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Effect of foliar application of salicylic acid on photosynthetic pigments and antioxidative enzymes of soybean plant

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Abstract

Salicylic acid is an endogenous plant growth regulator of phenolic in nature, which involves in the regulation of physiological processes in plants. The present investigation was carried out to study the effects of different concentrations (0, 10^{-4}, 10^{-5}, 10^{-6} M) of salicylic acid on vegetative characters, photosynthetic pigment, protein content, lipid peroxidation and antioxidative enzymes. Leaf samples were harvested on 45th, 60th and 90th days after sowing. Results showed that exogenous application of salicylic acid at concentration 10^{-6} M increases the vegetative characters and biochemical parameters which lead to significant rise in chlorophyll, protein and MDA content. However, SA at 10^{-4}M and 10^{-5}M did not exert a significant promotive effect as compared to SA at 10^{-6} M in retaining pigment loss, protein content and MDA content. Foliar spray with different concentration of SA also increased the activity of antioxidant enzymes like GPOX and SOD which prevent the plants from oxidative stress which is further produced by abiotic and biotic factors. Exogenous application of different concentration of SA (10^{-4}M, 10^{-5}M) also resulted in increased antioxidant activities but best response occurred at (10^{-6} M).

***Key words:** salicylic acid, soybean, photosynthetic pigment, protein, antioxidant enzymes.*

I. INTRODUCTION

Plant growth regulators can improve the physiological efficiency including photosynthetic ability and thereby helping in effective flower formation, fruit and seed development and ultimately enhance productivity of the crops (Solamani *et al*. 2001). Foliar feeding of plants can effectively supplement soil fertilization. It has been found that element foliar application is more influential compared to soil application (Kazemi 2013).

Salicylic acid (SA) is an endogenous plant growth of phenolic nature that possesses an aromatic ring with a hydroxyl group or its hormone plays a vital role in plant growth, ion uptake and transport (Hayat *et al.* 2010). Enhanced germination and seedling growth were recorded in wheat, when the grains were subjected to pre-sowing seed-soaking treatment in salicylic acid (Shakirova 2007). In cucumber and tomato, the fruit yield enhanced significantly when the plants were sprayed with lower concentrations of salicylic acid (Larque-Saavedra and Martin-Mex 2007).

Salicylic acid was also found to enhance the activities of antioxidant enzymes such as peroxidase (POD), superoxidase dismutase (SOD) and catalase (CAT), when sprayed exogenously to the drought stressed plants of tomato (Hayat *et al.* 2008) or to the salinity stressed plants (Szepesi *et al.* 2008; Yusuf *et al.* 2008). The exogenous SA application also enhanced the growth and photosynthetic rate in wheat (Hussein *et al.* 2007) under water stress. However, numerous studies have demonstrated that the effect of exogenous SA depends on various factors, including the species and developmental stage, the mode

of application and the concentration of SA used (Vanacker *et al.* 2001; Horvth *et al.* 2007). Fariduddin *et al.* (2003) also eported that the dry matter accumulation was significantly increased in *Brassica juncea*, when lower concentrations of salicylic acid were sprayed. However, higher concentrations of salicylic acid had an inhibitory effect. Khodary (2004) observed a significant increase in growth characteristic, pigment contents and photosynthetic rate in maize, sprayed with salicylic acid. Eraslan *et al.*, (2007) also reported that exogenous application of salicylic acid, enhanced growth, physiological process and antioxidant activity of carrot plants grown under salinity stress.

Soybean (*Glycine max* (L.) merril) is an important grain legume due to its high protein (35%), oil content (21%) and nitrogen fixing ability (17-24 kg/ha/yr.). Increasing plant productivity is one the main target in Indian Agricultural policy, which could be achieved through fertilization and plant growth regulators. However, very little is known about the effect of SA applied to foliage on plant growth and development and especially about the effect of SA on influencing the activities of antioxidative enzymes at different growth stages. Keeping in view the diverse physiological roles played by SA, the present research was undertaken to improve our understanding of the effect of the various concentrations of SA applied as foliar spray on the pigment and protein content, lipid peroxidation and activities of antioxidative enzymes in soybean at different growth stages.

II. MATERIALS AND METHODS

Seeds of Soybean (*Glycine max* L. var. Pusa- 9612) were collected from CCS Haryana Agriculture University, Hisar and were surface-sterilized with 5% (v/v) mercuric chloride solution and washed properly with double distilled water (DDW) before sowing. The experiment was set up in the experimental cage of Botany Department of Kurukshetra University, Kurukshetra. Five seeds per pot sown in earthern pots (30 cm diameter) lined with polythene having 5.0 kg of dune sand grown under natural light conditions during kharif season in July. The temperature conditions were 35 ± 2°C and 24 ± 2°C, during days and nights respectively; with mean relative humidity of 82 ± 5%.

After three weeks, the seedlings were thinned to two plants per pot and each treatment consisted of three replications in a complete randomized design (CRD). At the stage of 30 and 45 days after sowing (DAS), the foliage of the plants was sprayed uniformly either with double distilled water (control), or with different concentrations (10^{-4}, 10^{-5} or 10^{-6} mol/L) of SA dissolved in ethanol to elucidate the effect of exogenous SA on plants. The plants were sampled at 45, 60 and 90 DAS to assess various biochemical parameters.

Leaf sample (200 mg) was ground in chilled 80% acetone (AR grade) with 20 mg of $CaCO_3$ and centrifuged at 3000 g for 5 min. Absorbance of the filtrate was recorded at 645 and 663 nm for chlorophylls and at 480 and 510 nm for carotenoids depending upon respective peaks in their absorption spectra using a UV-Visible spectrophotometer (Specord-205, Analytic-Jena, Germany). Chlorophyll (Chl) amount was estimated with the formula of Arnon (1949). Carotenoid level was calculated by the method of Holden (1965).

Total soluble proteins were estimated according to the method described by Bradford (1976) using Coomassie Brilliant Blue G-250. Fifty mg of fresh leaf tissue (earlier stored in a freezer) was dropped boiling 80% ethanol (EtOH) on a water bath for a minute. The tissue along with EtOH was cooled to room temperature and homogenized. The extract was centrifuged at 10,000 g for 5 min. The residue was re-extracted with 5% perchloric acid followed by centrifugation at 10,000 g for 5 min. Five-

mL of 1N NaOH was added to the residue and maintained in warm water (40-50°C) with regular shaking for 30 min. The clear supernatant was used for further analysis.

Total peroxidase activity was measured by the method of Maehly (1954). Plant material (0.1 g) was homogenized with ice cold distilled water and centrifuged in a Remi centrifuge at 6000 g for 10 min. The supernatant was used as the enzyme source and final volume of the extract raised to 10 mL with ice cold double distilled water. The reaction set was prepared by mixing 2 mL each of enzyme source; phosphate buffer (pH 7.0); guaiacol (20 mM), and H_2O_2 (10 mM) in sequence. A blank set was prepared by mixing 2 mL of enzyme source; 2 mL of phosphate buffer (pH 7.0) and 4 mL of double distilled water. Blank, and reaction sets, were kept undisturbed at room temperature exactly for 10 min., then the absorbance was recorded in a spectrophotometer at 420 nm. Protein was estimated from the same extract following the procedure of Bradford (1976).

Fifty-mg of fresh leaf tissue was crushed in 2 mL of 0.1M EDTA- phosphate buffer, pH 7.8, containing K_2HPO_4 and EDTA and the final volume raised to 100 mL with double distilled water (DDW). This was centrifuged at 15000 g and the resultant supernatant used as crude extract. The reaction mixture was prepared by adding 0.1 mL of crude extract followed by 0.9 mL of DDW, 0.5 mL of 300 mM Na_2CO_3 (pH 10.2), 0.5 mL of 378 µM p-nitrobluetetrazolium chloride (NBT), 0.5 mL of 78 mM L-methionine and 0.5 mL of 7.8 µM riboflavin. The final reaction mixture was 3 mL. The reaction was carried out in test tubes at 25°C for 15 min under 100 µmol photon $m^{-2}s^{-1}$ PFD from fluorescent lamps. The initial rate of reaction, measured by the difference in increase in absorbance at 560 nm in the presence, and absence, of extract was proportional to the amount of enzyme. The unit of SOD activity was obtained as that amount of enzyme which under the experimental conditions caused a 50% inhibition of the reaction observed in the absence of enzyme (Giannopolitis and Ries, 1977).

The level of lipid peroxidation in samples was measured by estimating the malondialdehyde (MDA) present (Heath and Packer, 1968). Leaf samples (0.2 g) were homogenized in 3 mL of 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 15000 g for 15 min. To 1.0 mL aliquot of the supernatant, 2.0 mL of 0.5 % thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) was added. The mixture was heated at 95°C for 30 min in a water bath and then cooled in an ice bath. After centrifugation at 10000 g (Remi) for 10 min the absorbance of the supernatant was recorded at 532 nm. The value for nonspecific absorption of each sample at 600 nm was recorded and subtracted from the absorbance recorded at 532 nm.

Catalase activity (CAT) was determined by following the method of Aebi (1984). The reaction mixture was prepared by adding 1.5 ml of 50 mM HEPAS buffer 1.2 ml of 150 Mm H_2O_2 and 30 µl petal extract. In the reaction mixture without enzyme, no crude extract was added, instead of it 50 µl 50 mM HEPAS buffer was added. The change in absorbance was read at 490 nm in the test tube cuvette using uv- vis spectrophotometer. Specfic activity of catalase was expressed in term of per mg protein. Protein was estimated from the same extract following the procedure of Bradford (1976) as described earlier.

A mean of three readings was taken in every replication. In biochemical estimation, three aliquots were used for each replication. Statistical analysis was done using Statistical Packages for Social Sciences (SPSS) version 16.0. One-way ANOVA was used to test whether there was a significant difference in various estimations.

III. RESULTS

Result presented in Table 1 clearly state that, using SA as foliar treatment at different concentrations (10^{-4}, 10^{-5}, 10^{-6} M) increased all the studied growth characters like (Plant height, number of branches and number of leaves per plant as well as plant dry weight) of Soybean plant as compared with control plant. Treatment of plant with lower concentration of SA (10^{-6} M) gave the highest plant height and number of leaves per plant. They were increased by 55 % and 40 % respectively as compared to control at 45 DAS. On the other hand at 60 DAS plant height and number of leaves per plant increased by 44 % and 53% respectively as compared to control plants. Regarding plant dry weight, SA (10^{-6} M) had beneficial effect whereby it increased the dry weight about 15 % and 26 % at 45 and 60 DAS respectively. However, the magnitude of stimulatory effect declined at 60 DAS as compared to 45 DAS in almost all the growth parameters studied.

The effect of SA at different concentration (10^{-4}, 10^{-5}, 10^{-6} M) on photosynthetic pigment (chlorophyll a, chlorophyll b, total chlorophyll (a+b) and carotenoids) of Soybean plant are presented in Table 2. Exposure of the plant with different concentration of SA significantly increased chl. a, chl.b, total chl.(a+b) and carotenoids as compared to plants treated with double distilled water . The most pronounced effect was seen in the plants sprayed with 10^{-6} mol/L of SA. The increment in the pigments content in the tested plant increased up to 60 DAS and futher declined at 90 DAS in all the treatments. Foliar application of SA(10^{-6} M) enhanced pigment content by about 74 % and 61 % for total chlorophyll and cartenoids respectively at 90 DAS compared to control. Interestingly SA at higher concentration of 10^{-4} M was negligible in retaining the pigment content at all three stages.

Total soluble protein content in both control and treated plants has been depicted in Table. 3. A gradual increase in protein content as plants reached maturity were noticed in all groups up to 60 DAS where after a sharp decline was observed at 90 DAS. There had been an increase of about 48, 32 and 27 percent in SA (10^{-6} M), SA (10^{-5} M) and SA (10^{-4} M) treated plant respectively when compared to control at 90 DAS. It can be inferred from the result that the effectiveness of these concentrations were more at 60 and 90 DAS as compared to 45 DAS. In the present study Lipid peroxidation (MDA content) increased from 0.672 (n mol/g fresh wt.) to 1.25 (n mol/g fresh wt.) during 45 to 90 DAS in untreated soybean leaves. MDA content gradually and significantly increased by about 86 % from 45 to 90 DAS in control set. SA (10^{-6} mol/L) prevented the increase by about 10 percent from 45 to 90 DAS. The effect became more promotive whereby the same concentration of SA was able to prevent the rise by about 35 percent at 90 DAS. However foliar application of SA at (10^{-5} M) and (10^{-4} M) did not generate any significant response.

Exogenous application of lower concentration of SA increased the activity of antioxidant enzymes at different growth stages. The superoxide dismutase (SOD) activity increased up to 60 DAS and further declined in all the samples at 90 DAS. In the present study exogenous application of SA caused a significant increased in the SOD activity as compared to control plant. Among the three concentration (10^{-4}, 10^{-5}, 10^{-6} mol/L) of SA, 10^{-6} mol/L proved to be the best and significantly increased the values of SOD by about 80 % over that of the control at 90 DAS followed by SA (10^{-5} M) when it was able to increased the value by about 59% at the same stage. The Guaicol peroxidase (GPOX) activity increased from 45 to 60 DAS followed by decline at 90 DAS when plants nearing to maturity. Exogenous foliar application of SA promotes increase in the GPOX activity by about 72 % as compared to control plants from 60 to 90 DAS. Catalase activity increased by 70 percent at 60 DAS but further declined when plants reached to maturity at 90 DAS as compared to the control. Moreover, SA at 10^{-6} M

was able to increase the catalase activity by about 23 percent and about 9 percent at 60th and 90th DAS respectively as compared to the untreated plants. However, SA at 10^{-4} and 10^{-5} M had shown insignificant promotive effect in increasing catalase activity at all the stages.

IV. DISCUSSION

Exogenous application of plant growth regulators is considered effective technique for improving the plant productivity. Salicylic acid has been found to induce significant effects on various biological aspects in plants. Our results state that SA have stimulatory effects on vegetative growth parameters of Soybean plant. Application of SA leads to increase in number of branching and leaves per plant along with increase in the dry weight also. Regarding foliar application of SA, obtained results are similar to those describe by Salarizdah *et al.* (2012) on canola, Dawood *et al* (2012) on sunflower and Ali and Mahmoud (2012) on mungbean. In the present study SA showed elevated effects and leads to manifold increase in the level of biochemical and antioxidant enzymes. The chlorophyll content of Soybean leaves was increased due to foliar application of SA (Khan *et al.* 2003). Lower SA concentration was generally more effective in enhancing photosynthetic rate and biochemical parameters. Application of lower concentration of SA might leads to synthesis of more carbohydrate in treated plants. Chlorophyll pigments play a key role in light capturing for photosynthesis whose content forced a direct impact on the intensity of photosynthesis. The stimulatory effects of SA are in agreement with those of Barakat (2011) on wheat and Saeidnejad *et al.* (2012) on maize. However, declined in chlorophyll content under influence of SA in certain crops like *Vigna mungo* has been reported by Anandhi and Ramanujam, (1997). The reduction of total chlorophyll content occurs due to increased in activity of the enzyme chlrophyllase. The Role of SA deficiency is associated with reduced damage to the photosynthetic apparatus as well as chlorophyll level. MDA content was estimated to determinate the extent of lipid peroxidation. The data showed that increased level of MDA content was achieved from 45 to 60 DAS over that of control. It has been postulated that low level of the induced leakiness of membrane is caused by lipid peroxidation resulting from uncontrolled ROS increase (Rodriques-Rosales *et al.* 1999). Delvari *et al*. (2010) showed that pretreatment will decrease the level of lipid peroxidation induced by oxidative stress in basil plants. Agarwal *et al*. (2005) also showed that SA treatment of wheat leaves under water stress conditions resulted in less production of MDA. So, the lipid peroxidation induced by drought stress was ameliorated by SA treatments. Antioxidant enzymes of Soybean were increased in response to different concentration of SA. Oxidative stress generated in the plants can be removed with the help of antioxidative enzymes. It was found that application of low concentration of SA increased the activity of antioxidant enzymes like CAT, APOX, GPOX, SOD. This increase in the activity of antioxidant enzymes might be due to the regulatory role of SA at the level of transcription/Translation. Foliar spray of SA to soybean plant leads to significant increased in SOD and CAT activity. Among the enzymes measured here, CAT and SOD most effective in preventing cellular damage by converting superoxide anion to H_2O_2 and H_2O_2 to H_2O (Scan-Dalios 1993). It was found that increased SOD activity was accompanied by increase in CAT and POD because of high demands of H_2O_2 quenching. It was cleared that increment in SOD and POD simultaneously affect each other. First line of defense was provided by SOD against the cellular due to environmental stress along with its major superoxide scavenger. Catalase seems to be a key enzyme in SA induced stress tolerance. Tenhakan and Rubel (1997) and Rao et al. (1997) have reported that SA caused hypersensitive reaction or enhanced H_2O_2 produced leading to cell death was not associated with the inhibition of these H_2O_2 scavenging enzymes, similarly there was no inhibition of GPOX activity by SA thus conforming the results reported by Durner and Klessing (1995). So these antioxidant enzymes protect plant cell from oxidative damage by being scavenging of ROS. So from above discussion, it was observed that foliar

application of SA increased antioxidant enzymes activity (SOD, GPOX, CAT) which was further related to decrease in oxidative stress (H_2O_2). From above results, it was observed that foliar feeding of soybean (*Glycine max* L. merril) varieties Pusa-9612 with SA at lower concentration can stimulate the growth through increasing in the activities of antioxidant enzymes, preventing protein loss and enhancing photosynthetic pigment thereby increasing overall growth parameter of the plant. Thus SA at 10^{-6} M concentration can positively affect the growth of the Soybean plant as compared to 10^{-5} or 10^{-4} M application.

V. CONCLUSION

In conclusion, the exposure of Soybean plant to foliar application of SA resulted in protein increment along with enhancement in the photosynthetic pigments under normal growth condition. The results also indicate the role of SA in plant defense mechanism which can be seen in its role in enhancing the activities of antioxidant enzymes like SOD, POD, CAT and lowering the MDA content. Overall, SA at (10^{-6} M) compared to other concentrations under investigations has a stimulatory role in the growth and development of the soybean plant.

VI. ACKNOWLEDGEMENT

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Table 1- Effect of Salicylic acid on growth characters of Soybean (*Glycine max* var. Pusa-9612) at 45 and 60 DAS after sowing.

Treatments	Plant height (cm)	No. of Branching/plant	No. of leaves/plant	Plant dry wt. (g)
	45 DAS			
Control	42.3^{c} ±1.21	18.6^{c} ±0.52	17.6^{b} ±0.46	7.68^{b} ±0.22
SA(10^{-4}mol/L)	46.4^{c} ±1.84	19.4^{b} ±0.63	18.2^{b} ±0.60	7.79^{b} ±0.26
SA(10^{-5}mol/L)	53.2^{b} ±2.42	20.3^{b} ±0.53	21.4^{b} ±0.66	8.42^{ab} ±0.23
SA(10^{-6}mol/L)	65.6^{a} ±2.39	23.2^{a} ±1.04	24.1^{a} ±1.09	8.85^{a} ±0.45
LSD at 5 %	6.624	2.335	2.503	1.008
	60 DAS			
Control	52.5^{c} ±0.98	20.2^{c} ±0.58	18.4^{c} ±0.52	7.96^{b} ±0.22
SA(10^{-4}mol/L)	58.5^{bc} ±1.21	21.9^{ab} ±0.75	22.3^{b} ±0.75	8.55^{b} ±0.34
SA(10^{-5}mol/L)	64.2^{b} ±1.84	22.8^{ab} ±0.87	24.6^{b} ±1.10	8.82^{ab} ±0.40
SA(10^{-6}mol/L)	75.6^{a} ±2.19	24.7^{a} ±1.27	28.2^{a} ±1.45	10.04^{a} ±0.52
LSD at 5 %	8.994	2.949	3.309	1.263

Means with the same letters in each column are not significantly different

Table 2- Effect of Salicylic acid on Chllorophyll a, Chlorophyll b, total chlorophylls and carotenoids (mg g^{-1} fr.wt) of Soybean (*Glycine max* var. Pusa-9612) at 45, 60 and 90 DAS after sowing.

Days after Sowing	Treatments	Chl.a	Chl.b	Total Chl.	Carotenoids
45	Control	2.30^{c} ±0.07	0.92^{c} ±0.02	3.20^{c} ±0.09	1.35^{b} ±0.03
	SA(10^{-4}mol/L)	2.40^{bc} ±0.08	1.06^{c} ±0.04	3.46^{bc} ±0.13	1.33^{b} ±0.03
	SA(10^{-5}mol/L)	2.59^{ab} ±0.09	1.25^{b} ±0.05	3.85^{ab} ±0.17	1.37^{b} ±0.03
	SA(10^{-6}mol/L)	2.75^{a} ±0.06	1.52^{a} ±0.07	4.30^{a} ±0.19	1.50^{a} ±0.03
	LSD at 5 %	0.2691	0.1685	0.5089	0.1135
60	Control	2.83^{a} ±0.09	1.45^{c} ±0.04	3.51^{b} ±0.12	1.91^{c} ±0.06
	SA(10^{-4}mol/L)	2.86^{a} ±0.11	1.77^{b} ±0.06	3.63^{b} ±0.14	2.06^{bc} ±0.06
	SA(10^{-5}mol/L)	3.00^{a} ±0.23	1.88^{ab} ±0.08	3.89^{b} ±0.15	2.16^{ab} ±0.06
	SA(10^{-6}mol/L)	3.14^{a} ±0.08	2.06^{a} ±0.10	5.34^{a} ±0.15	2.37^{a} ±0.07
	LSD at 5 %	0.4675	0.2590	0.4680	0.2291
90	Control	1.06^{c} ±0.04	0.36^{c} ±0.02	1.45^{c} ±0.04	0.54^{bc} ±0.06
	SA(10^{-4}mol/L)	1.09^{c} ±0.04	0.41^{c} ±0.01	1.51^{c} ±0.05	0.62^{ab} ±0.04
	SA(10^{-5}mol/L)	1.29^{b} ±0.05	0.54^{b} ±0.02	1.84^{b} ±0.05	0.72^{a} ±0.04
	SA(10^{-6}mol/L)	1.64^{a} ±0.08	0.69^{a} ±0.04	2.53^{a} ±0.06	0.87^{a} ±0.05
	LSD at 5 %	0.1888	0.9995	0.1811	0.1667

Means with the same letters in each column are not significantly different

Table 3- Effect of Salicylic acid on the amount of protein (mg mg^{-100} fr.wt) and MDA content (n mol g^{-1} fr.wt) of Soybean (*Glycine max* var. Pusa-9612) at 45, 60 and 90 DAS after sowing.

Days after Sowing	Treatments	Protein	MDA content
45	Control	12.23^{c} ±0.89	0.672^{a} ±0.02
	SA(10^{-4}mol/L)	13.80^{bc} ±0.68	0.655^{b} ±0.03
	SA(10^{-5}mol/L)	14.75^{b} ±1.92	0.607^{c} ±0.05
	SA(10^{-6}mol/L)	16.77^{a} ±1.67	0.601^{c} ±0.07
	LSD at 5 %	1.640	0.0085
60	Control	16.26^{c} ±0.49	0.966^{a} ±0.02
	SA(10^{-4}mol/L)	19.51^{bc} ±0.75	0.966^{a} ±0.02
	SA(10^{-5}mol/L)	21.30^{ab} ±0.79	0.901^{b} ±0.04
	SA(10^{-6}mol/L)	23.38^{a} ±0.49	0.895^{b} ±0.06
	LSD at 5 %	2.082	0.016
90	Control	6.71^{b} ±0.37	1.25^{a} ±0.03
	SA(10^{-4}mol/L)	7.82^{b} ±0.38	1.23^{b} ±0.04
	SA(10^{-5}mol/L)	8.88^{b} ±0.37	1.20^{c} ±0.04
	SA(10^{-6}mol/L)	9.95^{a} ±0.49	1.02^{d} ±0.06
	LSD at 5 %	1.203	0.016

Means with the same letters in each column are not significantly different

Table 4- Effect of Salicylic acid on specific activity of guiacol peroxidase (mg^{-1} protein min^{-1}), SOD activity (U min^{-1} mg^{-1} protein) and CAT activity (U g^{-1} fr. wt.) of Soybean (*Glycine max* var. Pusa-9612) at 45, 60 and 90 DAS after sowing.

Days after Sowing	Treatments	GPOX	SOD activity	CATALASE
45	Control	0.578^{a} ±0.05	2.78^{c} ±0.08	16.12^{d} ±0.96
	SA(10^{-4}mol/L)	0.569^{a} ±0.04	2.86^{bc} ±0.07	17.67^{c} ±1.33
	SA(10^{-5}mol/L)	0.550^{b} ±0.03	2.94^{b} ±0.05	20.49^{b} ±1.97
	SA(10^{-6}mol/L)	0.521^{c} ±0.05	3.05^{a} ±0.08	22.58^{a} ±2.18
	LSD at 5 %	1.016	0.105	2.649
60	Control	0.957^{a} ±0.04	3.69^{d} ±0.04	27.42^{d} ±1.79
	SA(10^{-4}mol/L)	0.945^{ab} ±0.02	3.85^{c} ±0.02	29.61^{c} ±2.09
	SA(10^{-5}mol/L)	0.932^{b} ±0.02	3.95^{b} ±0.08	30.98^{b} ±2.43
	SA(10^{-6}mol/L)	0.901^{c} ±0.05	4.13^{a} ±0.05	31.25^{a} ±2.63
	LSD at 5 %	0.013	0.114	4.104
90	Control	1.65^{a} ± 0.07	1.14^{d} ±0.07	3.43^{c} ±0.09
	SA(10^{-4}mol/L)	1.56^{b} ±0.03	1.40^{c} ±0.01	3.67^{ab} ±0.17
	SA(10^{-5}mol/L)	1.40^{c} ±0.05	1.82^{b} ±0.04	3.93^{ab} ±0.16
	SA(10^{-6}mol/L)	1.11^{d} ±0.07	2.06^{a} ±0.04	4.12^{a} ±0.24
	LSD at 5 %	0.023	0.016	0.504

Means with the same letters in each column are not significantly different

