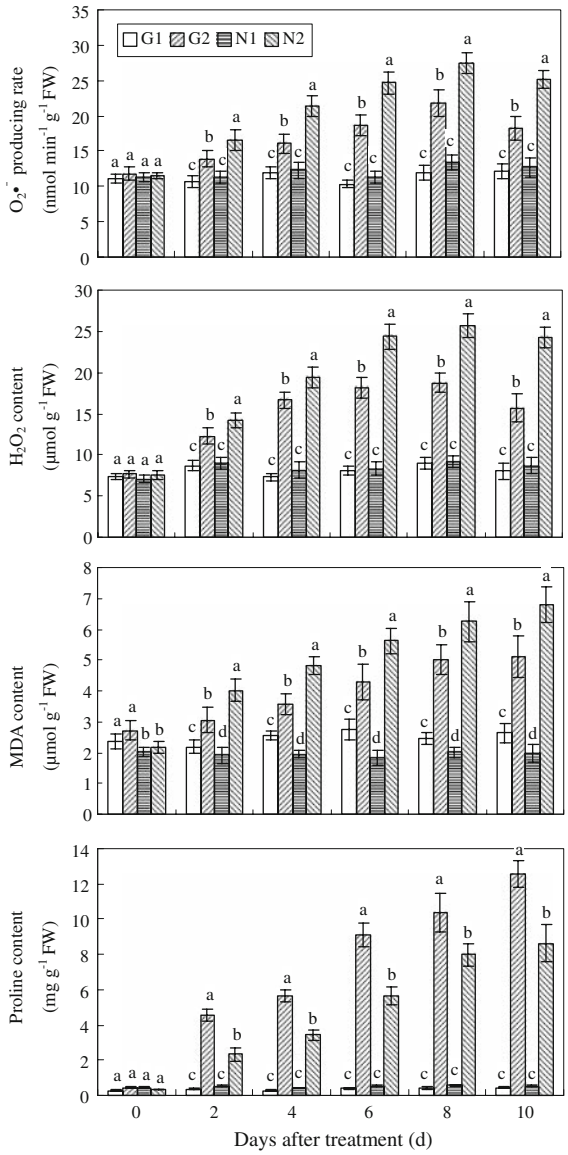

Fig. 2 Effects of $Ca(NO_3)_2$ stress on $O_2\bullet^-$ producing rate, H_2O_2, MDA and proline contents in roots of grafted and non-grafted tomato plants. Each histogram represents a mean value of three independent experiments, and the vertical bars indicate SE ($n = 3$). Significances were tested within the same day by one-way ANOVA. Different letters in each bar indicate significant differences ($P < 0.05$)

however, MDA content of N1 increased significantly during the whole stress period. On day 10, MDA content of G2 was 93.94% higher than that of G1, N2 was 246.43% higher than that of N1 from day 2, MDA content of N2 was significantly higher than that of G2.

In the absence of stress, there was no significant difference in proline content between G1 and N1 (Fig. 2). Under stress, proline contents of both G2 and N2 increased significantly throughout the whole stress period. On day 10, proline content of G2 increased 26.91-fold, N2 increased 15.61-fold from day 2, proline content of G2 was significantly higher than that of N2.

In the absence of stress (0 day), SOD, POD and CAT activities of G1 were significantly higher than those of N1 (Fig. 3). Under stress, SOD activities of both G2 and N2 increased significantly until day 8, then decreased on day 10. On day 10, SOD activity of G2 was 34.85% higher than that of G1 and N2 was 20.95% higher than that of N1. POD activities of G2 and N2

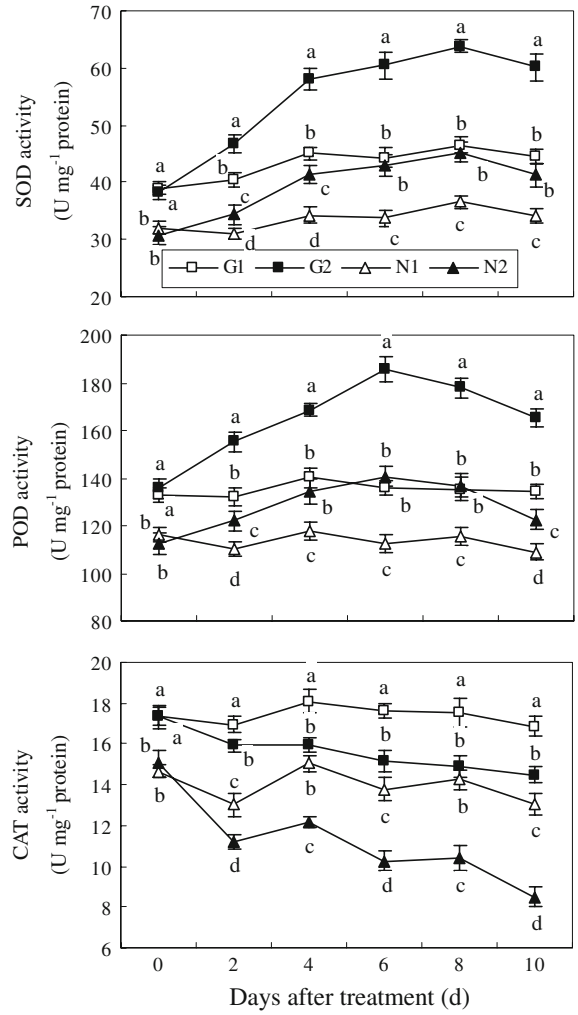

Fig. 3 Effects of $Ca(NO_3)_2$ stress on SOD, POD and CAT activities in roots of grafted and non-grafted tomato plants. Each value is the mean of three independent experiments, and the vertical bars indicate SE ($n = 3$). Significances were tested within the same day by one-way ANOVA. Different letters indicate significant differences ($P < 0.05$)

increased significantly until day 6, then decreased significantly on day 8 and 10. On day 10, POD activity of G2 was 23.12% higher than that of G1 and N2 was 12.32% higher than that of N1. CAT activities of G2 and N2 decreased significantly during the whole stress period. On day 10, CAT activity of G2 decreased 14.17% and N2 decreased 34.82%. During the whole stress period, SOD, POD and CAT activities of G2 were significantly higher than those of N2.

Effect of $Ca(NO_3)_2$ stress on ADC activity in roots of grafted and non-grafted tomato plants

In the absence of stress (0 day), there was no significant difference in ADC activity between G1 and N1 (Fig. 4). Under stress, ADC activity of both G2 and N2 increased significantly until day 6, then decreased on the day 8 and 10. On day 10, ADC activity of G2 was 21.90% higher than that of G1 and N2 was 13.73% higher than that of N1. From day 2, ADC activity of G2 was significantly higher than that of N2.

Effects of $Ca(NO_3)_2$ stress on free polyamine contents in roots of grafted and non-grafted tomato plants

Regardless of stress, the most abundant free polyamine in tomato roots was Spd. In the absence of stress (0 day), free Put, Spd and Spm contents of G1 were significantly higher than those of N1 (Fig. 5). Under stress, free Put, Spd and Spm contents in both G2 and N2 increased significantly, but the increase in

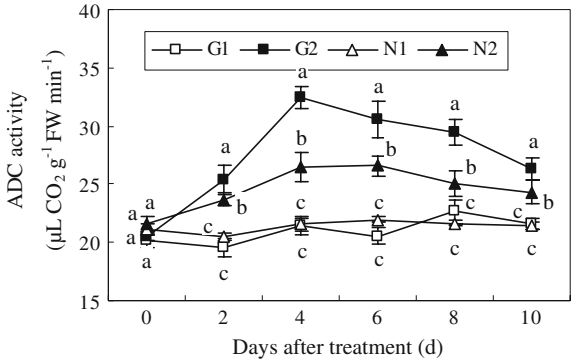

Fig. 4 Effects of $Ca(NO_3)_2$ stress on ADC activity in roots of grafted and non-grafted tomato plants. Each value is the mean of three independent experiments, and the vertical bars indicate SE ($n = 3$). Significances were tested within the same day by one-way ANOVA. Different letters indicate significant differences ($P < 0.05$)

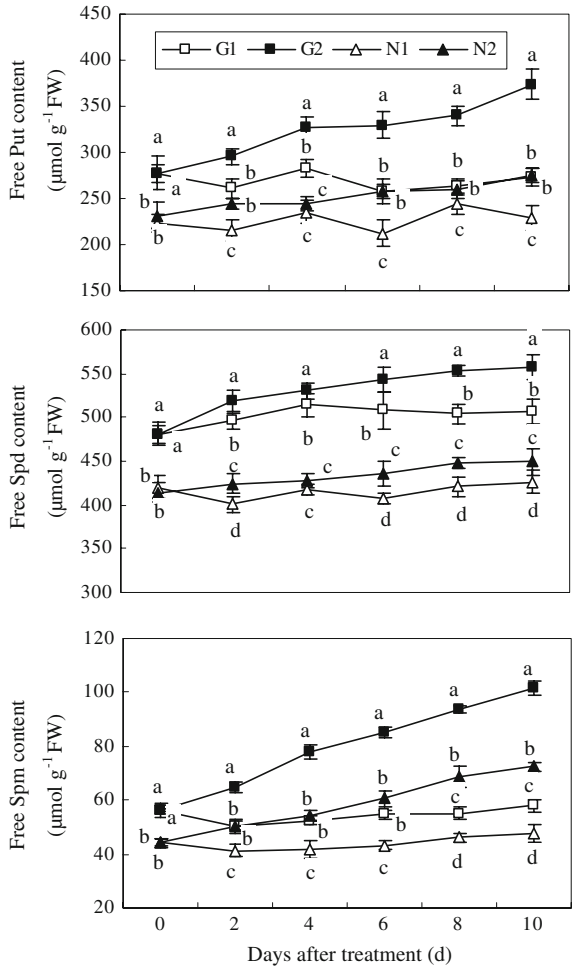

Fig. 5 Effects of $Ca(NO_3)_2$ stress on free Put, Spd and Spm contents in roots of grafted and non-grafted tomato plants. Each value is the mean of three independent experiments, and the vertical bars indicate SE ($n = 3$). Significances were tested within the same day by one-way ANOVA. Different letters indicate significant differences ($P < 0.05$)

N2 was slighter than that of G2. On day 10, free Put, Spd and Spm contents of G2 was 37.06, 10.14 and 76.02% higher than those of G1, and N2 was 20.29, 5.41 and 51.85% higher than those of N1. During the whole stress period, free Put, Spd and Spm contents of G2 were significantly higher than those of N2.

Effects of $Ca(NO_3)_2$ stress on conjugated polyamine contents in roots of grafted and non-grafted tomato plants

Unlike free polyamines, the most abundant conjugated polyamine in tomato roots was Put (Fig. 6).

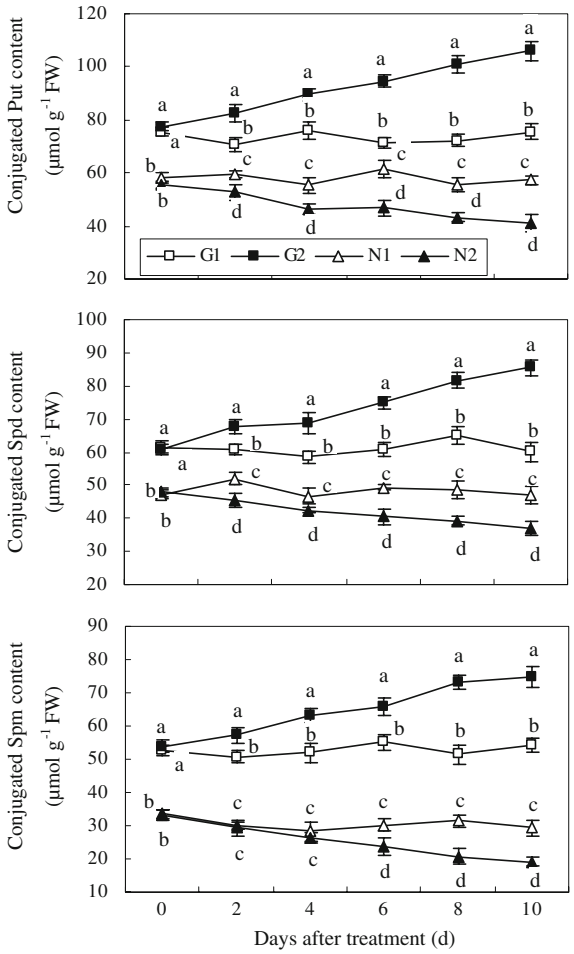

Fig. 6 Effects of $Ca(NO_3)_2$ stress on conjugated Put, Spd and Spm contents in roots of grafted and non-grafted tomato plants. Each value is the mean of three independent experiments, and the vertical bars indicate SE ($n = 3$). Significances were tested within the same day by one-way ANOVA. Different letters indicate significant differences ($P < 0.05$)

Also, conjugated polyamine contents were strongly affected by $Ca(NO_3)_2$ stress, but they showed significant difference in G2 and N2 during the stress. In the absence of stress (0 day), conjugated Put, Spd and Spm contents of G1 were significantly higher than those of N1. Under stress, conjugated Put, Spd and Spm contents of G2 increased significantly, however, conjugated Put, Spd and Spm contents of N2 decreased significantly. On day 10, conjugated Put, Spd and Spm contents of G2 increased 40.69, 42.45 and 37.15%, respectively, however, N2 decreased 28.87, 21.35 and 35.00%, respectively. During the whole stress period, conjugated Put, Spd and Spm contents of G2 were significantly higher than those of N2.

Effects of $Ca(NO_3)_2$ stress on bound polyamine contents in roots of grafted and non-grafted tomato plants

Like conjugated polyamine, the most abundant bound polyamine in tomato roots was Put (Fig. 7). In the absence of stress (0 day), bound Put, Spd and Spm contents of G1 were significantly higher than those of N1. Under stress, bound Put, Spd and Spm contents of G2 increased significantly, however, bound Put content of N2 stayed unchanged until day 6, then decreased slowly, no changes were detected in bound Spd and Spm contents. During the whole stress

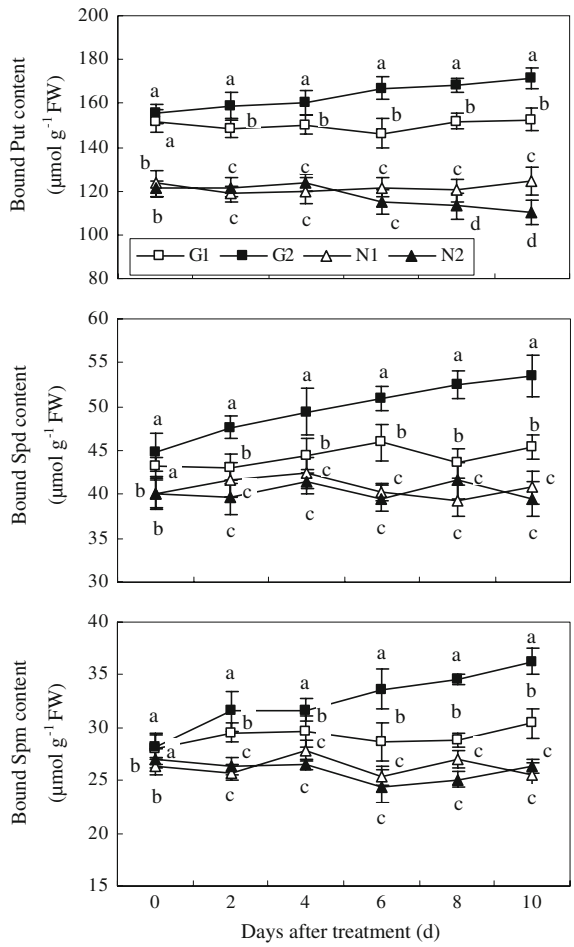

Fig. 7 Effects of $Ca(NO_3)_2$ stress on bound Put, Spd and Spm contents in roots of grafted and non-grafted tomato plants. Each value is the mean of three independent experiments, and the vertical bars indicate SE ($n = 3$). Significances were tested within the same day by one-way ANOVA. Different letters indicate significant differences ($P < 0.05$)

period, conjugated Put, Spd and Spm contents of G2 were significantly higher than those of N2.

Discussion

Most plant species show reduced growth, smaller leaves and stunted root systems when exposed to high nitrogen stress, and in severe cases this leads to the death of plant. Plants exposed to high nitrate levels will absorb excessive NO_3^- (Fig. 1). NO_3^- can increase the pH around the roots due to the efflux of HCO_3^- or OH^- from the roots and this pH increase outside the roots can considerably reduce Fe availability (Bar and Kafkafi 1992). Chlorosis in plant leaves can be caused by the insufficient uptake of Fe or by the failure of leaves to reduce Fe^{3+}, a process that is affected by the presence of NO_3^-, since the activity of chelate reductase (FeCH-R) located in the plasmalemma appears to be depressed at alkaline pH (Mengel et al. 1994). It is worth noting that too much assimilation product NH_4^+ is also harmful to plant. High level of NH_4^+ can inhibit the NR and NiR activities and cause decrease of cytokinins in the xylem sap, which will result in leaf expansion and impaired root growth (Walch-Liu et al. 2000). In addition, high nitrate levels in soil or nutrient solution will cause osmotic stress, which can cause oxidative damage and induce ROS. ROS are highly toxic and can damage many important cellular components, such as lipids, protein, DNA and RNA (Sainju et al. 2001). MDA is the direct production of lipid peroxidation and its content is often used as an indicator of the extent of lipid peroxidation. In this experiment, under high nitrate stress, NO_3^- and NH_4^+ levels in roots of grafted plants were significantly higher than those of non-grafted plants (Fig. 1), however, $O_2\bullet^-$ producing rate, H_2O_2 and MDA contents in grafted plants were significantly lower than those of non-grafted plants (Fig. 2), the biomass production reduction in non-grafted plants was significantly higher than that of grafted plants and the DW percentage of grafted plants was higher than that of non-grafted plants. All these results suggest that grafted plants were more tolerant to high nitrogen stress.

It has been well documented that stress tolerance of plants is associated with their ability to remove ROS (Senaratna et al. 1985). Antioxidant enzymes play an important role in removing ROS, when plants are subjected to osmotic stress, activities of a number of antioxidant enzymes are enhanced in order to eliminate ROS (Ruiz-Lozano 2003). As a major scavenger, SOD catalyzes $O_2\bullet^-$ to H_2O_2 and O_2, then POD and CAT catalyzes H_2O_2 to H_2O and O_2 (Zhu et al. 2006). Jagtap and Bhargava (1995) reported that antioxidant enzymes activities in drought-tolerant sorghum cultivars were significantly higher than that of drought-sensitive cultivars. Arbuscular mycorrhizal (AM) fungi, which could enhance antioxidant enzymes activities, could enhance citrus osmotic tolerance (Wu et al. 2006). Also, in many plant species, an increase of proline level under stress conditions is a common response of plants and this is a typical mechanism of the biochemical adaptation in living organisms subjected to stress (Delauney and Verma 1993). It has been suggested that proline may function as an osmoticum, a sink of energy and reducing power, a nitrogen-storage compound, a ROS-scavenger and a compatible solute that protects enzymes (Sánchez et al. 2002). In this experiment, under high nitrogen stress, SOD, POD activities and proline content of both grafted and non-grafted plants increased significantly under high nitrate stress (Figs. 2 and 3), suggesting that SOD, POD and proline were associated with nitrogen tolerance of tomato plants. The SOD, POD activities and proline content of grafted plants were significantly higher than those of non-grafted plants, suggesting that grafted plants had stronger ability to remove ROS.

It is well be known that free polyamines play important roles in stress tolerance of plants. Minocha et al. (2002) reported that pine foliar free polyamines were close correlated with the total foliar nitrogen contents, when foliar total nitrogen contents increased, free polyamine contents increased. Under high nitrogen stress, arginine, the precursor of Put has been shown to be present in higher levels in spruce and pine needles, consequently free polyamines levels of spruce and pine needles were significantly higher than those of control (Erisson et al. 1993). Corey and Barker (1989) reported that when subjected to high ammonium stress, free Put level of tomato leaves increased significantly. Free polyamines have been reported to be involved in the plant responses to osmotic stress by playing a role in the ROS-mediated damage caused by osmotic conditions (Zhu 2002). Under salt stress, polyamines could increase the activities of key enzymes involved

in oxidative stress such as SOD, glutathione reductase (GR) and ascorbate peroxidase (APOX) and decrease lipid peroxidation in Virginia pine, then improved growth and development was obtained in calluses and plantlets (Tang and Newton 2005). Addition of exogenous polyamines to osmotically stressed oat leaves retarded protein degradation, inhibited loss of chlorophyll and stabilized thylakoid membranes. In osmotically-stressed oat leaves, the degradation of cytochrome thylakoid proteins and the enzyme Rubisco could be avoided by addition of Spm to the incubation medium (Besford et al. 1993; Borrell et al. 1996). Otherwise, application of Spd to the plants partially prevented the damaging effects induced by UV-C (Campos et al. 1991). In this experiment, free polyamines contents of both grafted and non-grafted tomato plants increased significantly under high nitrogen stress, suggesting that free polyamines were involved in the promotion of nitrogen tolerance of tomato plants. The reasons why high levels of free polyamines may enhance nitrogen stress tolerance of tomato might be attributed to the following arguments. First, nitrogen has been reported to be stored in plant leaves in the form of nitrogen or arginine (Aber et al. 1995), arginine was the synthetic precursor of Put, high level of arginine could enhance the synthesis of polyamines, which contain 2–4 amido would reduce nitrogen or arginine levels of plants. Second, polyamines as polycation could neutralize the negative charges of NO_3^- and keep charge balance in cells. Third, polyamines acting as free-radical scavengers could remove ROS, then reduce the oxidative damage and maintain cell normal physiological function. In the present study, ADC activity (Fig. 4) and free polyamine contents in roots of grafted plants were significantly higher than those of non-grafted plants under nitrogen stress (Fig. 5), suggesting that grafted plants had stronger ability to synthesize polyamines, higher levels of polyamines will do favor to protect plants from nitrogen toxicity.

So far, though many studies reported the function of conjugated and bound polyamines in floral induction and reproductive processes, more and more reports pointed that they played important roles in plants tolerance to stresses. They could be the substrates or precursors of reactions of secondary metabolism or act as a nitrogen reservoir (Smith 1990). Martin-Tanguy (1997) have demonstrated that polyamines conjugated to cinnamic acids would be important in detoxicating phenolic compounds. Under stresses, the loss of plant membrane integrity may be due to accumulated phenolic and their oxidation products (Cakmak and Römheld 1997), the conjugating of polyamines to phenolic compounds could decrease the phenolic compounds levels, then maintain membrane integrity. Also, conjugated polyamines could effect protein directly at the level of protein synthesis or indirectly by influencing the properties of cell membranes and then enhance tolerance of tobacco plants to high-temperature stress (Königshofer and Lechner 2002). In addition, conjugated and bound polyamines have the potential to act as free radical scavengers to remove ROS and conjugated polyamines are good substrates for peroxidases, which utilize conjugated polyamines may remove H_2O_2 in the apoplast and then protect plant from ROS toxicity (Negrel and Lherminier 1987). It has been proposed that the protective effect of exogenous polyamines was dependent on their conjugated forms (Langebartels et al. 1991). Otherwise, the forming of conjugated and bound polyamines would maintain membrane integrity and DNA, RNA and protein stability. In this experiment, conjugated and bound polyamine levels in roots of grafted plants increased significantly under high nitrogen stress (Figs. 6 and 7), however, conjugated polyamine levels in roots of non-grafted plants decreased significantly and no obvious changes were observed in bound Spd and Spm levels. The reasons why high levels of conjugated and bound polyamines may enhance nitrogen stress tolerance might be attributed to the following arguments. First, acting as nitrogen reservoir, conjugated and bound polyamines could decrease nitrogen levels of plants and then promote the tolerance of plants to nitrogen stress. Second, acting as ROS scavenger, conjugated and bound polyamines could decrease ROS levels of plants and then protect plants from oxidative damage. Third, the conversion from free polyamines to conjugated and bound polyamines would do favor to maintain cell membrane integrity and DNA, RNA and protein stability. Fourth, the conversion from free polyamines to conjugated and bound polyamines could regulate free polyamines levels. However, the decrease of conjugated polyamines and bound Put in roots of non-grafted plants did not do favor to promote the tolerance to nitrogen toxicity.

In conclusion, the present study has shown that under high nitrogen stress, $O_2\bullet^-$ producing rate, H_2O_2 and MDA contents in non-grafted plants were significantly higher than those of grafted plants, and the biomass production reduction in non-grafted plants was more significant than that of grafted plants, suggesting that grafted tomato plants were more tolerant to high nitrogen stress. And the higher tolerance of grafted tomato plants was associated with higher antioxidant enzymes activities, higher proline content and higher free, conjugated and bound polyamine contents.

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