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Improving Effects of Mild Cold Stress and Salicylic acid on Growth and Physiology of Periwinkle (*Catharanthus roseus* Don.)

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Abstract
To study effects of salicylic acid (SA) treatments (0, 0.5, 1 and 2 mM) in two forms of seed priming (for 24 h) and spraying on seedling at 4-6 leaf stage an experiment was conducted on periwinkle (*Catharanthus roseus* Don.) with and without exposure to a mild cold stress of 8 °C (for 32 h). Seeds were sown in trays containing peat-based substrate (with 20% sandy loam field soil). Transplants of both groups were treated with mild cold. Seedlings were grown in greenhouse with an average day/night temperature of 25/20± 2°C until their blooming. Seed emergence rate and percentage, activities of catalase (CAT), guaiacol peroxidase (GPX), polyphenol oxidase (PPO), CAT gene expression, height and number of nodes per plant, and days to flowering were evaluated. Results showed that mild cold stress together with salicylic acid at 0.5 mM (as either seed priming or spray on seedling) was the best treatment to accelerate the flowering, and improve growth parameters and antioxidant enzymatic activities. Thus, mild cold stress enhanced the positive effects of SA treatments on cold acclimation of periwinkle (particularly when associated with spraying, which is a simple administration method). Accordingly, it might be recommended for its seedling production and cultivation in temperate climates to prevent late spring frost damages.

Keywords: Antioxidant Enzymes, medicinal ornamental plant, stress, plant growth regulator(s).

Abbreviations: SA, salicylic acid; **CRD**, Completely randomized design; **CAT**, catalase; **GPX**, guaiacol peroxidase; **PPO**, polyphenol oxidase; **ERI**, Emergence rate index;

Introduction

Periwinkle (*Catharanthus roseus* Don.) is a perennial ornamental and medicinal plant with more than 130 unique indole-alkaloid and terpenoid compounds (Van der Heijden et al., 2004). Although Periwinkle is native to tropical regions and has sensitivity to cold stress (Dole and Wilkins, 1999), it is cultivated in different areas and occasionally exposed to harmful late spring cold, which could either positively or negatively influence the plant stability and production. Temperature is an important environmental factor, which varies seasonally and it is subjected to unpredictable and daily oscillations. The cold stress impact on plant

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is influenced by minimum stress temperature and its duration, plant growth stage, and physiological conditions. To keep their growth and development, plant species should be able to cope with the seasonal imbalances and temperature fluctuations (Thomashow et al., 1999). The specific roles of additional safeguarding strategies to avoid any damage provoked by late cold spring (i.e., fulfillment of a certain amount of winter chilling to break dormancy and/or a certain photoperiod) have been controversially discussed for some woody plants (Körner and Basler, 2010; Chuine et al., 2010). However, new evidence revealed that chilling may be more important than photoperiod (Basler and Körner, 2012; Laube et al., 2014; Polgar et al., 2014).

Cold tolerance is naturally induced with the gradual adaptation of plant to the low but above freezing temperatures (Thomashow et al., 1999). This adaptation is achieved through multiple changes in the gene expression, decreased or ceased growth, changes in the lipid composition of the membrane, some change in the composition of compatible solutes and increase in cellular antioxidants (Nayyar et al., 2005). These changes are all directly involved in stress tolerance and thus considered as active ingredients to cope with environmental stresses, particularly cold stress.

Cold stress increases the concentration of reactive oxygen species (ROS), causing oxidative stress in plants, which known as secondary stress. Cold-tolerant plant genotypes are more able to remove ROS and other harmful factors, due to the production of higher levels of antioxidants (Verma and Dubbey, 2003). Antioxidant enzymes (e.g., ascorbate peroxidase, catalase, and superoxide dismutase) together with guaiacol peroxidase and other enzymatic and non-enzymatic antioxidants have an important role in balancing synthesis and degradation of ROS. Therefore, antioxidant response and other capabilities induced in plant due to mild cold stress and their interactions with oxidative stress damages could be effective to cope with cold stress in plants.

Salicylic acid (SA) is considered to be a plant hormone-like substance, which plays an important role in the regulation of some plant processes such as plant growth and development, seed germination, flowering, fruit yield, glycolysis, membrane structure, uptake, photosynthetic rate, stomatal conductance, chlorophyll content, stress tolerance, and heat production in thermogenic plants (Belkhadi et al., 2010). SA also increases tolerance to high temperatures in some plants such as potatoes and beans and effectively protects these plants against water stress and increases plant growth under stress conditions (Senaratna et al., 2000). It has been reported that salicylic acid can increase leaf number and leaf area in plants such as violets (Martin-Mex et al., 2001). The inhibitory effects of exogenous SA application were recorded for concentrations mostly over than 1 mM. It is confirmed that SA efficiency depends on the concentration and differs among plant species and growth stages (Hayat et al., 2010; Xie et al., 2007).

It is supposed that mild cold stress and SA application through influencing the quality of protein translation, priming of seed metabolism, synthesis of antioxidant enzymes, and mobilization the seed storage proteins could alter the morphological and physiological traits of plants and seed vigor (Rajjou et al., 2006; Horváth et al., 2007). Therefore, this study aimed to evaluate the effects of mild cold and SA application methods (seed priming or seedling spraying with different concentrations of SA) on the response of periwinkle to cold stress, its enzymatic activities, and the plant acclimation regarding some morphological and biochemical characteristics.

Material and Methods

A factorial experiment based on CRD was conducted to evaluate the effect of salicylic acid (SA) either as seed priming or spraying on seedlings at 4-6 leaf stage on

periwinkle (*Catharanthus roseus* Don.), furthermore, the effect of mild cold on some morph-physiological characteristics of this plant were investigated. For this purpose, before sowing, periwinkle seeds (150 seeds) primed with SA solution at four different concentrations (0, 0.5, 1 and 2 mM) for 24 hours (Farooq et al., 2008). The rest (150 seeds) hydroprimed for the same time and were taken as the control group. The seeds were rinsed three times with distilled water and dried back closer to their original moisture level with tissue paper at room temperature. They were then sealed in polythene bags and stored in a refrigerator at 5 °C until use (Lee and Kim, 2000). All of the seeds were sown in trays (with 72 cells) containing peat-based substrate (with 20% sandy loam field soil). A given part of transplants grown from hydroprimed seeds were also sprayed with different concentrations of SA solution at 4-6 leaf stage on seedlings.

The second experiment factor that was mild cold treatment, applied at seedling stage for both SA treated groups (seed priming or spraying at seedling stage) with 8 °C for 32 hours in an incubator where control was also considered thoroughly. Sampling for enzymatic measurements was performed for a number of plants at four to six-leaf stages at a given time for all treatments corresponding to the 8^{th} hour after performing the cold treatment (i.e. 40 h after SA treatment as spraying). The rest of the transplants were transferred to the greenhouse with an average day/night temperatures of 25/20 ± 2 °C, until flowering stage to assess the other traits such as days to flowering, plant height, and number of nodes.

Thereafter, seed emergence rate and percentage were computed at emergence stage. CAT, GPX, PPO, and CAT gene expression evaluated 8 hours after performing the cold treatment and days to flowering, height and number of nodes were measured at the end of the experiment.

Emerged seeds were counted daily during days 5 to 16. Emergence percentage was computed based on the seeds emerged on the last day. Emergence rate index (ERI) was computed according to the method described by Maguire's (1962) by the following equation:

$$ERI=\sum G_n/D_n$$

where G_n is the percentage of total viable seeds counted as emerged and removed on day n and D_n is number of days after sowing (n equals the given day after sowing). Sampling for enzymatic measurements was performed for a number of plants at four to six-leaf stages at a given time for all treatments corresponding to the 8th hour after application of cold treatment (i.e. 40 h after SA treatment as spraying). The rest of the plants were transferred to the greenhouse until flowering stage to assess the other traits such as days to flowering, plant height, and number of nodes. Enzymatic assays were performed according to the method described by Chang and Koa (1988), where 0.5 gram of fresh leaf from each treatment was totally pulverized and then 6 ml of extraction buffer (containing 0.5 M Tris-HCl (pH=7), 3 mM of $MgCl_2$, and 1 mM EDTA) was added. Thereafter, the solution was centrifuged at 10,000 rpm at 4 °C for 15 minutes, and the supernatant solution was removed for measuring enzymes. All enzymes were measured using s spectrophotometer (Shimadzu UV 180).

CAT activity was measured at 25 °C and at a wavelength of 240 nm according to the method described by Colowick and Kaplan (1984). The materials were included 3000 µl of phosphate buffer solution (50 mM), 50 µl of hydrogen peroxide (30%), and 50 µl of enzyme extract. The activity was recorded for 5 minutes at 20-second intervals.

GPX activity was also measured at 25 °C according to the method described by Chance and Maehly (1955) at a wavelength of 470 nm. The materials for this measurement were included 3000 µl of 50 mM phosphate buffer (pH=7), 10 µl of

hydrogen peroxide (30%), 3 µl of 200 mM guaiacol solution, and 50 µl enzyme extract. The activity was recorded at 20-second intervals for 5 minutes and uptake increased with time. The activity of GPX was computed in milligrams based on the absorption of orange tetraguaiacol protein concentration.

PPO was evaluated according to the method described by Kar and Mishra (1976). The reaction medium was contained 50 µl of 100 mM pyrogallol, 3000 µl of 50 mM phosphate buffer (pH=7), and 50 µl of the enzyme extract. The enzyme activity was measured at 420 nm based on the amount of oxidized pyrogallol and reported as absorption per minute per milligram protein.

Total RNA was extracted using an extraction buffer that contained 300 mM Tris HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, CTAB 2%, PVPP 2%, spermidine trihydrochloride 0.05%, and β-mercaptoethanol 2% (Reid et al., 2006). First strand cDNA was synthesized from 2 µg of total RNA using the iScript cDNA synthesis kit (Bio-rad, Hercules, CA) according to the manufacturer's instructions. The ribosomal protein gene was used as a reference (Table 1). Real-Time PCR reactions were performed using HiFi SYBR® Green Master Mix Kit in the LightCycler 96 (Roche). The specific primers used for Real-time PCR are given in Table 1, respectively. Two primer pairs of T16 and G10h genes were designed according to periwinkle data (El-Domyati et al., 2014; Goklany et al., 2009). Since there is no information regarding CAT in periwinkle, the primers were designed through multiple alignments of CAT genes in different reported species and based on the conserved region. Data from this phase was analyzed relatively using comparative CT method ($\Delta\Delta C_T$ method); where ΔCT1 was for target or test sample, ΔCT2 was for control or calibrator sample, CT1 was the CT value for target gene, and CT2 was the CT value for housekeeping gene of the same sample. Control sample prepared from SA and mild cold untreated plants for comparative expression results of both seed and seedling samples, where:

ΔCT1= CT1 -CT2; ΔCT2= CT1 -CT2; $C\Delta\Delta_T$=ΔCT1 -ΔCT2.

The range of fold-differences in the amount of target, normalized to housekeeping gene and relative to a calibrator, was calculated as $2^{-\Delta\Delta C}{}_T$.

Statistical analysis was performed using SAS and mean comparisons was carried out using Duncan's multiple range test ($P<0.05$).

Table 1. Specific primers used for Real-Time PCR

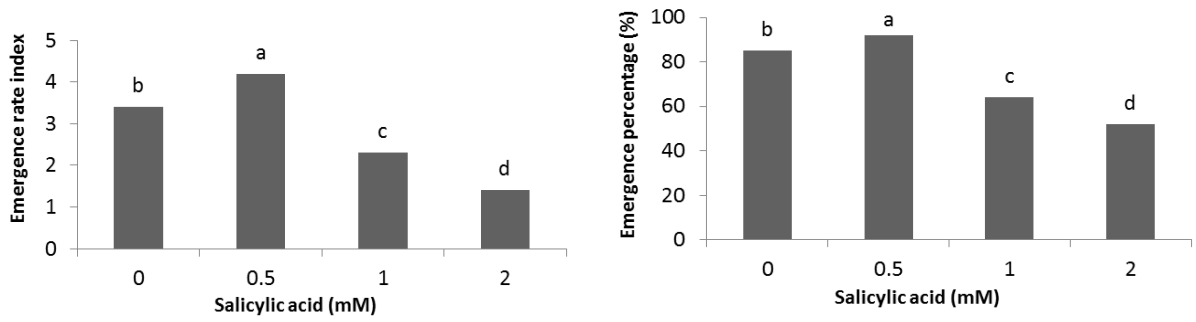
Explanation	Gene type	Forward primer	Reverse primer
Ribosomal protein gene	RPS9	TCCACCATGCCAGAGTGCT	ACCACCAGATGCCTTCTTCG
Catalase	CAT1	CACCGTCTTGGACCAAACTA	CATCCCTGTGCATGAAGTTCAT

Results and discussion

Plant growth

The effect of salicylic acid (SA) treatment as seed priming on seedling emergence rate index and percentage was significant. The highest emergence rate index (4.2) and emergence percentage (92%) was obtained in treatment containing 0.5 mM salicylic acid, and these indices were declined as SA concentrations were increased (Figure 1). Accelerated seed germination and plantlet emergence was observed once the seeds were treated with 0.5 mM SA, which might be due to the role of the product that stimulates α-amylase enzymatic activity.

Xie et al. (2007) reported the inhibitory effect of SA at higher concentrations (1 mM) through regulation of α-amylase in barley seeds. In the plant life cycle, the seed and seedling stages are key developmental stages determining the final yield of crops, both of which are very sensitive to environmental stresses (Koornneef et al., 2002). Seed



(A) **(B)**

Fig. 1. Effects of different concentrations of salicylic acid on (A) emergence rate and (B) emergence percentage of periwinkle. Different letters show significant differences (P≤0.05) among treatments.

germination have three steps: water imbibition, cell elongation, and increase in cell number (Toole et al., 1956). In some seeds, besides water absorption, some other factors are also needed for completion of germination. Increase in enzymatic activities and the subsequent increased respiration causes breaking down of stored materials (De Villiers et al., 1994). Plant defense mechanisms are principally studied on mature plant organs, and there is very scarce information about the onset of defense mechanisms at the level of seed germination. Increased photosynthetic rate has been reported as an effect of SA priming in mustard plant (Fariduddin et al., 2003). The quality of protein translation, priming of seed metabolism, synthesis of antioxidant enzymes, and mobilization of seed storage proteins were concerned as some processes that might be affected by SA and are likely to improve seed vigor (Rajjou et al., 2006).

Results of current experiment showed that the height and number of nodes per plant reduced at 2 mM SA treatment compared to the lower concentrations. However, 0.5 mM salicylic acid was the best concentration to increase plant height and number of nodes, particularly when accompanied by cold treatment (Table 2, Figure 2).

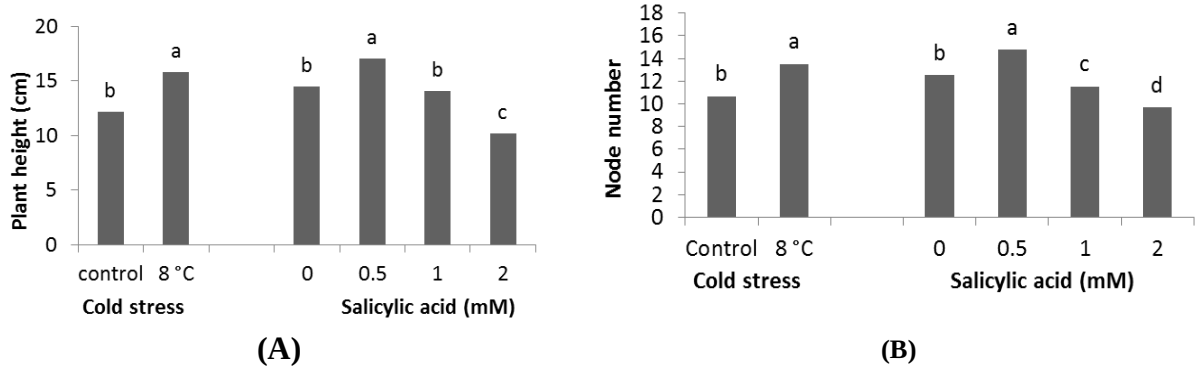
Plant growth in the term of height and node number, was accelerated following the application of 0.5 mM SA when SA-treated transplants exposed to a mild cold (Table 2). Increased concentration of SA negatively influenced the growth parameters. It is clear that plant height and number of nodes are closely correlated (Brevendan et al., 1978). The positive effect of low concentrations of SA on yield has already been observed in other plants. For instance seed priming by 0.5 mM SA in wheat (Senaratna et al., 2000) and 0.01 mM SA in alfalfa (Drazic et al., 2006) improved plant growth by increasing cell division and enlargement of the root cells. SA, usually at low concentrations, increases the growth and resistance of plants to stresses (Dat et al., 2000). It seems that the improved stem growth at low temperatures is due to the change in sink organs. Low temperatures increase leaf dry matter, so more nutrients can be available to stem for elongation and growth (Roh, 2005).

On the other hand, at high temperatures, plant respiration rate and demand for carbohydrates and assimilates stored in the plant for growth, are increased to compensate for materials consumed in respiration. Generally, the plant demands more assimilate in warm conditions. Therefore, a higher consumption of storage materials and a reduction in plant height is expected at high temperatures (Kamenetsky et al., 2003).

Table 2. Means comparisons of the interactive effects of cold and salicylic acid on some morph-physiological traits and activity of antioxidant enzymes including catalase (CAT, Abs_{240} min^{-1} g^{-1} protein), guaiacol peroxidase (GPX, Abs_{470} min^{-1} g^{-1} protein) and polyphenol oxidase (PPO, Abs_{420} min^{-1} g^{-1} protein) in periwinkle.

Treatment period	Mild Cold	Salicylic acid (mM)	Days to flowering	Plant height (cm)	Node number	CAT (Abs_{240})	GPX (Abs_{470})	PPO (Abs_{420})
Seed	Control	0	104.3cd	12.3e	11d	0.33e	0. 53f	0.024e
		0.5	98f	17.8ab	15bc	0.44d	0.70d	0.037c
		1	106.6bc	11.8ef	10.6de	0.34e	0.52f	0.020e
		2	106bc	9.5fg	9ef	0.21f	0.44g	0.014f
	Cold	0	98.3f	16bc	13.6c	0.63b	0.80c	0.053b
		0.5	93.6g	19.8a	17a	0.72a	0.96a	0.062a
		1	100.3ef	15bcd	10.6de	0.64b	0.80c	0.054b
		2	101def	11ef	10.6de	0.44d	0.64e	0.035cd
Seedling	Control	0	104ed	13de	11.6d	0.32e	0.52f	0.024e
		0.5	99f	13.6cde	11.6d	0.34e	0.53f	0.025e
		1	106.3cb	12ef	9ef	0.33e	0.52f	0.024e
		2	113.6a	8g	7.3f	0.35e	0.50f	0.023e
	Cold	0	99.3f	16.6b	13.6c	0.57c	0.77c	0.050b
		0.5	93g	17b	15.3abc	0.70a	0.93b	0.059a
		1	101def	17.6ab	15.6ab	0.61b	0.77c	0.050
		2	108b	12.6e	11.6d	0.41	0.62e	0.031d

Different letters show significant differences (P≤0.05) among treatments.



(A) **(B)**

Fig. 2. The effects of cold stress (8 °C for 32 h) and different concentrations of salicylic acid on (A) plant height and (B) node number of periwinkle. Different letters show significant differences (P≤0.05) within each group of treatments.

The results also revealed that SA, mild cold, and application period significantly affected the number of days to flowering. Mild cold caused a significant decrease in the number of days to flowering compared to untreated control plants (Figure 3). The shortest days to flowering (93 days; over 10 days less than that of the control group) was found in plants treated with 0.5 mM SA (whether applied on seeds or seedlings) and grown under mild cold (Table 2). Low temperatures make changes in various plant physiological aspects leading to internal auxin biosynthesis and flowering (Xu et al., 2007). Seed priming was more effective than spraying on seedlings to decrease the number of days to flowering stage (Figure 3).

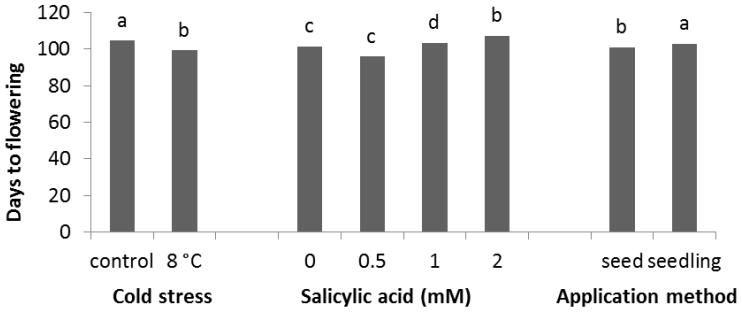


Fig. 3. The simple effect of cold stress (8 °C for 32 h): different concentrations of SA and application method on days to flowering of periwinkle. Different letters show significant differences (P≤0.05) within each group of treatments.

Effects of seed priming on decreasing the number of days to flowering is indicative of the influence of early root growth, plant deployment, and optimal use of nutrients by the plant. SA at 0.5 and 2 mM had the highest and the lowest effects on shortening the flowering time, respectively. Salicylate effects on flowering processes have been documented by various researchers (Dekock et al., 1974). The improving effects of SA on flower bud formation and vegetative growth of soybean have been previosly reported, furthermore, pod formation was accelerated about 2-5 days (Kumar et al., 2000), indicating that SA plays a role in the regulation of flowering process in plants.

Flowering time of seedlings that were treated with SA at 0.5 mM together with a mild cold was accelerated than other plants. In addition, spraying SA at 2 mM at four to six-leaf stages without cold treatment delayed the flowering time than the other treatments. Since a single treatment might be preferred from an economic standpoint or for time saving purposes, hereafter the simple effects of the treatments were further analyzed.

Antioxidant enzymes and gene expression

Results showed that SA and cold treatments had significant effects on the activities of antioxidant enzymes such as CAT, PPO, and GPX. Mild cold stress compared to the control, 0.5 mM of SA compared to the higher concentrations, and application of SA as seed priming compared to spray on seedlings resulted in higher CAT, PPO, and GPX activities (Figure 4). 0.5 and 2 mM SA had the highest and the lowest impact on the activity of antioxidant enzymes, respectively. SA has a dual effect on the plant metabolism. At low concentrations, it activates antioxidant enzymes, induces scavenging system for ROS, and as a result leads to improvement of stress resistance (Mutlu et al., 2009). In contrast, at high concentrations, not only it acts as an antioxidant, but also it acts as an oxidant that can influence cellular components, thereby having negative effects on the plant metabolism (Fariduddin et al., 2003). For example, SA pretreatment of corn seeds resulted in increased activity of new isozyme ascorbate peroxidase (enzyme neutralizing H_2O_2) and reduced ethylene production (Janda et al., 1999). In another study, the effect of SA at 0.5 mM on plants treated with paraquat was studied. It was reported that the previous treatment of plants with SA for 24 hours caused a significant increase on the activities of antioxidant enzyme such as superoxide dismutase, peroxidase, ascorbate peroxidase, ascorbate hydro-reductase, and catalase, as a result increase their resistance to paraquat (Ananieva et al., 2002). It was also reported that 1 mM SA on young wheat plants elevated the activities of superoxide dismutase, ascorbate peroxidase, catalase, and NADPH oxidase, as antioxidant enzymes (Agarwal et al., 2005).

Cold treatment resulted in increased activities of CAT, PPO, and GPX (Figure 4). Although most of the time there is a direct positive correlation between gene expression and its protein product, sometimes the lower expression causes a higher product activity and vice versa. Therefore, study of a gene and its products in all possible levels (genome, transcriptome, proteome, etc.) would be informative.

The highest relative CAT gene expression was observed in plants grown from seeds primed with 0.5 and then with 1 mM of SA and treated with a mild cold stress (8 °C for 32 h; Figure 5A). However, the effect of mild cold stress and SA treatment at seedling stage on relative CAT gene expression was insignificant (Figure 5B). Relative gene expression level of control samples of seedling stages (without cold stress) was considerably higher under 0.5 mM of SA which was completely different from those obtained on control plants of seed-primed group even with SA treatment, where expression levels were very negligible (Figure 5A and B). Janda et al. (2007) has already showed an increase in the content of antioxidants and other metabolites in a short cold hardiness and acclimation period especially when exogenous SA applied at suitable concentrations. Catalase is an enzyme important for the breakdown of hydrogen peroxide produced under stressful conditions. Under stress condition, its new isoforms are produced and the content of the background isoforms increases (Srivalli et al., 2003).

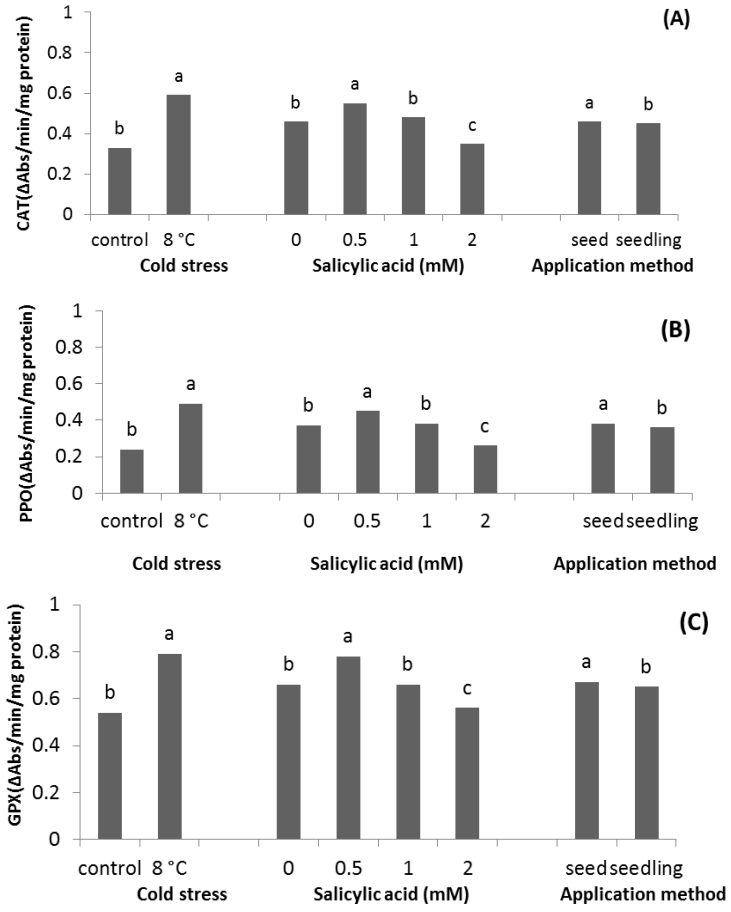


Fig. 4. Simple effect of cold stress (8 °C for 32 h), different concentrations of SA and application method on (A) activity CAT; (B) PPO; and (C) GPX in periwinkle. Different letters show significant differences (P≤0.05) within each group of treatments.

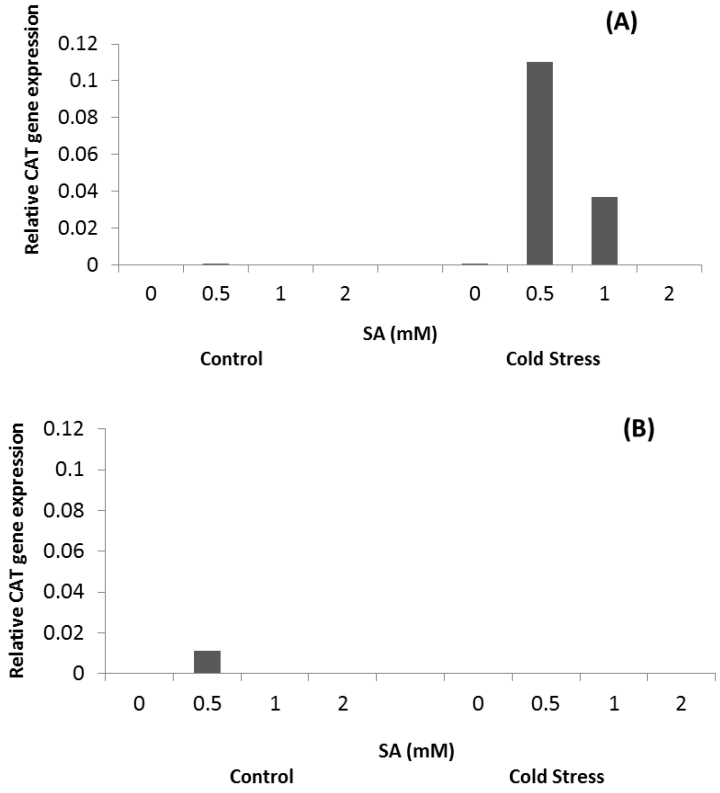


Fig. 5. Relative CAT gene expression on periwinkle under mild cold (8 °C for 32 h) as affected by SA treatment at (A) seed and (B) seedling stages.

The activity of catalase varies among plant species. During mild cold, the basic activity of the enzyme differs among species and varies based on the time course of cold stress (Lukatkin, 2002). Low temperatures increase the amount of phenolic compounds and polyphenol oxidase activity. Most studies indicated that the main role of antioxidant enzymes in scavenging cells from reactive oxygen species, and the important ones of these enzymes are peroxidase, catalase, and polyphenol oxidase (Yong et al., 2008). Increase in the activity of PPO and CAT enzymes are reported under cold and heat stress (Cervilla et al., 2009).

increased activity of peroxidase has been reported in wheat 48 hours after cold treatment and the activity of this enzyme decreases after 72 hours of exposure to cold stress (Yordanova and Popova, 2007). Yong *et al.* (2008) also reported an increase in antioxidant enzymes in a short term exposure to cold temperatures and a decline was observed in the long-term hardening. In the study of antioxidant defense system of winter wheat during the initial stages of cold acclimation period, a significant increase in the synthesis of peroxidase and catalase was observed on the second day after the administration of cold stress at 2 °C (Apostolova and Yaneva, 2006).

GPX is also an antioxidant enzyme in plants, along with other antioxidant enzymes such as ascorbate peroxidase and catalase, which are responsible for the protection of the plants against oxidative stress. Hydrogen peroxide is removed from the apoplast and vacuoles by these enzymes, and ascorbic acid is used as an electron donor. Moreover, for non-living environments, phenolic substances, such as guaiacol, are used as redox mediator.

Ascorbate peroxidase is a key component of the scavenging system in the cytosol and chloroplast of higher plants for the removal of hydrogen peroxide. Catalase converts hydrogen peroxide into water. In the current study on periwinkle (*Catharanthus roseus* Don.) increase in the activity of antioxidant enzymes was observed following cold stress. It has been shown that H_2O_2 is accumulated in the Arabidopsis cells which exposed to cold and therefore the activities of APX, GR, and GPX as antioxidant enzymes were increased (O´Kane et al., 1996). Furthermore, when rice plants were placed at 5 °C of cold stress, activity of APX and CAT enzymes were sharply increased (Kuk et al., 2003).

Conclusion

According to the obtained results, hardening with mild cold application of 0.5 mM SA regardless of application stage (seed or seedling) accelerated the flowering time in periwinkle, which is a positive trait for ornamentals. Plant growth, measured as height and the number of node, was increased by application of SA at 0.5 mM (but not at higher concentrations) that was administered as seed priming (but not on seedlings) in plants hardened with mild cold. Mild cold together with 0.5 mM SA also caused increase in the activity of antioxidant enzyme such as CAT, PPO, and GPX, regardless of application stage (seed or seedling). Therefore, priming of periwinkle seeds with 0.5 mM SA and hardening of seedlings at 4 to 6-leaf stage at 8 °C for 32 h can be recommended for its seedling production and cultivation in temperate climates to prevent late spring frost damages.

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