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Effect of Salicylic acid application on morphological, physiological and biochemical characteristics of *Cyclamen persicum* Miller

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ABSTRACT

Persian cyclamen is a perennial geophytes plant which has a special position between ornamentals due to its charm, diversity and winter flowering period. As this flower's quality and quantity is affected extensively by its nutrition and environmental factors, and regarding the various influences of salicylic acid (SA) in plant performance, this experiment was conducted to evaluate the external application of salicylic acid on the quality and quantity of this Iranian native flower. Therefore cyclamen plants were sprayed with 0, 0.5, 1 and 1.5 mM salicylic acid and their morphological, physiological and biochemical features were studied. Studied morphological features were: bud stage days, blooming days, total flowering days, total flowers, leaf area and number. Studied physiological features were: fresh weight, dry weight, relative water content and membrane stability index of petals. This was while for biochemical features superoxide dismutase, catalase activity beside protein, spermidine and spermine content of petals at five different flowering stages were studied. Morphological results indicate the beneficial effect of external SA application on flowering and vegetative growth of Persian cyclamen. SA application significantly delayed bud anthesis, increased blooming days, total flowers and total flowering days. Although SA application did not have a great impact on total number of leaves, but it increased leaf area. Relative water content, dry and fresh weights were increased by exogenous SA application. Membrane stability index was also similarly improved. SA improved antioxidant enzyme activity by increasing the activity of free radical scavengers such as SOD and CAT during all flowering stages. Protein and spermine content increased in all SA treatments. Spermine increment was only observed up to stage 3. This was while spermidine level was only improved in 1.5 mM concentration.

Keywords: Catalase, flower life, persian cyclamen, spermidine, spermine, superoxide dismutase.

INTRODUCTION

Persian cyclamen (*Cyclamen persicum* Miller. from the Primulaceae family) is an Iranian perennial geophyte ornamental plant with various applications such as pot and bedding plant [1]. This flower is gaining its popularity within the floriculture business due to its diverse attractive appearance and its winter time flowering [2]. As cyclamen flowers during winter, changes in nutrition and environmental conditions have a great impact on the quality and quantity of this flower [1]. Plant growth regulators and growth regulating compounds also affect the performance of this flower.

Salicylic acid (SA) (2-hydroxybenzoic acid) is a simple natural phenolic signaling molecule, which plays an important role in regulating a number of physiological processes in plants [3, 4]. As its external application affects many plant physiological processes, some have recognized it as a plant growth regulator [5]. It has been known that

exogenous SA application influences lipid peroxidation, chlorophyll fluorescence, antioxidant enzyme activity [6] and signaling pathogen-induced disease resistance [7]. One of the major roles for this compound is its impact on plant stress reaction and delaying senescence.

There are various reports on the role of SA in reducing stress effects. SA reduces the effect of drought [8], heat [9, 10], cold [11], salinity [12], disease [13] and heavy metal stress [14, 15] by affecting the activity of some free radical scavenger enzymes such as: SOD [16], CAT [17], PFOX [16] and proxidase [12]. It has also been shown that SA increases vase life by improving the antioxidant system and reducing oxidative stress damages during rose flower senescence [18]. According to our knowledge, there are no reports on the effects of exogenous SA application on cyclamen physiology and quality. Therefore this paper focuses on the influence of different SA treatments on quantity and quality of *Cyclamen persicum* to investigate if SA is involved in the regulation of antioxidant enzymes and quality of this native flower during its flowering period.

MATERIALS AND METHODS

Plant Material:

Cyclamen persicum Miller. Plants were prepared from a commercial cyclamen production greenhouse in Golzar, Tehran, Iran. Plants were treated with SA in a completely randomized design which was factorial arranged for the biochemical part. The main factor was application of 10ml SA on apical meristem of each plant. Applied SA solutions were 0, 0.5, 1, and 1.5 mM SA. The second factor in the biochemical part was spraying time according to flowering stage (Fig. 1). As seen in Fig.1, flowering stages were: a) Goose neck (uncolored bud), b) Bud bending (bud gaining color), c) Petal elongation, d) pre-anthesis (one petal is not open), and e) Full anthesis.

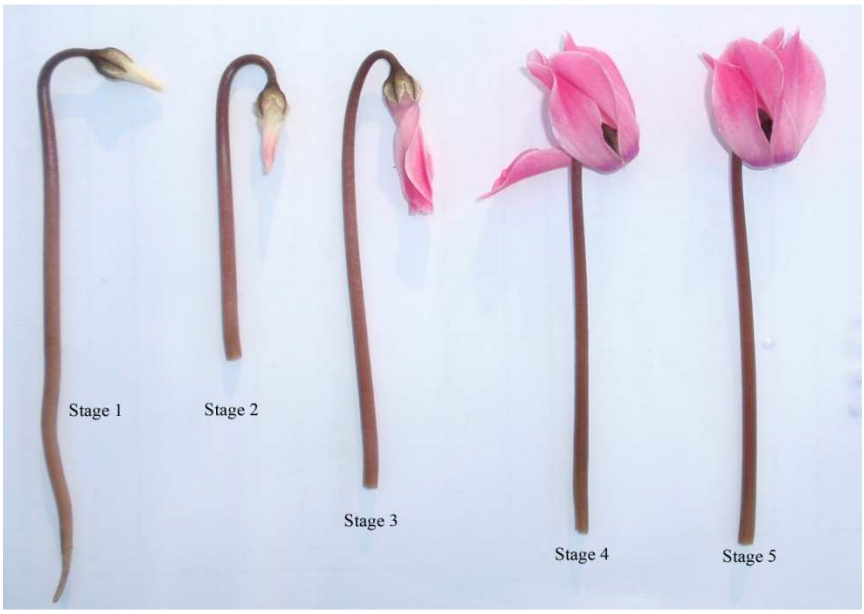

Fig. 1: Flowering stages of Persian Cyclamen.

Considering the required time for flower induction, the first appeared flower after 30 days from SA spraying was marked to indicate the first SA influenced flower. Morphological and physiological studies were conducted after this period and sample taking for biochemical analysis was conducted on the 45th day of spraying.

Morphological features:

In this part some flowering behavior and vegetative growth indexes were studies. Flowering related characteristics were: a) Bud stage days, b) Blooming days, c) Total flowering days, and d) Total flowers. This was while vegetative growth indexes consisted of leaf area and total number of leaves. Leaf area was measured by scanning three mature leaves with Leaf Area Meter: Am200.

Physiological features:

Petal fresh weight:

Petals of three flowers which were at the flowering stage of 5 (full anthesis) were freshly weighted in each treatment.

Petal dry weight percentage:
Fully developed flowers at flowering stage of 5 (full anthesis) were selected and their petals were dried at 80 ºC for 48 h in an electric oven and weighted subsequently in order to obtain dry weight percentage.

Petal relative water content:
Relative water content (RWC) was calculated by considering fresh and dry weight of petals at the flowering stage of 5 (full anthesis) in the bellow formula.

$$RWC\ (g) = (FW\ (g)-DW\ (g))/DW\ (g)$$

Membrane stability index:
For each treatment, petal samples were cut into discs of uniform size (1 cm^3 each) and placed in 10 ml of double distilled water in two set of falcon tubes. One set was kept in a hot water bath at 40 ºC for 30 min and its conductivity recorded as C1 using a conductivity meter. The electric conductivity of the second set was measured after keeping them in a boiling water bath (100 °C) for 15 min and recorded as C2. The membrane stability index (MSI) was calculated as:

$$MSI = [1–(C1/C2)] \times 100 \qquad [19].$$

Biochemical features:
For biochemical analysis, sampling was conducted at five flowering stages in 4 replication and flower petals at each stage were analyzed to evaluate the effect of SA application on various biochemical aspects. The Studies biochemical futures consisted of antioxidant enzyme activities (superoxide dismutase and catalase), polyamins (spermine and spermidine) and protein content.

Antioxidant enzyme activities:
For extracting the antioxidant enzymes, fresh petals (1 g) were ground in an ice cooled tissue grinder in 5 ml of 50 mM cooled phosphate buffer (pH 7.8). The homogenate was centrifuged at 4 ºC and 15000 *g* for 30 min [20]. After centrifugation, the supernatant (crude extract of petals) was used to determine superoxide dismutase and catalase enzyme activities, which were measured at 25 ºC.

Catalase activity was determined by following the consumption of H_2O_2 (extinction coefficient 0.0394 $mMcm^{-1}$) at 240 nm for 30 s. The assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 ml petal extract in a 3 ml volume. Then the reaction was initiated by adding the enzyme extract. Changes in absorbance of the reaction solution at 240 nm were read every 20 s. Unit was defined as nmol H_2O_2 decomposed per 1 min [21].

Superoxide dismutase activity was determined by measuring its ability to inhibit the photo reduction of nitro blue tetrazolium (NBT) with 50 mM Tris–Ca–codylic sodium salt buffer (at a pH of 8.2), containing 0.1 mM EDTA. The reaction mixture was composed of 0.055 mM NBT, 1.42% Triton X-100, 16 mM pyrogallol and enzyme extract (50 mg protein). The unit was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% per 1 min [21]. The activity of CAT and SOD was expressed on protein basis.

Proteins analysis
Hundred milligrams of fresh petal tissue were ground with liquid N_2, then 0.3mL of extraction buffer consisting of 0.1 M Tricine pH 8.1, 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 5 mM EDTA, 10 mM dithiothreitol, 1.0 mM phenazine methosulphate, 2 mM benzamidine, and 0.01 mM leupeptin were added and centrifuged for 10 min at 19000 g at 4ºC [22]. Total protein was determined according to Bradford [23].

Spermine and Spermidine analysis
Extraction was conducted by using one gram of lyophilized petal tissue with using 3 mL 0.2 N perchloric acid [24]. 1 mL 0.6 mM 1,6-hexanodiamine was added to the extraction solution as an internal standard. The later was first homogenated and centrifuged for 20 min at 12000 *g*. Obtained supernatant was consequently used for analysis of soluble polyamins. Dansylation was performed by mixing 200 µL saturated sodium bicarbonate and 400 µL dansyl chloride (10 mg/mL acetone) with 100 µL of the polyamin extract obtained from petal tissue. The mixture was vortexed for 30 sec, incubated at 60 C for 1 h, and then 100 µL (100 mg/mL H_2O) of proline was added. Dansylated polyamins were extracted with 500 µL toluene, dried in a stream of N_2 at 60 C, and resuspended in 200 µL of acetonitrile for HPLC analysis. Polyamines were separated on a reverse phase C18 column (20×0.4 cm) packed with a 5 µm Hypersil ODS resin. Polyamines were eluted from the column at a flow rate of 1.5 ml/min with a gradient of 60% to 90% acetonitrile in 25 min [24]. Polyamines in the extracts were quantified by comparison of peak areas

with those of the standard Spermine (SPM) and Spermidine (SPD) and their concentration was expressed as µg/g of dry tissue.

Statistics:
Data were analyzed by one way ANOVA using MSTAT-C software and means were compared by the least significant difference (LSD) test at the 0.05 and 0.01 probability level (*P*=0.05 and 0.01).

RESULTS AND DISCUSSION

Morphological features:
Bud stage days: SA application on Persian cyclamen significantly increased bud stage days compared to control (Table 1). With SA spray concentration increment bud anthesis was delayed. The most delay in bud anthesis was observed in SA 1.5 mM. Increase in bud stage days was only significant at concentration of 1 mM and higher. Although there is no report on the impact of SA on cyclamen bud development, but it has been shown that preharvest application of SA increases bud length of some rose cultivars such as 'Poison' [25].

Blooming days: Generally SA application increased blooming days compared to control (Table 1). The increment in blooming days increased up to 1 mM concentration and decreased afterwards resulting 1 mM treated flowers to have the highest blooming days of 23.67 days. This was while there was not a significant difference within different SA treatments. Like our findings, previous findings have showed the beneficial effect of SA treatment on vase life of various flowers and delay of flower senescence. Hatamzadeh et al. [26] results show treatment of 'Wing's Sensation' gladiolus spikes with SA, delays petal senescence. Hashemabadi and Zarchini [25] have also reported preharvest SA treatment of 'Poison' rose to increase flower vase life. Beneficial effects of SA treatment on other flowers such as lily [27], lisianthus [28], carnation [29] and rose [30, 31] have also previously been reported which are in accordance with our report on flower senescence delay.

Total flowering days: Total flowering days increased significantly by SA application. This was while there was not a significant difference between SA concentrations; and total flowering days decreased by SA concentration increment (Table 1). This caused 0.5 mM SA treated flowers to have the longest flowering days. Although there is no report on the impact of SA on cyclamen flowering period, but it has been shown that SA application can improve flower size. In a study Sabzi et al. [31] found that SA application increased flower diameter of 'Utopia' rose.

Total flowers: Total flowers per plant increased significantly up to 8 flowers per plant by SA application (Table 1). The most number of flowers was observed at 1 mM concentration of SA. By SA application, number of flowers increased up to 45.33 flowers per plant in 1 mM SA treated flowers and decreased afterwards. This resulted in the lowest number of flowers in 1.5 mM SA treatment. SA is known to stimulate and increase flowering in various species. Like our findings, Mady [32] has reported that SA foliar application significantly increases number of flowers in tomato (cv. 'Super Strain') during 2 successive seasons.

Leaf area: Unlike flowering aspects which almost all had an increase up to a certain concentration and subsequently a decrease, leaf area showed an increasing trend by SA application (Table 1). With SA concentration increment, leaf area increased up to 549.6 mm^2 in 1.5 mM SA treatment. There was not a significant increase in leaf area until applied SA concentration reached a limit of 1.5 mM. This resulted in the highest area of 3019.3 mm^2 for this kind of cyclamen. Confirming our findings, Mady [32] observed that leaf area significantly increased by foliar application of SA in tomato. Chandra et al. [33] have also observed an increase in leaf length of several cow pea cultivars by SA application.

Total leaves: SA application did not have a great impact on total number of leaves (Table 1). As seen in table 1, there was not a significant difference between different SA concentrations in number of leaves. On the other hand there was a slight increase in total leaf numbers at 1 mM SA concentration, which was reduced and reached the initial number of control. This was while Mady [32] reported a significant increase in number of leaves per plant in SA treated tomato.

Generally, it seems that although SA increased number of flower per plant, it had influenced flower development by delaying it and increasing bud stage days. It has also increased blooming days and cyclamen's total flowering days. On the other hand, its impact on persian cyclamens vegetative growth has been little; as there has not been any significant differences between SA treatments and control in number of produced leaves. Also there has not been a significant difference in leaf area of control and SA treatments up to 1 mM concentration.

Table 1. Effect of salicylic acid treatment on morphological characteristics of *Cyclamen persicum* L.

Treatment	Bud Stage Days	Blooming Days	Total Flowering Days	Total Flowers	Leaf Area (mm^2)	Total Leaves
Control	16.33 b[†]	21.53 b	37.86 b	37.33 c	2469.7 b	30.33 a
Salicylic Acid 0.5 mM	17.63 ab	23.33 ab	41.33 a	43.66 ab	2655.7 ab	30.33 a
Salicylic Acid 1 mM	18.00 a	23.67 a	41.30 a	45.33 a	2833.0 ab	33.66 a
Salicylic Acid 1.5 mM	18.10 a	22.63 ab	40.73 a	40.66 b	3019.3 a	30.66 a

[†] *Means followed by the same lower-case letters are not significantly different at the 0.01 probability level using Least Significant Difference (LSD) test.*

Physiological features:

Fresh and dry weight:

As seen in table 2, SA application increased both fresh and dry weight of petals in Persian cyclamen (Table 2). The increasing trend for fresh and dry weight was in accordance to concentration increment. This resulted 1.5 mM SA treated flowers to have the most weight gain. Weight gain increment was not significant for dry weight, while for fresh weight only 1.5 mM SA treatment caused significant weight gain. Like our findings, application of SA on 'Pattriot' chrysanthemum increased fresh weight [34]. Previous reports show that SA application suppress fresh weight decline. Hatamzadeh et al. [26] reported that SA application on 'Wing's Sensation' gladiolus suppresses postharvest decline of fresh weight. Treatment of cut 'Cheers' anthurium with 2 mM SA also resulted in lower fresh weight loss compared to control. This was while like our experiment, lower concentrations were not efficient [35]. Like fresh weight, dry weight has been improved by SA application in most reports. Like our findings, Mady [32] observed that in tomato application of SA will result in leaf dry weight increment. Chandra et al. [33] found the same result as ours for dry weight in several cow pea cultivars.

Relative water content:

Although there was not a significant difference, petal relative water content was also improved by SA application (Table 2). This was while SA application significantly increased petal water content of 'Pattriot' chrysanthemum flowers [34]. In their study, SA also increased relative water content of leaves. In our experiment, the highest water content was 9.323 g which was seen in 1.5 mM SA treated flowers after which 1 mM SA treated flowers were placed. The least relative water content was seen in 0.5 mM SA treatment.

Membrane stability index:

Results indicate that membrane stability index of Persian cyclamen was also improved by SA application (Table 2). With SA concentration increment, membrane stability index increased. This was while increase of SA application up to 1 mM did not result a significant improvement. Like relative water content, significant improvement was only seen at 1.5 mM SA treated flowers. Similar to our findings it has been reported that SA application significantly improves membrane stability of various flowers such as: lily [27], lisianthus [28], white carnation [29] and rose [30]. In anthurium flowers, electrolyte leakage from flower spath significantly decreased by 15 min dip treatment with 2 mM SA solution [35].

Table 2. Effect of salicylic acid treatment on physiological characteristics of *Cyclamen persicum* L.

Treatment	Fresh Weight (g)	Dry Weight (g)	Relative Water Content (g)	Membrane Stability Index (%)
Control	1.647 b[†]	0.172 a	8.524 ab	77.91 b
Salicylic Acid 0.5 mM	1.694 b	0.192 a	7.822 b	80.77 ab
Salicylic Acid 1 mM	1.845 ab	0.195 a	8.461 ab	83.44 ab
Salicylic Acid 1.5 mM	2.030 a	0.200 a	9.323 a	84.09 a

[†] *Means followed by the same lower-case letters are not significantly different at the 0.01 probability level using Least Significant Difference (LSD) test.*

Biochemical features:

Antioxidant enzyme activities:

Catalase activity: In control flowers, CAT activity increased initially up to flowering stage of two and decreased afterwards as flowers developed and aged (Fig. 2). This was while SA application increased CAT activity throughout all flowering stages compared to control. CAT activity increment trend showed an increase up to stage four and a sharp decrease at flowering stage of five. Within SA treatments, there was an increase in CAT activity with SA concentration increment. This resulted an activity of 44.87 Unit/mg protein in 1.5 mM SA treated flowers at stage 4. At flowering stage 5, CAT activity in SA treated flowers was 10 folds more than control. In 'Cheers' anthurium, Promyou et al. [35] observed that treatment with SA caused an increase in spath CAT activity compared to control. Similar to our findings for cyclamen, they observed that CAT activity trend showed an initial increase until day 8 which was flowed by a decrease afterwards.

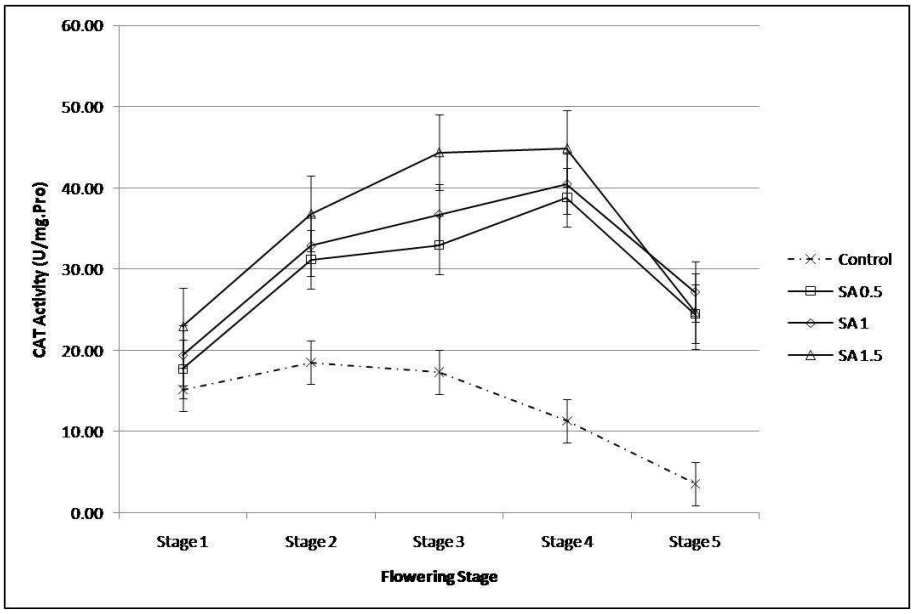

Fig. 2. Effect of SA application on CAT activity during different flowering stages of Persian cyclamen.

Superoxide dismutase activity: In control flowers, SOD activity was almost constant during flowering stage one and two. At flowering stage 3, there was an increase in SOD activity and a sharp decrease afterwards until flowering stage 5. This was while SA treated flowers showed a higher SOD activity throughout the flowering stages. The SOD activity trend showed a sharp increase in activity until flowering stage 3, after which there was a slow decrease until stage 4 and a sharp decrease at stage 5. Within SA treated flowers, SOD activity increased with SA concentration increment. The SOD activity increment recorded the highest activity of 28.97 Unit/mg protein at flowering stage 3 in 1.5 mM SA treated flowers. Even at flowering stage 5, SA treated flowers had a 4 fold higher SOD activity (Fig. 3). Previously it has been reported that SA manipulates SOD activity in different ways. Like our findings, most research reported an increase in SOD activity by SA application. Zamani et al. [30] reported that SOD activity of *Rosa baccara* increases by SA application. Gerailoo and Ghasemnezhad [18] found that application of SA as vase solution significantly increases SOD activity in petals of 'Yellow Island' rose compared to control.Similar reports were published by Kazemi and Shokri [28] for 'Blue' lisianthus and Kazemi et al. [29] for 'Pink' carnation. Although there was an initial increase in SOD activity, during vase life and aging of 'Yellow Island' rose, SOD activity decreased. Surprisingly similar to our findings, even at the end of vase life SOD activity was more than control [18]. Zamani et al [30] obtained trend for changes of SOD activity in *Rosa baccara* was also similar to our findings, indicating an increase and a decrease afterwards.

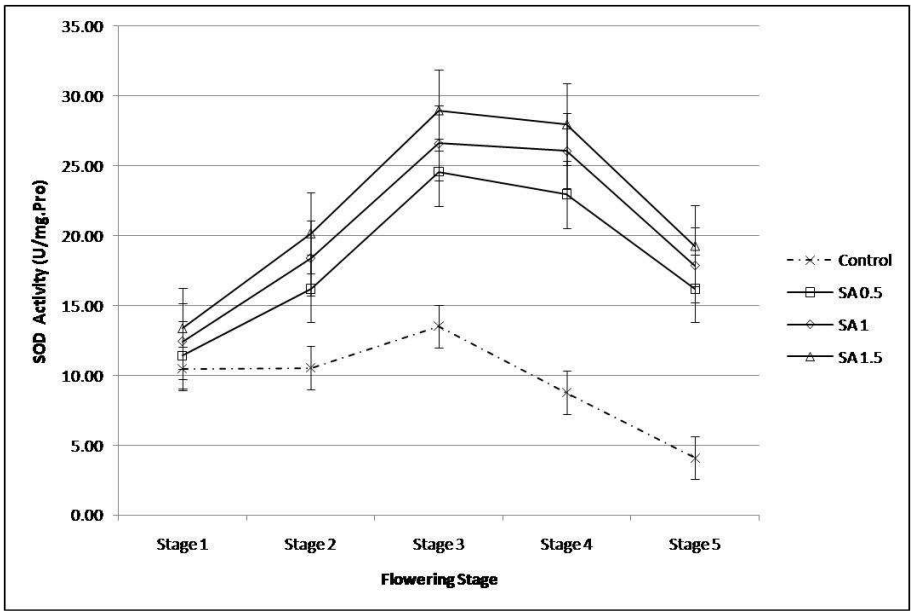

Fig. 3. Effect of SA application on SOD activity during different flowering stages of Persian cyclamen.

Proteins analysis:

Generally, SA application increased protein content compared to control (Fig.4). The most protein increment was seen at the flowering stage of three with 36.93 mg/gDW. As seen in Fig. 4, protein content in flower petals of Persian cyclamen had an increasing trend until flowering stage of three, after which it decreased. Throughout flowering stages, SA significantly increased protein content. This increment had a positive correlation with SA concentration increment. Similar to our results, Hatamzadeh et al. [26] saw that SA application on 'Wing's Sensation' gladiolus significantly increases protein levels during different flower developmental stages (from bud stage to senescence). Although during vase life and senescence protein content decreases, but Gerailoo and Ghasemnezhad [18] observed that supplying 'Yellow Island' cut rose flowers with SA, increases petal protein content with a similar trend as Persian cyclamen in our experiment.

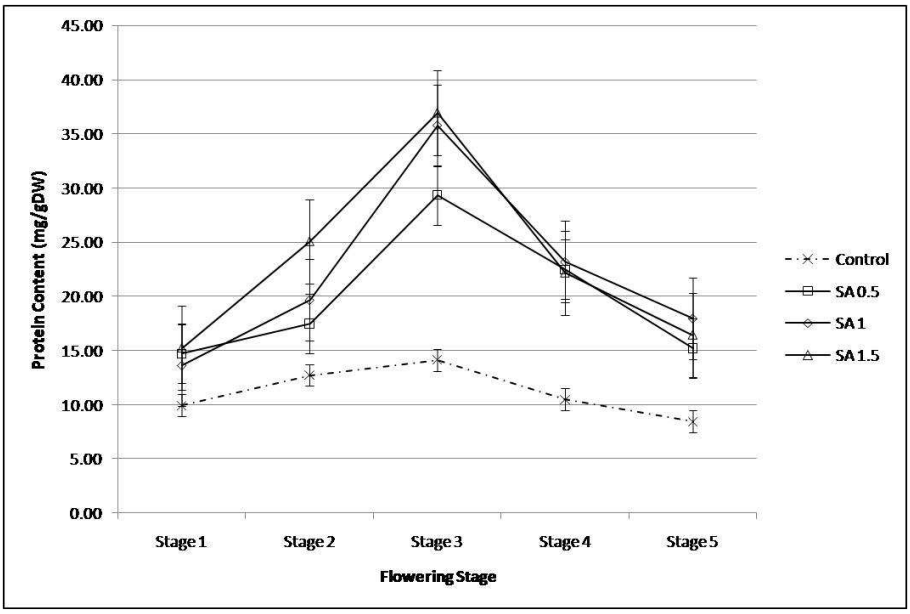

Fig. 4. Effect of SA application on protein content during different flowering stages of Persian cyclamen.

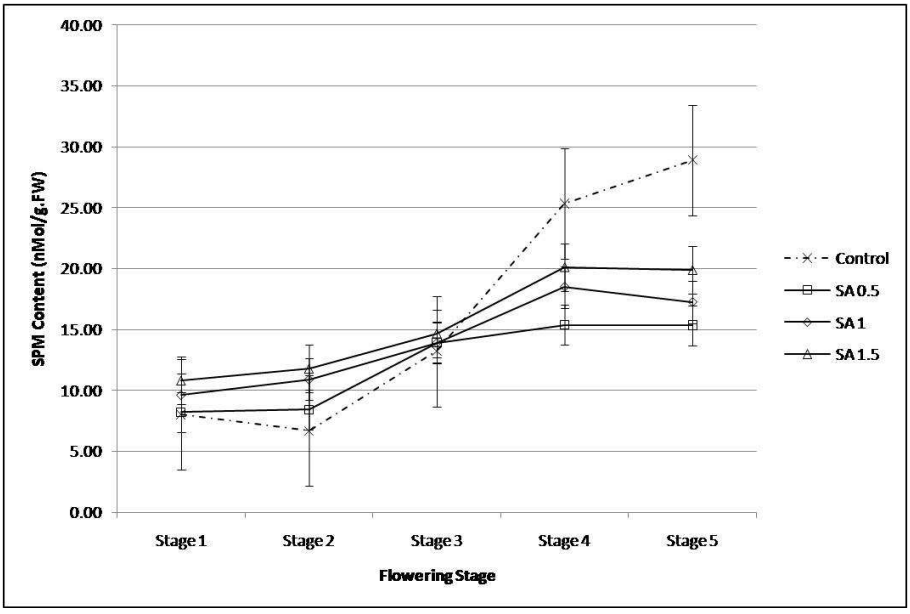

Fig. 5. Effect of SA application on spermine content during different flowering stages of Persian cyclamen.

Spermine and Spermidine analysis

Spermine content: During the early stages of flowering stages of 1 and 2, control flowers had a lower spermine content compare to SA treated flowers (Fig. 5). At flowering stage 3 there was a sharp increase in spermine content which resulted to similar spermine content as SA treated flowers. The increasing trend of spermine continued until the final stage of flowering (stage 5). This caused control flowers to have the highest spermine content at a value of

28.78 nMol/gFW at stage 5. In SA treated flowers, spermine content had a slow increasing trend throughout the experiment until stage 4, after which there was a slow decrease in spermine content. In SA treated flowers, with concentration increment, spermine levels increased. Wang and Zhang [36] reported that exogenous SA influence endogenus polyamine levels and different SA concentrations have different effects. Similar to our study, they found that SA significantly increased spermine content compared to control. This was while Nemeth et al. [37] reported a decrease in spermine content after SA application in leaves of maize.

Spermidine content: In all treatments spermidine content showed an increasing trend and subsequently a decreasing trend (Fig. 6). In all SA treatments, spermidine content increased until stage 3 after which there was a decrease trend. Although there was not a significant difference between 0.5 and 1 mM SA applications, spermidine content increased with SA concentration increment. The highest spermidine content was 40.73 nMol/gFW at flowering stage of 3. In control flowers similar trend as SA treated flowers was observed, but unlike SA treated flowers spermidine increment continued until stage 4 and spermidine content was always lower than 1.5 mMol SA treatment. Nemeth et al. [37] reported that in general exogenous SA application will increase endogenous polyamine content. Janad et al. [38] have also reported that application of suitable SA concentrations will results in an increase in polyamine synthesis in various plants. This was while in our experiment this finding was only seen for spermidine and at a concentration of 1.5 mM SA. Nemeth et al. [37] report that there were various responses by different polyamines. For example SA application caused a significant increase in putrescine content, while spermidine content decreased. Similar to our findings, Nemeth et al. [37] also found that leaf spermidine content increased after SA treatment.

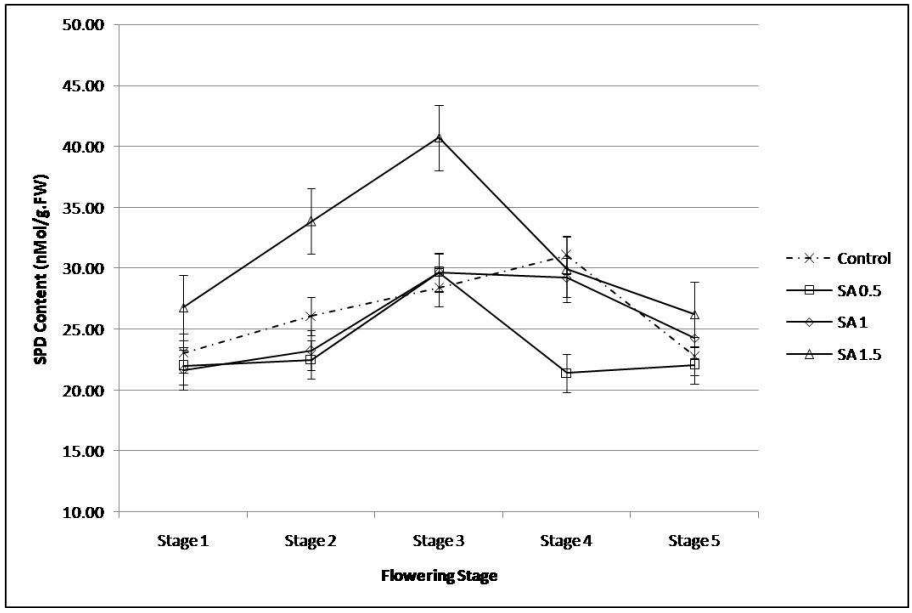

Fig. 6. Effect of SA application on spermidine content during different flowering stages of Persian cyclamen.

CONCLUSION

Generally, SA treatment of Persian cyclamen improved various morphological, physiological and biochemical features of this flower. From the morphological point of view, SA did not have a significant impact on vegetative aspects while it significantly improved flowering. Physiological features were also improved by SA application, but the changes were not significant. From biochemical point of view, SA improved antioxidant enzyme activities by increasing CAT and SOD activity. Protein content was also significantly increased throughout different flowering stages. Beside that polyamine content also increased to some extent. Considering the mentioned different aspects, foliar exogenous SA application is recommended for commercial Persian cyclamen production.

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