

The role of nitric oxide in priming-induced low-temperature tolerance in two genotypes of tomato

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Abstract

The present study was conducted to evaluate the contribution of nitric oxide (NO) to the priming effects on seed germination, seedling vigour and low-temperature tolerance of two tomato cultivars (*Solanum lycopersicum*, cv. Cherry and Falcato) under four different constant temperatures (10, 15, 20 and 25°C). Treatments included priming in a polyethylene glycol 6000 (PEG 6000) solution (osmo-priming) or presoaking in sodium nitroprusside (SNP) solution, a donor of NO, for 24 h at 25°C. Suboptimal temperatures reduced seed germination and subsequent seedling growth, as well as amylase activity, in both cultivars, but Falcato was more sensitive to low temperature than Cherry. Both osmo-priming and SNP treatments enhanced germination capacity, germination index, seedling root and shoot length, α - and β -amylase activities and sugar content under sub-optimal temperatures, especially in Falcato. We observed similarities between the modes of action of the two priming treatments. Both treatments increased seed NO content, whereas their positive effects on germination and subsequent seedling growth under low-temperature stress was reversed by the addition of methylene blue (MB), a scavenger of NO. This is the first report that suggests that positive effects of priming are most likely mediated through NO signalling.

Keywords: germination, low-temperature tolerance, nitric oxide (NO), osmo-priming, tomato

Introduction

Nitric oxide (NO), a highly reactive, membrane-permeable free radical, is a widespread intracellular

and intercellular messenger with a broad spectrum of regulatory functions in many physiological processes. In the past 20 years NO has been reported to be involved in various key physiological processes of plants, including seed germination, leaf senescence, ethylene emission, stomatal closure, and various plant responses to biotic and abiotic stresses, such as salinity, drought and ultraviolet (UV)-B-radiation (Neill *et al.*, 2003; Delledonne, 2005). It has been reported that molecules such as NO can enhance germination and/or break dormancy in seeds (Beligni and Lamattina, 2000; Beligni *et al.*, 2002; Neill *et al.*, 2003; Simontacchi *et al.*, 2004; Zhang *et al.*, 2005; Bethke *et al.*, 2007) and significantly impact plant growth and development (Lamattina *et al.*, 2003; Neill *et al.*, 2003; del Rio *et al.*, 2004). Gouvea *et al.* (1997) reported that exogenously applied sodium nitroprusside (SNP), as an NO donor, can enhance elongation growth in maize root segments, and application of methylene blue (MB), which inhibits NO production and/or NO action in plants, could reverse these responses.

Gould *et al.* (2003) reported that heat, osmotic and salinity stresses induced a rapid increase in NO production in tobacco cells. Furthermore, application of SNP alleviated the toxic effects caused by heat stress in weed calluses by decreasing hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) contents and increasing the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) (Song *et al.*, 2006). The positive effect of NO on alleviation of oxidative stress was also found after plant exposure to cold (Zhao *et al.*, 2009) and drought (Mata and Lamattina, 2001).

Like many other crop plants of tropical or subtropical origin, cultivated tomato (*Solanum lycopersicon* Mill.) is sensitive to chilling as well as freezing temperatures. Temperatures below 10°C inhibit growth, and those under 6°C may cause irreparable damage (Geisenberg and Stewart, 1986). Since the particular climatic conditions of the Mediterranean permit off-season production, tomato is often sown in winter or in early spring in cold greenhouses, plastic tunnels and seedbeds, or directly in the field. In early

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sowing, periods with suboptimal temperatures close to the minimum germination temperatures are frequently encountered. In these conditions, seedling emergence may be reduced, delayed and spread over time (Leskovar and Sims, 1987).

Treatments to enhance seed vigour have been proven to be very effective in achieving rapid and uniform seed germination. The priming treatment may constitute a useful tool in overcoming the problems described above and, thus, assuring a high probability of successful establishment for each seed planted. Since some tomato genotypes are more sensitive to cold than others, it is possible that priming could make these genotypes germinate as fast as the cold-tolerant ones. Furthermore, it could also improve the performance of the cold-tolerant ones (Dahal *et al.*, 1990).

Chen and Arora (2012) proposed a model illustrating the cellular physiology of priming-induced stress tolerance, which is likely achieved via two strategies. First, seed priming sets in motion germination-related activities, such as respiration (Corbineau *et al.*, 2000), endosperm weakening (Dahal *et al.*, 1990), embryo enlargement and transcription and translation (Schwember and Bradford, 2010), that facilitate the transition of quiescent dry seeds to germination and improve germination potential. Second, priming imposes an abiotic stress on seeds that represses radicle protrusion but stimulates stress responses, e.g. the accumulation of late embryogenesis abundant (LEA) proteins (Capron *et al.*, 2000), potentially inducing cross-tolerance. Together, these two strategies constitute a 'priming memory' in seeds, which can be recruited upon a subsequent stress exposure to mediate greater stress-tolerance of germinating primed seeds (Chen and Arora, 2012). Seed priming accelerates germination and improves seedling uniformity in many crops (Cayuela *et al.*, 1996; Benamar *et al.*, 2003; Farooq *et al.*, 2006), especially where they are grown under unfavourable environmental conditions (Dahal *et al.*, 1990; Tiryaki *et al.*, 2009; Amooaghaie, 2011).

Currently, the major focus of priming research is on optimizing protocols to improve germination performance. However, only a few biochemical studies have investigated enzymatic antioxidant activities during and after priming (Bailly, 2004; Amooaghaie *et al.*, 2010; Chen *et al.*, 2010b; Amooaghaie, 2011). Even fewer attempts have been made to understand the cellular and molecular mechanisms and signalling pathways of stress tolerance induced by priming (Chen and Arora, 2012).

The objective of the present study was to investigate the effects of exogenous SNP and osmopriming on germination, seedling vigour and suboptimal temperature tolerance of the two tomato genotypes Falcato and Cherry. Furthermore, a possible signalling role of NO in priming-induced responses was investigated.

Materials and methods

Plant material and treatments

Seeds of two tomato genotypes (Falcato, Cherry), were used to investigate the effects of priming on seed germination, seedling vigour and low-temperature tolerance. Seed treatments included osmopriming in polyethylene glycol solution (PEG 6000; Shanghai Chemical Reagent Co. Ltd, Shanghai, China) at 300 g l^{-1} , $200 \mu\text{M}$ sodium nitroprusside (SNP) as NO donor (Merck, Darmstadt, Germany), $100 \mu\text{M}$ methylene blue (MB; Fluka, Shanghai, China), $200 \mu\text{M}$ SNP + $100 \mu\text{M}$ MB, PEG + $100 \mu\text{M}$ MB, and distilled water as control (Con). For osmopriming, seeds were primed with continuous aeration. After 24 h at 25°C , the seeds were removed, rinsed in distilled water, wiped free of water and air-dried at 25°C for 24 h. For SNP or MB treatments, seeds were presoaked for 1 d in these solutions. As controls, seeds were soaked in distilled water for 1 d and then transferred to Petri dishes immediately.

Laboratory seed germination tests were conducted in incubators in 9-cm Petri dishes (50 seeds each per plate and three replicates for each treatment) on a layer of filter paper moistened with distilled water. Seeds were incubated in controlled-growth chambers at 10, 15, 20 and 25°C . Seed germination was counted daily and counts continued until no further germination occurred for three consecutive days. Seeds were considered germinated when radicles had protruded for more than 2 mm. Germination rate (GR) and germination index (GI) were calculated as described by the Association of Official Seed Analysts (AOSA) (1983), using the following formulae:

$$\text{GI} = \sum Gt/Dt$$

$$\text{GR} = \sum (n/t)$$

in which Gt is the number of germinated seeds in t days; Dt is the number of corresponding germination days; n is the cumulative number of germinated seeds in t days.

Root and shoot length were measured 7 d after the time of radicle protrusion. The vigour index was determined by the following equation:

$$\text{Vigour index} = \text{VI} = \text{seedling length} \\ \times \text{germination percentage.}$$

Biochemical analysis

All biochemical parameters (amylase activity, sugar content and NO content) were measured 12 h after incubation before any radicle protrusion took place.

Measurement of α - and β -amylase and soluble sugars

Alpha- and β -amylase activities of seeds at different temperatures were determined using the method of Bernfeld (1955), based on the reduction of free maltose, resulting from the enzymatic hydrolysis of starch, by 3,5-dinitrosalicylic acid, resulting in the formation of orange-coloured 3-amino-5-nitrosalicylic acid, which was determined colorimetrically at 540 nm. The activity of both enzymes was expressed as μ moles maltose (g seed)⁻¹. Soluble sugars were analysed by the anthrone method (Fales, 1951).

NO measurement

NO production was determined according to the method described by Zhou *et al.* (2005) with slight modifications. Samples were ground in a mortar and pestle in 3 ml of 50 mM cold acetic acid buffer (pH = 3.6, containing 4% zinc diacetate). The homogenates were centrifuged at 10,000 g for 15 min at 4°C. The supernatant was collected. The pellet was washed with 1 ml of extraction buffer and centrifuged as before. The two supernatants were combined and 0.1 g of charcoal was added. After vortexing and filtration, the filtrate was collected. A mixture of 1 ml of filtrate and 1 ml of Griess reagent was incubated at room temperature for 30 min. Another identical filtrate which was pretreated with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide potassium salt (cPTIO), a specific NO scavenger, for 15 min, was used as a control. Absorbance was assayed at 540 nm. NO content was calculated by comparison with a standard curve of NaNO₂.

Statistical analysis

Seed germination and seedling development experiments were performed as a factorial experiment with completely randomized design with three replicates. Data significance was assessed by analysis of variance and the differences between treatment means were compared by Duncan's multiple range test.

Results

Seed germination and seedling vigour

The significant interaction of genotype \times temperature is determined by the different responses of genotypes at low temperatures. In control treatments, germination percentage (GP) at 25°C was similar for the two genotypes (100% for both) but at 10°C the difference was significant (31.0% for Falcato and 50.3% for Cherry). GP was not affected significantly at 20°C in Cherry (91.6%) as compared with 25°C, but it was significantly reduced at 15 and 10°C, to 78 and 50.3%, respectively ($P < 0.05$). In contrast, in Falcato, GP was

reduced significantly at 20, 15 and 10°C to 75, 56 and 31%, respectively ($P < 0.05$). A similar trend was observed for GI and GR in both genotypes in response to temperature reduction (Fig. 1). However, both priming and SNP pretreatment were able to partly alleviate low-temperature reduced seed germination, which was consistent with the responses of other germination parameters, such as GI (Fig. 1).

A similar trend was observed for root and shoot length of the seedlings and, consequently, the vigour index (VI) in both genotypes in response to temperature reduction. Osmopriming and SNP were effective in promoting greater root and shoot length of seedlings and VI than the control at 10°C, especially for Falcato (Fig. 2). Although GP, GI, GR, root and shoot length and VI were higher in Cherry than Falcato in all treatments and at all temperatures, osmopriming and SNP improved these parameters to a larger extent in Falcato than Cherry (Figs 1 and 2). For example, at 10°C, SNP increased shoot and root length of Falcato seedlings by 0.65 and 1.1 cm, respectively, but in Cherry seedlings by 0.4 and 0.6 cm respectively (Fig. 2).

Alleviation of tomato seed germination and seedling growth inhibition as conferred by osmopriming or SNP pretreatment, could be reversed by the combination of osmopriming, or SNP, plus MB, a blocker of NO action (Figs 1 and 2).

Sugar content and amylase activity

Both α - and β -amylase activities and sugar contents declined significantly in germinating tomato seeds under low-temperature stress as compared to the control ($P < 0.05$, Fig. 3). In contrast, both osmopriming and SNP pretreatments brought about an obvious increase in α - and β -amylase activities and these responses were accompanied by an accumulation of soluble sugars, especially at 10°C (Fig. 3). Application of MB to the osmopriming and SNP solutions reversed these responses.

NO production driven by priming and SNP pretreatments

The results above demonstrated that priming-induced responses were sensitive to the NO scavenger. To further confirm whether above priming-driven responses were related to NO signalling we determined NO contents of the seeds. Low-temperature treatment (10°C) enhanced NO levels moderately (Fig. 4), whereas osmopriming and SNP pretreatment produced a very strong increase in NO content that was significantly higher than that of the sample under low-temperature stress alone. When MB was added to the osmopriming or SNP solutions, the rate of NO generation was dramatically inhibited, reaching the

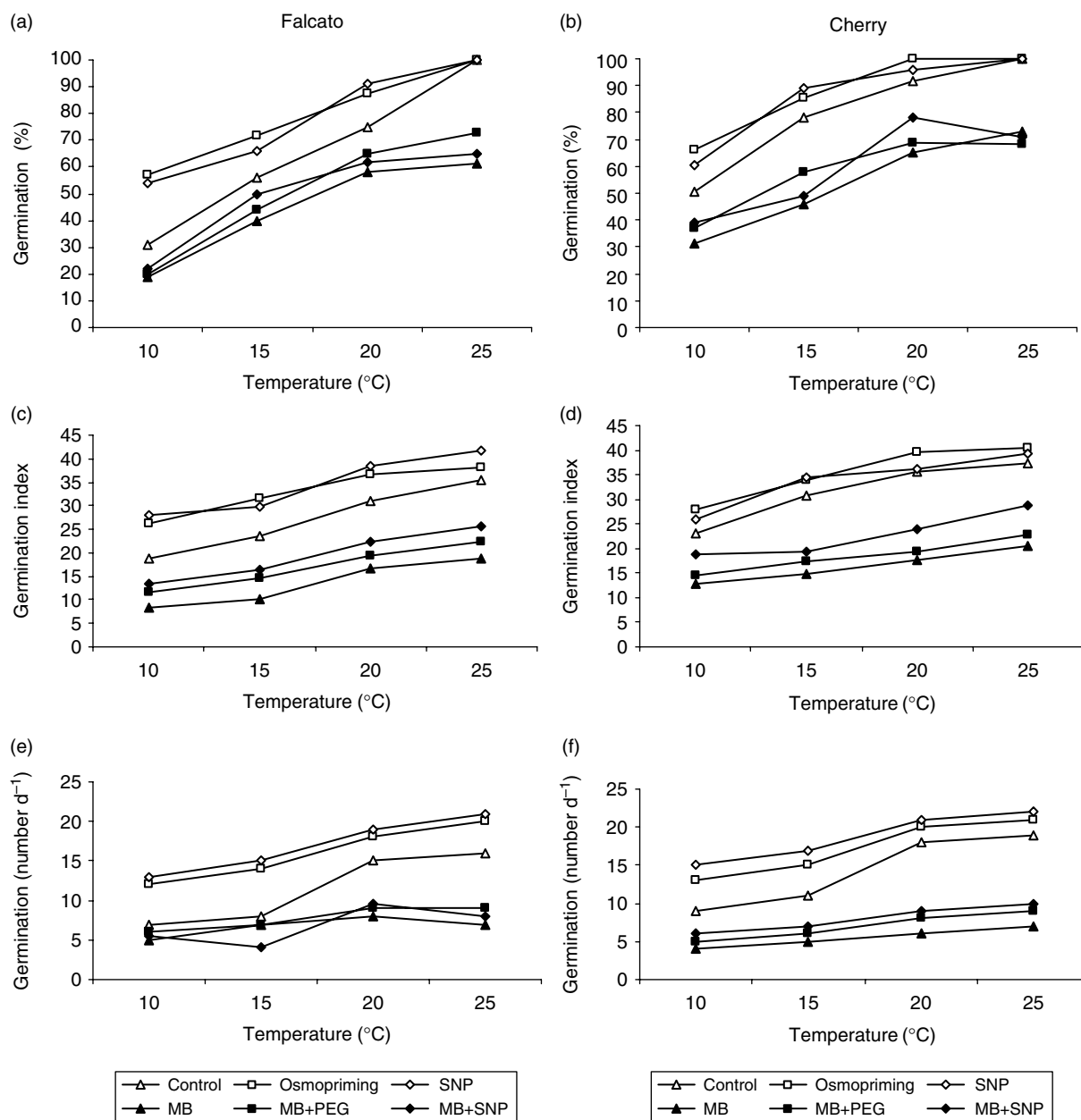


Figure 1. The effects of osmopriming (PEG) and sodium nitroprusside (SNP) and methylene blue (MB) treatments on germination percentage, germination index and germination rate of two tomato genotypes at various temperatures.

basal level of low-temperature stress alone. Interestingly, we also noticed that MB pretreatment alone significantly resulted in the decrease of NO production, in comparison with low-temperature stress alone and even compared with the control sample at 25°C ($P < 0.05$), suggesting that endogenous NO might be involved in tomato seed germination under both normal and low-temperature stressed conditions. Osmopriming and SNP increased NO content more in Falcato than Cherry (Fig. 4), although higher NO content was observed in Cherry in all treatments.

Discussion

Most commercial genotypes of tomato (*Solanum lycopersicum* Mill.), are sensitive to low germination temperatures. Germination temperatures range from a minimum of 8–10°C to a maximum of 35–38°C with an optimum between 20 and 25°C (Geisenberg and Stewart, 1986), which was consistent with our data showing an optimum temperature of 25°C. With reducing the temperature to 10°C, the controls showed a concomitant reduction in GP, GI and VI, as well as in

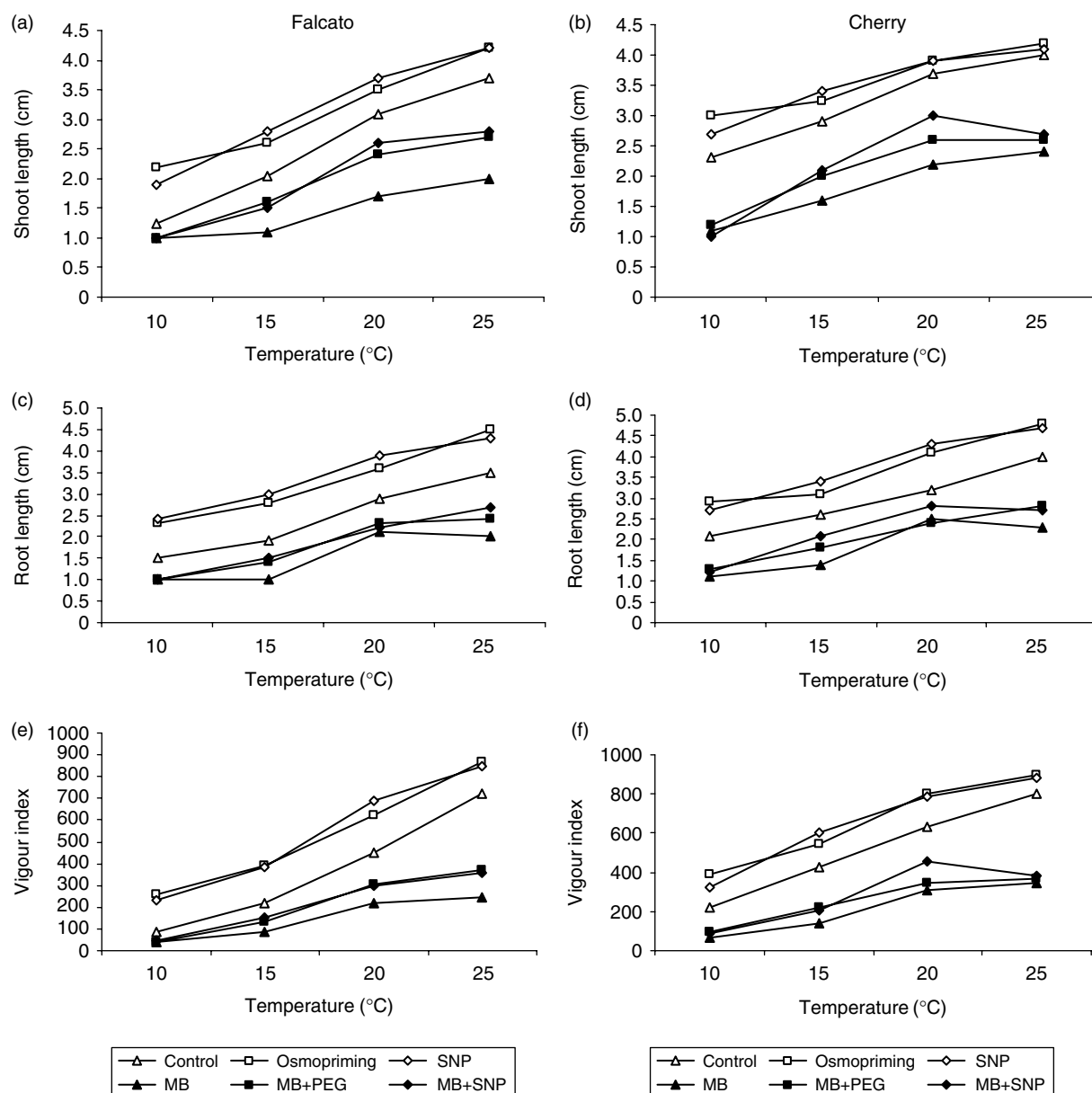


Figure 2. The effects of osmopriming (PEG) and sodium nitroprusside (SNP) and methylene blue (MB) treatments on shoot and root length of seedling and vigour index of two tomato genotypes at various temperatures.

α - and β -amylase activities, and sugar content in both genotypes. Falcato proved to be more sensitive to low temperature than Cherry. The differences between the two genotypes were likely due to their differing genetic background.

Priming and SNP induced earlier and more uniform germination and stronger seedlings at all temperatures, as indicated by higher GP, GI and VI, but their effect on these parameters was more pronounced at suboptimal temperatures. Both SNP and osmopriming treatments increased amylase activities and levels of soluble sugar in seeds. These sugars would be readily utilized to support the energy and carbon metabolism that are operational during

seed imbibition. SNP and osmopriming appeared to stimulate seed germination via a similar biochemical process. Both treatments enhanced amylase activities and seeds contained more soluble sugars, being able to more readily support metabolic activities and consequently resulting in a higher rate of emergence, which correlated with α -amylase activity and GI. Enhancement of amylase activity and sugar content by SNP (Zhang *et al.*, 2005; Xu *et al.*, 2011) and priming (Farooq *et al.*, 2006) have been reported by other authors.

Correa-Aragunde *et al.* (2006) have demonstrated that NO modulates the expression of cell-cycle regulatory genes in tomato and plays a central role in

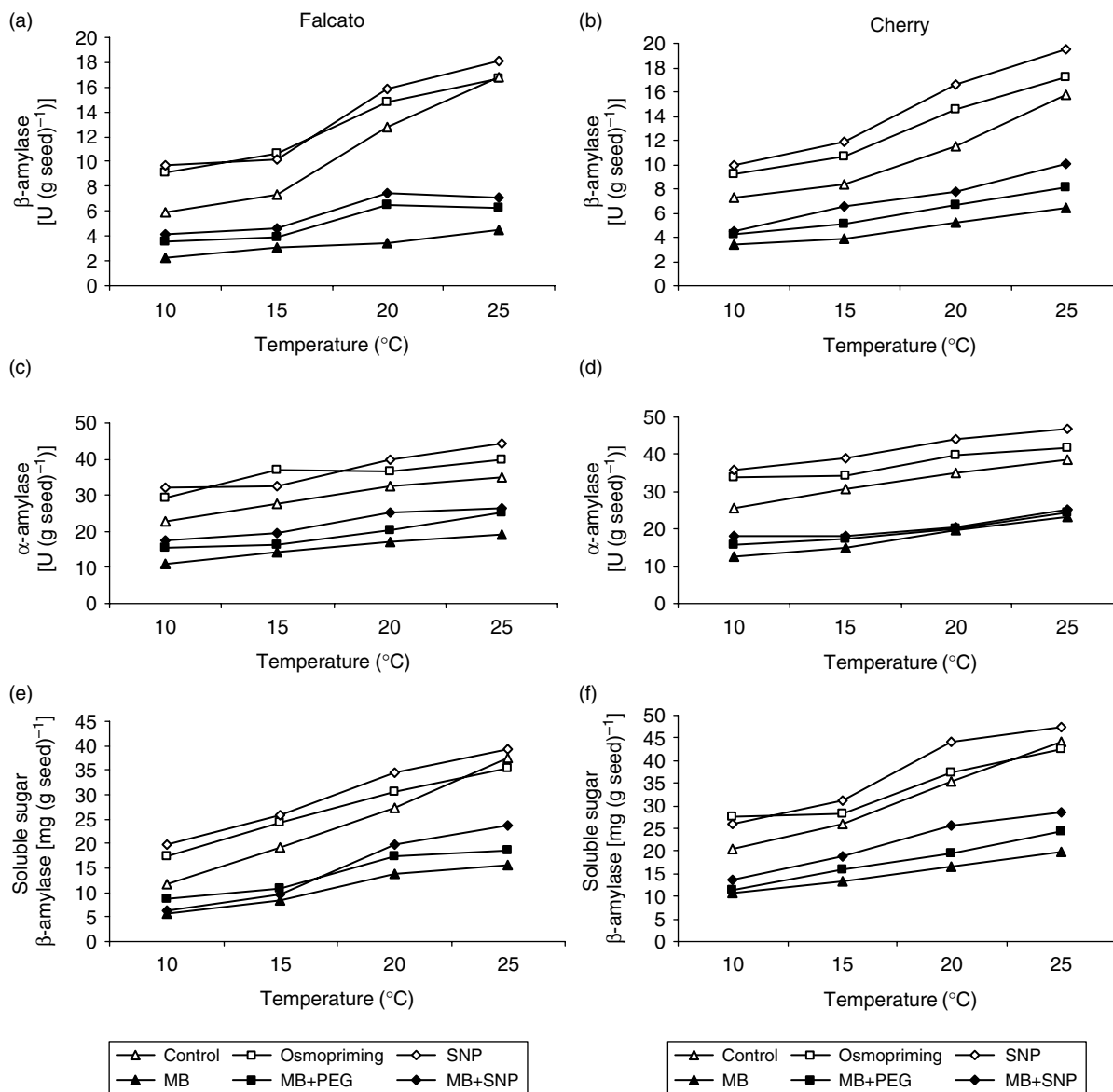


Figure 3. The effects of osmopriming (PEG) and sodium nitroprusside (SNP) and methylene blue (MB) treatments on α - and β -amylase activities and soluble sugar content in seeds of two tomato genotypes at various temperatures.

determining lateral root development, allowing further understanding of the components that control plant cell differentiation and proliferation. Therefore, the NO-induced increase in seedling growth under suboptimal temperature conditions may have been due to an increase in cell division and/or cell enlargement, although these phenomena were not examined in the present study. Similarly, differential display during maize germination has detected an earlier expression in primed germinating seeds (as compared to unprimed) of *ZmAA9-24*, a gene encoding a protein believed to regulate cell expansion and elongation (Cruz-Garcia *et al.*, 2003). Our data showed that no difference was observed in seed germination between control and osmopriming and SNP pretreatments at 25°C, but osmopriming and SNP increased GI, GR and VI.

Probably, seeds can produce just adequate amounts of NO required for germination at 25°C and these treatments probably shortened the time to achieve the required NO levels for seed germination and subsequent seedling growth. It has been reported that exogenously applied NO can enhance germination or break seed dormancy (Beligni and Lamattina, 2000), and when no dormancy breakage is required, greater germination rates have been observed by supplementation with an NO donor (Kopyra and Gwóźdz, 2003).

Under low-temperature conditions, SNP and osmopriming resulted in the enhancement of seedling growth of both tomato genotypes, but the enhancement in growth of the low-temperature-sensitive genotype (Falcato) was higher than that of Cherry. Wu *et al.* (2011) reported that SNP-induced

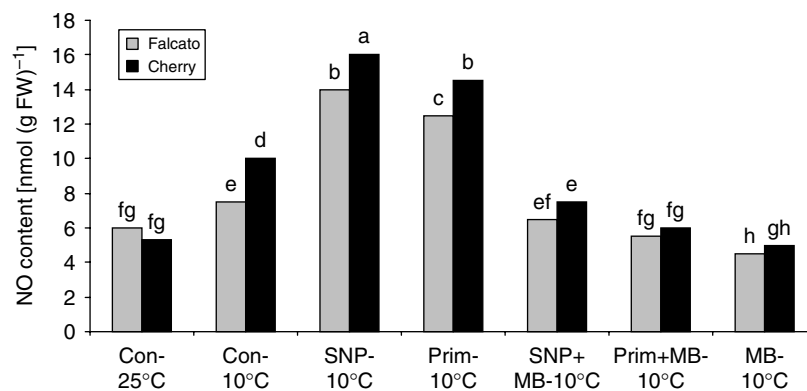


Figure 4. The effects of osmopriming (Prim) and sodium nitroprusside (SNP) and methylene blue (MB) treatments on NO content in two tomato genotypes at various temperatures. Con, control.

enhancement of growth of a salt-sensitive tomato genotype (Hufan2496) was higher than that of a salt-tolerant genotype (Hufan1480). In addition, external NO could act as a regulator of antioxidant intervention strategy, in preventing oxidative stress in response to cadmium stress in barley seedlings in a genotype-dependent pattern (Chen *et al.*, 2010a). On the other hand, suboptimal temperatures increased the NO content of the controls. Enhancement of endogenous NO content has been reported for plants under cold (Zhao *et al.*, 2009), heat (Song *et al.*, 2006) and salinity (Xu *et al.*, 2011) stresses. NO acts as an important signal in responses against abiotic stress. Therefore, under stress conditions, higher levels of NO are required for the maintenance of cell homeostasis, and once NO is endogenously generated or gets inside the cell from an exogenous source, it enhances stress tolerance (Neill *et al.*, 2003; Delledonne, 2005). Our results suggest that SNP- and osmopriming-induced suboptimal temperature tolerance in seeds is associated with higher NO contents. Similar roles of SNP have been suggested for lettuce seeds in the darkness (Beligni and Lamattina, 2000), and in salinity-stressed (Zheng *et al.*, 2009) and copper-stressed (Hu *et al.*, 2007) wheat seeds. Furthermore, SNP- and osmopriming-mediated responses appeared to be NO dependent, as they were blocked by treatment with MB, a scavenger of NO. The MB effect on reduction of NO content and reversal of beneficial effects of SNP on salt tolerance has also been reported for maize (Zhang *et al.*, 2006) and wheat (Xie *et al.*, 2008) seedlings. The beneficial effects of seed priming on germination under stress conditions have been observed in tomato (Dahal *et al.*, 1990; Cavallaro *et al.*, 1994), spinach (Chen *et al.*, 2010b) and white clover (Tiryaki *et al.*, 2009). However, to our knowledge, the present paper is the first report on the effect of osmopriming on NO content of seeds and blocking of the priming responses by MB. Our results highlight the probability of a role of NO signalling in priming responses.

Two mechanisms may explain the protective action of NO against oxidative damage. First, NO might act on reactive oxygen species (ROS) directly, to form peroxynitrite which is less toxic, thus limiting cellular damage (Martinez *et al.*, 2000) and, second, it might act as a signal in activating ROS-scavenging enzyme activities under abiotic stress (Neill *et al.*, 2003; Delledonne, 2005; Zhou *et al.*, 2005). NO plays an important role in resistance to salt, drought, temperature (high and low), UV-B and heavy metal stress (Lamattina *et al.*, 2003). Song *et al.* (2006) found that application of SNP and *S*-nitroso-*N*-acetylpenicillamine, both NO donors, dramatically alleviated heat-stress-induced ion leakage, growth suppression and decrease of cell viability in callus of reed under heat stress, and enhanced the activities of SOD, CAT, APX and POD. Similarly, several authors have reported that priming enhanced accumulation of non-enzymatic compounds and activities of detoxifying enzymes (e.g. SOD, APX, CAT) and improved membrane integrity under stress conditions (Sivritepe, 2008; Amooaghaie *et al.*, 2010; Chen *et al.*, 2010b; Amooaghaie, 2011; Sun *et al.*, 2011). These findings suggest that enzymatic and metabolic activities that are the basis of priming-induced seed invigoration and low-temperature tolerance, are probably promoted via NO signalling.

Taken together, our results suggest that endogenous NO plays a key role in priming-mediated responses, although we have not explored the potential source of NO driven by priming. Therefore, further investigations should address this question.

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