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Salicylic acid-induced germination, biochemical and developmental alterations in rye (*Secale cereale* L.)

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Abstract – Salicylic acid (SA) is one of the endogenous plant growth regulators that modulate various metabolic and physiological events. To evaluate the exogenous SA-induced germination, biochemical and developmental alterations, different concentrations (10, 100, 500 and 1000 μM) of SA were applied to rye (*Secale cereale* L.) seeds in hydroponic culture conditions for 15 days. The observations revealed that seed germination and root elongation were stimulated in 10 μM SA treatment, however they were inhibited in higher concentrations (100 and 500 μM) of SA. Furthermore, there was no germination in 1000 μM SA. The analysis of antioxidant enzymes revealed that although superoxide dismutase activity increased, catalase activity decreased in comparison to control. Besides, lipid peroxidation and peroxidase activity increased in 10 μM SA, whereas they decreased in higher concentrations. Similarly total chlorophyll content increased in 10 μM SA, but it decreased in 100 and 500 μM SA treatments. Moreover anthocyanins and carotenoids increased after SA treatment. In conclusion, exogenous SA application causes developmental and biochemical alterations in rye.

Key words: antioxidant enzyme, lipid peroxidation, photosynthetic pigments, salicylic acid

Introduction

Agricultural crops and wild flora are faced with a variety of intense environmental stress factors causing considerable economic losses worldwide (Tuzhikov et al. 2011). Plant defense is controlled through various type of protein and non-protein signaling molecules. Most phytohormones, such as ethylene, abscisic acid, jasmonic acid and salicylic acid (SA), have important roles as defensive molecules in the signaling pathways (Vicente and Plasencia 2011, War et al. 2011).

SA is one of the endogenous plant growth regulators that belong to a diverse group of plant phenolics (Pandey et al. 2013). It affects metabolic and physiological events in relation to growth and development in plants. It has long been known that SA regulates seed germination, adventitious root formation, photosynthetic reactions, cellular respiration, thermogenesis, flower formation and anthesis, seed production and senescence (Raskin 1992, Singh and Usha 2003, Khodary 2004, Vicente and Plasencia 2011). Numerous researches indicated that SA content increases under various types of oxidative stress conditions in plants (Larkindale and Knight 2002, War et al. 2011). SA mediates the recognition of pathogens and activation of defense pathways from local to distal infected tissue inducing systemic acquired resistance (An and Mou 2011). It also modulates plant defense under abiotic stress conditions, being an important signaling molecule (Borsani et al. 2001, Muñoz-Sanchez et al. 2013). SA regulates the activity of enzymatic antioxidants such as superoxide dismutase (SOD), polyphenol oxidase (PPO) and peroxidase (POD) in response to excessive production of reactive oxygen species (ROS). However, SA inhibits the activity of some enzymatic antioxidants, such as ascorbate peroxidase (APX) and catalase (CAT), leading to excess ROS accumulation as signaling molecule (Hayat et al. 2008).

Based on the regulatory and defensive role of SA in multiple developmental processes, exogenous application of SA has attracted attention in induction of plant defense against biotic and abiotic stress factors. However, SA controls plant growth and development, depending on the applied dose and species. According to previous research, a broad range of exogenous applied SA doses (10 nM to 10 mM) was effective in different plant species. (Janda et al. 1999, Shakirova et al. 2003, Vicente and Plasencia 2011). It has been reported

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that higher concentrations may cause inhibitory effects on plant growth and development (Hayat et al. 2008). The objective of the study is to investigate the effects of different concentrations of SA on growth, antioxidant enzymes and photosynthetic pigment content in rye (*Secale cereale* L.), an important crop worldwide.

Material and methods

The rye (*Secale cereale* L. cv Aslım 95) seeds were provided from the Bahri Dağdaş International Agricultural Research (Konya, Turkey). The surface-sterilized seeds were germinated in Petri dishes with nutrient solution with or without SA (10, 100, 500 and 1000 μM) in a plant growth room set at 23±2 °C temperature, 45-50% relative humidity and a light intensity of 70 μmol (photon) $m^{-2} s^{-1}$ (day/night: 16/8). The nutrient solution was a modified version of Hoagland's solution (pH 6.0) including 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 30 μM Fe(III)-EDTA and standard Hoagland micronutrients (Hoagland and Arnon 1950). All experiments were conducted three times and twenty five seeds were used for each repetition and experimental groups.

The germinated control and SA treated seeds were counted and germination index was calculated. At the end of 15 days primary root length was measured and relative root growth was calculated according to Schildknecht and De Campos Vidal (2002).

Hydrogen peroxide (H_2O_2) content was assayed according to Junglee et al. (2014). Control and SA treated roots (300 mg) were homogenized with 2 mL of the extraction buffer (0.1% trichloroacetic acid, 1 M KI, 10 mM phosphate saline buffer) and centrifuged at 12000 g for 15 min at 4 °C (Sigma 3K18). The supernatants were incubated in total darkness for 20 min and then measured at 390 nm, spectrophotometrically. The standard curve was used to calculate the H_2O_2 content.

Control and SA treated roots (100 mg) were homogenized with 1 mL of cold sodium-phosphate buffer (PBS, 50 mM, pH 7.0). Homogenates were centrifuged at 14000 rpm for 20 min at +4 °C. The supernatant was stored in ice for enzymatic assays. Superoxide dismutase (SOD) activity was determined by the method of Cakmak and Marschner (1992). The reaction mixture containing 2 mL of substrate buffer (100 mM pH 7.0 PBS, 2 M Na_2CO_3, 0.5 M EDTA, 300 mM L-methionin, 7.5 mM nitro blue tetrazolium, 0.2 mM riboflavin) and 2 μL of the supernatant was incubated under 15 W fluorescent lamps for 10 min and measured at 560 nm, spectrophotometrically. Peroxidase (POD) activity was evaluated by the method of Birecka et al. (1973). The reaction mixture containing 1.5 mL of substrate buffer (0.1 M PBS pH 5.8, 5 mM H_2O_2, 15 mM guaiacol) and 10 μL of enzyme extract was measured immediately for 2 min at 470 nm, spectrophotometrically. Catalase (CAT) activity was assayed as described by Cho et al. (2000). The reaction mixture containing 1 mL of substrate buffer (20 mM PBS pH 7.0, 6 mM H_2O_2) and 25 μL of enzyme extract was measured by the decrease in absorbance for 2 min at 240 nm, spectrophotometrically.

Lipid peroxidation (LPO) was evaluated by determining the concentration of malondialdehyde (MDA) (Cakmak and Horst 1991). Control and SA treated roots (200 mg) were homogenized with 1 mL TCA (0.1%) and centrifuged at 12000 g for 20 min at +4 °C. The reaction mixture containing 1 mL of substrate buffer (0.6% thiobarbituric acid in 20% TCA) and 250 μL of enzyme extract was incubated for 30 min at 95 °C. The mixture was cooled immediately on ice and centrifuged at 12000 g for 10 min. The supernatant was measured at 532 and 600 nm.

Total chlorophyll, chlorophyll *a*, *b* and carotenoid contents were determined according to Arnon (1949). Control and SA treated leaves (0.5 g) were homogenized with 15 mL acetone (80%) and centrifuged at 3000 g for 10 min at +4 °C. The supernatant was measured at 470, 645 and 663 nm, spectrophotometrically.

The anthocyanin content, one of the non-enzymatic antioxidants, was assayed according to Rabino and Mancinelli (1986). Control and SA treated leaves (0.5 g) were extracted in 10 mL methanol:HCl (99:1, v/v) and centrifuged at 12000 rpm for 10 min at +4 °C. The supernatant was measured at 530 and 657 nm, spectrophotometrically.

Statistical analysis was performed using one way analysis of variance (ANOVA), (SPSS 21.0 software). The significance of the applications was designated at the $P < 0.05$ level using the Tukey's test. All data presented are means ± SD.

Results

To determine the dose-dependent effects of salicylic acid (SA), rye seeds were germinated in Hoagland solution with or without of SA (10, 100, 500 and 1000 μM). According to our results the seed germination percentages were 26.67% in control, 41.33% in 10 μM, 22.67% in 100 μM and 17.33% in 500 μM (Fig. 1A). No germination was recorded at the highest concentration of 1000 μM. The germination percentages revealed that higher concentrations of SA reduced the seed germination; however 10 μM stimulated the germination in comparison to control. Similarly, relative root elongation was decreased at higher concentrations, but it was stimulated at 10 μM SA. Root elongation was 10.28% in control, 13.66% in 10 μM, 5.93% in 100 μM and 3.73% in 500 μM SA after 15 days (Fig. 1B).

To assess the oxidative stress after SA application hydrogen peroxide (H_2O_2) content and some antioxidant enzyme activities were evaluated after 15 days. Based on our results, the content of H_2O_2, generated after univalent reduction of superoxide radicals was reduced by 25.5% in 10 μM SA. However, it was increased by 2.5% in 100 μM and 16.7% in 500 μM SA with regard to control (Fig. 2A). One of the antioxidant enzymes, SOD is responsible for catalyzing the reduction of superoxide anions into H_2O_2. After SA application SOD activity increased by 8.1% in 10 μM, 49.3% in 100 μM and 61.9% in 500 μM with respect to control after 15 days (Fig. 2B). According to our results, after SA application significant reduction was observed in CAT activities suggesting the inhibition of H_2O_2 breakdown to water (Fig. 3A). The reduction was 60% in 10 μM, 50% in 100 μM and 35% in 500 μM as compared to control.

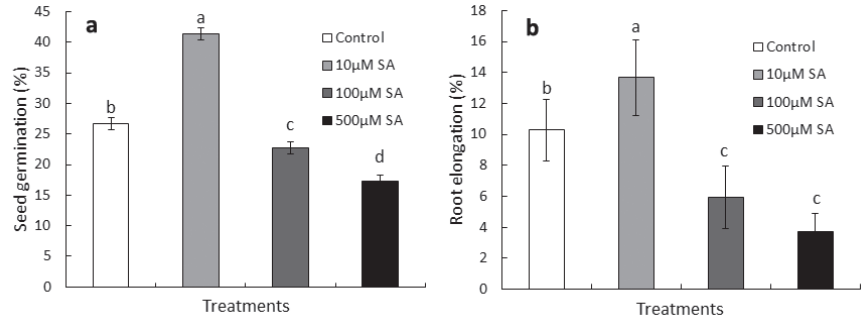

Fig. 1. Seed germination (A) and root elongation (B) of rye treated with different concentrations of SA (10, 100 and 500 μM) after 15 days. The data with different letters are significantly different according to Tukey's test at $P < 0.05$ for independent samples. Results are expressed as mean ± SD.

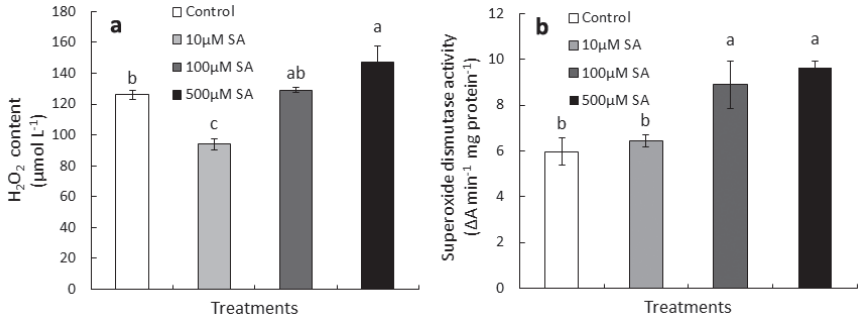

Fig. 2. H_2O_2 content (A) and SOD activity (B) of rye roots after 15 days of treatment with different concentrations of SA (10, 100 and 500 μM). The data with different letters are significantly different according to Tukey's test at $P < 0.05$ for independent samples. Results are expressed as mean ± SD.

Lipid peroxidation (LPO) was monitored by the malondialdehyde (MDA) level. Whereas 500 μM SA application decreased the MDA content by 46.2%, the lower concentrations increased the MDA content by 53.9% in 10 μM and 7.7% in 100 μM referring to lipid peroxidation (Fig. 3B). Although 10 μM SA application increased POD activity by 56.3%, it was decreased by 31.3% in 100 μM and 37.5% in 500 μM compared to untreated control (Fig. 4A).

To assess the possible effects of SA on photosynthetic pigments, they were quantified after 15 days (Tab. 1). The results revealed that SA application increased chlorophyll *a* content. Based on our results the most significant increase was observed by 5.3% in 10 μM SA solution. However, chlorophyll *b* content was decreased dose-dependently. Correlated with chlorophyll *b* reduction, total chlorophyll decreased in 100 and 500 μM SA applications. Conversely, after 10 μM SA application, total chlorophyll increased by 3%. In addition, the content of carotenoids, which are lipid soluble antioxidants functioning in oxidative stress tolerance, increased in all SA applications. One of the non-enzymatic antioxidants, anthocyanin, in the class of flavonoids increased by 36% in 10 μM, 2.4 fold in 100 μM and 2.9 fold in 500 μM, compared to control (Fig. 4B).

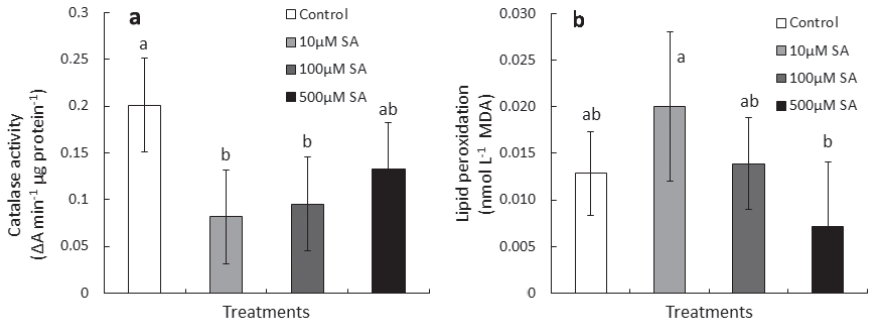

Fig. 3. Catalase (CAT) activity (A) and lipid peroxidation (B) of control and SA-treated (10, 100 and 500 μM) rye roots after 15 days of treatment. The data with different letters are significantly different according to Tukey's test at $P < 0.05$ for independent samples. Results are expressed as mean ± SD.

Tab. 1. Total chlorophyll, chlorophyll *a*, *b* and carotenoid contents (mg mL^{-1}) in rye leaves treated with different concentrations of SA (10, 100 and 500 μM) after 15 days. The data with different letters are significantly different according to Tukey's test at $P < 0.05$ for independent samples. Values represent means ± SD.

Treatment	Chlorophyll *a* (mg mL^{-1})	Chlorophyll *b* (mg mL^{-1})	Chlorophyll *a/b*	Total chlorophyll (mg mL^{-1})	Total carotenoids (mg mL^{-1})
Control	302.32^{c}±1.04	167.1^{a}±1.07	1.81	469.41^{ab}±1.58	89.53^{b}±0.7
10 μM SA	318.36^{a}±1.09	163.59^{a}±1.28	1.95	481.95^{a}±1.41	95.90^{ab}±0.35
100 μM SA	304.69^{c}±1.04	159.73^{ab}±0.79	1.91	450.30^{b}±1.61	97.42^{a}±0.24
500 μM SA	308.62^{b}±0.53	146.37^{b}±0.89	2.11	454.99^{b}±1.12	94.96^{ab}±0.42

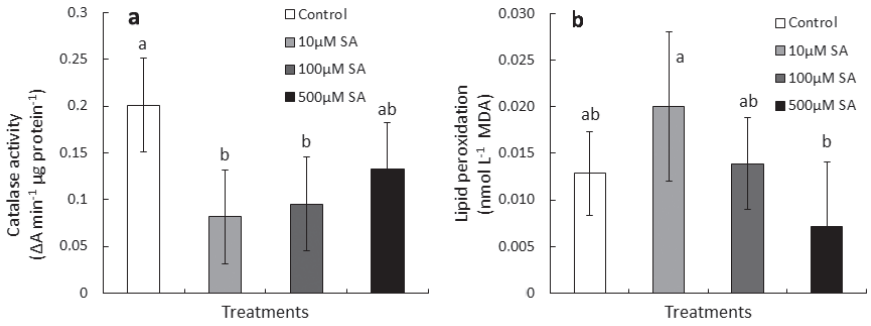

Fig. 4. Peroxidase activity (A) and total anthocyanin (B) of control and SA-treated (10, 100 and 500 μM) rye roots after 15 days of treatment. The data with different letters are significantly different according to Tukey's test at $P < 0.05$ for independent samples. Results are expressed as mean ± SD.

Discussion

Salicylic acid (SA), which is one of the phytohormones, has various regulatory roles in plant metabolism (Raskin 1992, Popova et al. 1997). It has been proposed that SA has an important role in bioproductivity, defense and resistance in plants (Hayat and Ahmad 2007). It has been reported that application of SA promoted seed germination, root and shoot growth in soybean, wheat, gloxinia, violet and *Brassica juncea* plants in a dose dependent manner (Gutiérrez-Coronado 1998, Fariduddin et al. 2003, Shakirova et al. 2003, Hayat et al. 2005, Martín-Mex et al. 2015). This situation can be explained by SA treatment stimulating plant growth by stimulating mitotic activity (Shakirova et al. 2003). Although most of the studies mentioned that the applied doses between 10^{-10} and 10^{-5} μM stimulated growth and development, the higher doses caused inhibitory effects (Pancheva et al. 1996, Pancheva and Popova 1998). In the presented study 10 μM SA stimulated seed germination and root growth but the higher concentrations (100 and 500 μM) were inhibitory after 15 days, consistently with the previous results. Moreover we observed no seed germination in the highest concentration of 1000 μM in rye.

Under appropriate conditions, ROS generation and scavenging are balanced in the cells. Cellular homeostasis is managed by complex signal transduction pathways (Dutilleul et al. 2003). It has been reported that under biotic and abiotic stresses endogenous SA is accumulated and this accumulation indicates that in stressed plants SA plays a crucial role as a signaling molecule in the management of cellular redox homeostasis (Apel and Hirt 2004). Oxidative stress induces the generation of ROS such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) in plant cells. The ROS scavengers include enzymatic and non-enzymatic antioxidants operating in the different cellular organelles (Noctor and Foyer 1998, Hernandez et al. 2001). H_2O_2 is generated from reduction of $O_2^{\bullet -}$ by superoxide dismutase (SOD) which is one of the key enzymatic antioxidants. Excess H_2O_2 causes oxidative stress and catalase (CAT) can directly catalyzes its decomposition into O_2 and H_2O (Quan et al. 2008). As we presented in our study, although 10 μM SA application induced a slight increase in SOD activity, it was increased progressively in 100 and 500 μM SA. Consistent with the SOD activity results, H_2O_2 levels were increased in 100 and 500 μM SA. Although CAT levels increased depending on the SA dose, all activities remained lower than that of the control suggesting the inhibition of H_2O formation. It is widely known that SA restricts CAT activity and stimulates an increase in H_2O_2 level as has been shown (Chen et al. 1993, Janda et al. 2003). There is also evidence for a complicated relationship between H_2O_2 and SA, which stimulate each other (Rao et al. 1997, Dat et al. 2000).

Peroxidase (POD) activity is a common response to various types oxidative stress factors. Numerous research results indicated that SA-controlled reduction in H_2O_2 levels is related to elevated POD activities (Wang et al. 2004). Our results revealed that POD activity increased in 10 μM SA, inducing the reduction of H_2O_2, but decreased in higher concentrations. This situation suggests that higher concentrations may inhibit POD enzyme activity via alterations in enzyme and/or biochemical pathways. On the other hand SOD may control the integrity of membrane structures of the cell cul-

minating in the processes of lipid peroxidation (LPO) deactivation (Zenkov et al. 2001). In the present study, LPO increased in 10 μM SA because of slight SOD activity. However, in higher SA concentrations LPO decreased according to the increased SOD activity. Horváth et al. (2007) also indicated that exogenous SA application causes a rapid transient increase of ROS including superoxide anions ($O_2^{\bullet-}$). Considering our results, inefficient SOD activity, which is responsible for reduction of $O_2^{\bullet-}$ to H_2O_2, may result $O_2^{\bullet-}$ accumulation in 10 μM SA culminating in LPO.

A widely known effect of SA is that it raises the photosynthetic pigment levels (Khodary 2004). In the present study total chlorophyll content was increased in 10 μM SA, but it was decreased in higher concentrations, suggesting a decrease in photosynthetic capacity. However total carotenoids were increased, representing close rates in applied concentrations. Similarly, Pancheva et al. (1996) reported that chlorophyll content decreased after different concentrations (100 μM to 1 mM) of SA application in barley leaves. In addition, Moharekar et al. (2003) indicated that different concentrations of SA (5, 10, 50, 100 and 200 mg kg^{-1}) activated the synthesis of carotenoids and xanthophylls but reduced the level of total chlorophyll in wheat and moong. According to Szepesi et al. (2009) 10^{-4} M SA did not cause any reduction in total chlorophyll, but the amount of carotenoids was slightly increased in *Solanum lycopersicum*. Higher SA concentrations may decrease the chlorophyll content due to the SA-induced excess ROS accumulation and the consequent inhibition of plant growth and development (Ma et al. 2013). In photosynthetic organisms, carotenoids serve as ROS scavenger photoprotectants, having a role in ROS scavenging and LPO suppression (Gill and Tuteja 2010). Anthocyanins are flavonoid pigments located in vacuoles responsible for coloring fruits and flowers (Grotewold 2006). They are considered to be non-enzymatic antioxidants under stress conditions (Kovinich et al. 2012). The function of stress-induced anthocyanins is thought to be that of ROS scavengers (Hatier and Gould 2009, Agati et al. 2012). Szepesi et al. (2008) indicated SA pre-treatments increased the accumulation of anthocyanins in both the presence and absence of salt stress. According to our results SA application induced an increase in anthocyanin content as well in carotenoids due to SA-induced ROS accumulation.

Conclusion

Some progress in understanding the effects of SA has been obtained under normal and stressful conditions. Although the pathway of signal regulation under biotic-abiotic stress factors in plant tolerance is still complicated, it is known that SA behaves as a signaling molecule, triggering a cascade of protective reactions. According to our results, exogenous SA application causes alterations to germination, antioxidant enzymes and photosynthetic pigment content in rye, depending on the dose. At low concentration SA increased the germination rate, the elongation of roots and enhanced the photosynthetic pigment contents. Higher concentrations resulted in H_2O_2 accumulation due to increased SOD and decreased CAT activities and concomitantly they decreased the germination rate and root growth. This comprehensive study may help to improve our knowledge of the complex mechanism of SA action, principally in relation to the doses applied. In conclusion exogenous SA application at an appropriate dose may be a possible approach for the control of growth and environmental stress response considering enzymatic and non-enzymatic antioxidant activity.

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