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The effect of Salicylic acid on physiological characteristics of Lolium grass (*Lolium perenne* cv. "Numan") under drought stress

Seyedeh mahsa Hosseini[1*], Mohsen Kafi[2], Masoud Arghavani[3]

[1]*Department of Horticulture (Ornamental Plants), Faculty of Agriculture, Science and Research Branch, Islamic Azad University, Iran*

[2]*Department of Horticultural Sciences, University of Tehran, Iran*

[3]*Department of Horticultural Sciences, University of Zanjan, Iran*

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Abstract

Salicylic acid is involved in plant responses to abiotic stress such as drought, cold, heavy metal poisoning, heat and osmotic stress. This study carried out to investigate the effect of salicylic acid on physiological characteristics of Lolium grass (*Lolium perenne* cv. "Numan") under drought stress. A factorial experiment in a completely randomized design with four replications was performed. The treatments consisted of three salicylic acid levels (0.0, 0.75, and 1.5 mm) and drought stress consisted three levels (50, 75, and 100 percent of field capacity). Salicylic acid Foliar application at 0.75 and 1.5 mm levels increased the content of chlorophyll a, b and reduced electrolyte leakage, proline accumulation and antioxidant enzyme activity, which suggests that salicylic acid, to reduce the negative impacts of drought stress.

* **Corresponding Author:** SeyedehMahsa Hosseini✉ hossainim3@gmail.com

Introduction

One of the main components of Land scape are cover plants and grass is one of the world's most important cover plants. Ryegrass is a perennial, trample-resistant and cold grass, and is an important ingredients in the production of mix grass seeds which is due to its relatively good growth and establishment rate (Amiri Nasab *et al*., 2013). Salicylic acid or Ortho-hydroxy benzoic acid is a phenolic compounds in plants which is considered as a hormone-like substance that has an important role in plant growth regulation. This material exists in plant in small quantities (milligrams per gram of fresh weight or less), in free-form and Glycosyl form (Raskin., 1992). Salicylic acid plays an important role in the regulation of various physiological processes such as growth, plant evolution, ion absorption and transport, stomatal closure, photosynthesis, growth rate and germination depending on the concentration used, plant, species, developmental period and environmental conditions (Raskin., 1992; Senaratna *et al*., 2000). Salicylic acid also is counted as an endogenous signal molecule in plant resistance to environmental stresses (Elwan *et al*., 2009). This substance plays an important role in plants coping with biotic and abiotic stresses and initial reports indicate that salicylic acid in plants has the role of regulation against wide range of oxidative stresses (Deef., 2007). The mechanism of action of salicylic acid against stress is relevant to its role in the regulation of antioxidant enzymes and compounds containing active oxygen species in plant (Stevens *et al*., 2006). Low concentrations of salicylic acid leads to an increased tolerance to drought damage in tomatoes and beans, although its high concentrations did not impact the results (Senaranta *et al*., 2000). Moreover, Salicylic acid enhances the production of pigments such as anthocyanin, carotenoid and chlorophyll (Khodary., 2004). The external application of salicylic acid enhanced plant growth and photosynthesis in wheat under drought stress conditions (Hussein *et al*., 2007). Salicylic acid is readily transmitted from treatment location (leaves and roots) to other locations and subsequently the associated response incidences (Alaey *et al*., 2011).

Drought is one of the most important environmental factors limiting production, quality and sustainability of grass which its water needs is always considered, since meeting these requirements in the warmer months of the year in the arid and semi-arid is very difficult and expensive (Huang., 2008). Drought stress stops grass growth and decreases their quality (Zhang *et al*., 2004). Drought stress usually occurs when the available water in the soil reduces and climatic conditions cause more water reduction by evapotranspiration (Jaleel *et al*., 2007). Water scarcity is one of the most important abiotic stress that has a negative effect on growth and yield (He *et al*., 2005). Severe water stress results in inhibition of photosynthesis, Metabolism disorders and eventually death of the plant (Jaleel *et al*., 2008a).Additionally, drought stress causes reduction in plant growth by a variety of effects on physiological and biochemical processes such as photosynthesis, respiration, ion uptake, transport, carbohydrates, food metabolism and growth parameters, that the severity of damage depends on the duration of drought stress and different growth stages (Farooq *et al*., 2008).

The first section of cells damaged against drought stress is the cell membrane which loses its integrity leading to an increase in electrolyte leakage (Jaleel *et al*., 2008b).Thus use of plant growth regulators to increase resistance to stress, especially drought stress is recommended (Hayat *et al*., 2010).

Proper management and understanding the physiological and biochemical responses of grass to drought stress conditions play an important role in minimizing grass problems in arid and semi-arid areas. The aim of this study was to evaluate the effect of salicylic acid on physiological characteristics of perennial grasses (*Lolium perenne* L.) under drought stress.

Materials and methods

To evaluate the effect of salicylic acid on physiological characteristics Lolium grass under drought stress, an experiment was performed in greenhouses located in Tehran during the 2013-2014. Grass Seeds Planted in

Pots (28 cm height and 18 cm span), filled with a mixture of soil (loam) and Manure. A factorial experiment in a completely randomized design with four replications was established. After planting the seeds, grass were daily irrigated with enough water until the beginning of treatment. Until the start of treatment, grass was evenly detopped with scissors in 5-6 cm height once in every two weeks and allowed to fully establish.Grass feeding performed regularly once a week with irrigation water and Crystallon fertilizer with 20:20:20 NPK ratio and the concentrations of three grams per liter.

Application of Salicylic acid and drought stress

With full deployment grass, Salicylic acid solution sprayed on the grass which were detopped that day before treatment in three concentrations (0, 0.75, and 1.5 mM). The solution application with simultaneous drought stress performed once every two weeks, six times during the test with foliar application by spraying the leaves uniformly. Drought stress was imposed in three levels of maintaining soil moisture at field capacity as control treatment (100% of field capacity), Mild water deficit stress at 75% field capacity and relatively strong water stress at 50% of field capacity. Electrolyte leakage, chlorophyll a and b, proline accumulation and antioxidant enzyme activities were measured in six innings.

Measure electrolyte leakage

In order to calculate the electrolyte leakage (Wang & Huang., 2004) method was used with slight modification so that the leaf samples (about 0.1 g wet weight) was washed with distilled water and were placed on a shaker in 20 ml of distilled water for 24 hours.Then the electrical conductivity of the solution was measured. Subsequently, test tubes containing the samples were placed in boiling water for 20 minutes at 100 °C and then cooled at room temperature. Then measured the electrical conductivity of the solution. electrolyte leakage was calculated as a percentage, by dividing the initial electrical conductivity the electrical conductivity of dead cells.

EC1/EC2×100

Leaf chlorophyll measurements

Chlorophyll measurements was conducted according to (Hiscox & Israelsta., 1979) method with slight modification. So that chlorophyll is extracted by soaking 0.1 g of fresh samples in 10 ml of dimethylsulfoxide at 65 °C in the darkness for 30 min. Absorbance of the extract was measured at 663 and 645 nm by spectrophotometer and the chlorophyll content was calculated using the (Arnon., 1949) formula as follows:

$C_b = 0.0229\ D_{645} - 0.00468\ D_{663}$

$C_a = 0.0127\ D_{663} - 0.00269\ D_{645}$

$C_T = C_a + C_b = 0.0202\ D_{645} + 0.00802\ D_{663}$

Proline measurement

In order to measure proline (Bates *et al.*, 1973) method was used with slight modification. Briefly, 0.1 g of fresh samples crushed using liquid nitrogen and was homogenized with 2 ml of 3% Sulfosalicylic acid. After 20 minutes of centrifugation at 15000 rpm intensity, 0.5 ml of supernatant with 2 mL of Ninhydrin acid and 2 ml acetic acid, the reaction was placed in boiling water for one hour and it ended in ice. By adding 2 ml of Toluene, colored liquid absorption containing proline was measured at a wavelength of 520 nm with a spectrophotometer, and proline level was calculated using the standard plots.

Measuring the antioxidant enzymes activity

Preparation of enzyme extract

Extraction buffer contained 0.1 M (100 mM) sodium phosphate buffer with 7.5 pH and 1 mM AS and 0.5 mM Na2-EDTA respectively. 1.0 gram of fresh tissue with 4 cc extraction buffer was gritted in a mortar on ice, and then centrifugation (centrifuge machine model 303k) was performed at 4°C for 30 min with 13000 rpm intensity and the supernatant were collected, distributed in eppendorf tubes and was kept at -20 °C temperature.

Superoxide dismutase activity was examined based on the (Misra., 1979) method. After preparing enzyme extracts, SOD activity was determined by measuring the ability of each extract to inhibit photochemical reduction reaction of Nitro Blue Tetrazolium.

The reaction solution for measuring SOD activity contained 50 mM phosphate buffer with 7.5 pH, 13 mM methionine, 75 mM Nitro Blue Tetrazolium (NBT), 0.1 mM EDTA and 2 mM Riboflavin. The enzyme reaction mixture was kept in complete darkness. 2.5 ml of SOD reaction medium plus 200 µl enzyme extract were under fluorescent for 10 minutes. Two sets of tubes containing 2.5 ml of reaction medium plus 200 µl extraction buffer was prepared, which one set placed in darkness, and the other set placed under a fluorescent lamp along with samples for 10 minutes. After 10 min, all samples were transferred to darkness and samples absorption was measured at 560 nm by spectrophotometry. The device was reset to zero using the tube containing extraction buffer (without enzyme extract) and was placed in the darkness was zero (as Blank) and then absorption of the tube containing reaction medium and extraction buffer placed under light was measured by spectrophotometer and considered as a control. Then absorbance of each sample (containing enzyme extract) was measured in nm 560. The difference between the absorbance of samples containing enzyme extract and absorbance of the control sample actually represents a spontaneous Formazan establishment reaction inhibited by SOD. Therefore, the percent decrease in absorbance was measured for each sample. One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of Nitro Blue Tetrazolium reducing. This method is based on the conversion of NBT to Formazan in the presence of light and color detection. And if there was superoxide dismutase enzyme in the reaction medium, it would prevent the reaction and reduce the rate of dye formation and color detection. Finally, the SOD activity were calculated for all samples based on enzyme units for per mg protein (Unit Eg-1fw).

Catalase enzyme activity were examined based on (Luck., 1974) method. For this purpose, 900 ml of the reaction mixture (a solution of 10 mM hydrogen peroxide in the buffered saline phosphate without PVP) and 100 µl of enzyme extract was shed in (Kut Spectophotometer) and after the addition of hydrogen peroxide (H_2O_2) in the reaction mixture, reduction caused by the decomposition of H_2O_2 due to action of catalase was measured immediately, at a wavelength of 240 nm for 1 min by a spectrophotometer. Enzyme activity in the reaction mixture after 1 minute is calculated as follows using the extinction coefficient (0.395 $mMol^{-1}cm^{-1}$).

Enzyme activity = (ΔOD *A)/ (ε *V* P)

Statistical analysis

Data analysis was performed using SAS 9.2 software, means were compared using Duncan's multiple range test at the 5% level, and Excel software was used for diagramming.

Results and discussion

The results from data variance analysis showed that electrolyte leakage, chlorophyll a and b, proline accumulation and activity of catalase enzyme and superoxide dismutase influenced by different levels of salicylic acid, different levels of drought stress, drought stress and salicylic acid interaction were significant in 1 % statistical class (table 1).

Table 1. Effect of salicylic acid, drought stress and drought stress and salicylic acid interaction on physiological characteristics of (*Lolium perenne* L.).

Variation Source	Degree of freedom	electrolyte leakage	Chlorophyll a	Chlorophyll b	Proline	Catalase enzyme	superoxide dismutase enzyme
Salicylic acid	2	28.3692**	23.5263**	23.4474**	38.6130**	92.9107**	72.9979**
Drought stress	2	31.2738**	22.3500**	22.2044**	61.6535**	93.8570**	63.7109**
Salicylic acid* Drought stress	4	3.3779**	1.6159**	1.6370**	79.804**	11.3636**	5.7132**
Experimental error	27	0.0283	0.0822	0.0961	4.96	0.0882	0.0829
Coefficient of Variation (%)	-	5.14	5.61	7.21	5.55	5.88	6.60

Electrolyte leakage

Electrolyte leakage is among the important physiological parameters in evaluating water stress. In the carried out test, drought stress increased the electrolyte leakage in Lolium grass. Comparison of the mean of the data relating to salicylic acid, showed significant difference between used concentrations in two groups and control group. In both normal and stressed conditions, by increasing the amount of salicylic acid electrolyte leakage leakage was reduced and salicylic acid prevents the extreme increase of electrolyte leakage in treatments containing stress. Electrolyte leakage was significantly more than other levels at 50% of field capacity, which the use of salicylic acid could lead to a reduction in all levels (Figure 1). Using salicylic acid by increasing the amount of and putrescine polyamines, spermidine and spermine and other protective compounds of cell, enhances and stabilize cell membrane stability index of the controlled permeability of the membrane of electrolyte leakage of leaves has been used to prevent (Rajou *et al*., 2006).Investigations have shown that salicylic acid prevents damage to membrane fatty acids, reduces membrane permeability and protects Thylakoid membrane in times of drought stress and this likely effects when the amount of hydrogen peroxide is reduced. On the other hand, salicylic acid decreases cellular membrane electrolyte leakage in tomato plants (Stevens *et al*., 2006). Similar results reported by Khan *et al* (2010) which agrees the findings of this study.

Chlorophyll a and b

The results of this study indicated that increasing drought stress decreased the amount of chlorophyll a and b. Using salicylic acid significantly increased the amount of chlorophyll a and b. Comparison of the mean of the data relating to this hormone between two concentrations used and the control group is significant. In both normal and stressed conditions chlorophyll increased by increasing the amount of salicylic acid, and chlorophyll prevents the extreme decline in treatments with stress (Figures 2 and 3). Drought stress reduces the resistance of the chloroplast, chloroplast structure damage, reduction of chlorophyll synthesis enzyme activity (glutamate ligase) and increased activity of destructive enzymes proline manufacturer (glutamate kinase) and abnormal regulation of stomatal opening and closing (Jalili Marandi., 2010). Therefore, treatment with salicylic acid can help prevent these events. Moreover, with the development of chloroplast and increased glutamate ligase activity and improving indexes and mechanisms associated relating to chlorophyll synthesis those will be improved (Abraham *et al*., 2008). Regulating stomatal opening and closing mechanism and maintaining the structure, caused by treating with this plant growth regulator could be reasons of the increase in chlorophyll (Khan., 2010; Rajou *et al*., 2006). This is an accepted fact that salicylic acid has the potential to make changes in a wide range of metabolic responses in plants, such as photosynthesis. Photosynthetic pigments in wheat seedlings increased, which the seeds were treated at low concentrations of salicylic acid (Hayat *et al*., 2010). Spraying a small amount of salicylic acid and acetyl salicylic acid on leaves lead to increased photosynthesis and yield in soybean and maize (Wajahatullah *et al*., 2003). The results matches with the results of Huang (2008).

Leaf proline contents

The results of this study indicate that increased levels of drought stress, increased levels of proline and proline showed significant differences between the levels of drought stresses. Salicylic acid had a significant effect on proline content in both 0.75 and 1.5 mM concentrations under the drought stress. In both normal and stressed conditions, increasing the amount of salicylic acid reduced the proline accumulation. (Figure 4).

Plants exposed to drought stress attempt to produce organic osmolites such as proline. Proline as a solution, decreases the osmotic potential, maintains cell swelling, stabilize and protects membrane system, prevents protein breakdown, and ultimately reduce the negative effects of stress. As a result, it prevents further water loss in drought stress conditions, and lessens damage caused by free radicals (Kafi *et al*., 2009).

Due to the moderating effect of salicylic acid on oxidative stress, this hormone triggers stomatal closure and protect cells water against stress. Thereby by avoiding situations causing stress by plant growth regulator, salicylic acid, the plant does not produce Proline, so no Proline is accumulated (Jalili Marandi., 2010). Salicylic acid also regulates proline in shoots and roots of pepper plants and affects its growth (Elwan and El-Hamahmy., 2009). Similar results obtained by (Steinke and Stier., 2004), which agrees with the findings of this study.

Activity of antioxidant enzymes

In the present study, by increasing the levels of drought stress, levels of antioxidant enzymes, superoxide dismutase and catalase also increased, and the levels of these antioxidant enzymes showed significant differences between the levels of stresses. In both 0.75 mM and 1.5 mM concentrations, salicylic acid has a significant effect on both the antioxidant enzymes SOD and CAT under drought stress, and in both normal and stressed conditions by increasing the amount of salicylic acid, the antioxidant enzymes declined (Figure 5 and 6). Drought stress stimulates Reactive Oxygen Species (ROS) generation including superoxide (O_2^-), the single oxygen (O_2), hydroxyl (OH) and hydrogen peroxide (H_2O_2) (Alaey *et al.*, 2011).Reactive oxygen species damage the proteins, lipids, carbohydrates and nucleic acid. Plants have enzymatic and non-enzymatic defensive systems for the elimination and detoxification of oxygen species, which SOD struggling through converting H_2O_2 to water and CAT struggling through the conversion of H_2O_2 to water and oxygen are samples of these defensive systems (Bian and Jiang., 2009). Salicylic acid growth regulator act such as non-enzymatic antioxidants such as ascorbate, glutathione, tocopherols and carotenoids under stress conditions. They begins to operate under osmotic stress, which leads to the activation of ROS and prevents its damaging effects (Reezi *et al.*, 2009). Salicylic acid serves as signaler in the development of systemic resistance acquisition and has an important role in the regulation of antioxidant enzymes.

Due to the moderating effect of salicylic acid on oxidative stress, this hormone triggers stomatal closure and protect cells water against stress. Thereby by avoiding causing stress conditions by the plant growth regulator, salicylic acid, the plant does not produce antioxidant enzymes. Therefore Salicylic acid is produced under water stress conditions in the presence of salicylic acid, compared to those without (Jalili Marandi., 2010). The results of this study has been consistent with the results of (Kazemi *et al.*, 2011) and inconsistent with the results of (Moba *et al.*, 2007).

The general conclusions

The results show that under stress conditions the application of salicylic acid as a foliar spray Result in improved biochemical and physiological characteristics of perennial grass so that the negative impacts of stress is reduced and turf resistance is added. Thus, considering the low cost of salicylic acid and no adverse environmental effects, more water deficit is viable and recommended by application of this hormone.

Indices

F1: 100 % of field capacity; F2: 75 % of field capacity; F3: 50 % of field capacity.

S1: control; S2: 0.75 mM salicylic acid; S3: 1.5 mM salicylic acid.

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