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ORIGINAL ARTICLE

The effect of *Azospirilium* bacteria and Salicylic Acid effects on drought stress tolerance in *Ocimum basilicum L.* Medicinal plant

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ABSTRACT

Drought stress is one the most important problems with growing plants in arid and semi-arid regions of the world including Iran. On this basis, an experiment was conducted with factorial design based on complete block design with three replications in 2012 to examine the effect of Azospirillum bacteria and salicylic acid on relieving the drought stress in Ocimum basilicum L. medicinal plant. The examined factors comprised different levels of drought stress (soil moisture at the level of 100, 66, 33 percent of field capacity), inoculation and non-inoculation of Azospirillum bacteria and two acid salicylic concentrations (0 and 0.75mM). The results showed that the drought stress, Azospirillum incubation and application of salicylic acid had a significant effect on the growth parameters, electrolyte leakage, photosynthetic pigments and the amount of proline in the plant. In drought conditions, the growth parameters and the amount of chlorophyll content were decreased but the amount of proline and electrolytes leakage were increased. Incubation of the plant by Azospirillum bacteria and application of salicylic acid decreased the drought effects and resulted in increasing of the growth, chlorophyll content and proline amount to increase and reduced the electrolytes leakage. In most of the assessed traits the effect of Azospirillum bacteria was more than salicylic acid.

Key words: *growth parameters, electrolytes leakage, proline, water deficit stress, chlorophyll.*

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INTRODUCTION

In general, any factor or a combination of environmental factors undermining the potential genetic growth of the plant is called stress. Drought stress is caused by lack of balance between evapotranspiration and rainfall. In fact, drought is caused by stressful conditions like low rainfall, high temperature, and wind. Plants reaction to the corresponding conditions is different and primarily depends on the plant's growth stage at which drought occurs [1].

Drought is a distinctive characteristic of Iranian geography and there is no escape from this natural and unchangeable phenomenon. On the one hand, energy, water, and food resources are constantly decreasing. So, instead of just focusing on the problems caused by drought, we should try to tackle them. In modern agriculture, some methods proposed for mitigating the adverse effects of drought stress on plants include introducing resistant cultivars, identifying and transferring resistant genes into plants by genetic engineering methods, applying proper agricultural methods, protective tillage, using windbreakers and mulch, proper selection of cultivars and crops, density and suitable planting date and so on [2].

in addition to the above-mentioned methods, using some additives materials such as plant materials, manure, compost, and mineral materials like polymers which are capable of absorbing and keeping water [3], as well as applying some creatures coexisting with such plants as fungus, bacteria [4], and plant

growth regulators [5] can be considered as logical methods of ameliorating adverse effects of drought stress on plants.

It is to use biological technologies such as inoculation seeds with different kinds of bacteria by which plants to be protected from adverse effect of stressful conditions. Application of *Azospirillum* inoculation, as one of the modern approaches to increase plant tolerance to stressful conditions, has been become prevalent because of its prolific effects on fixing molecular nitrogen and producing growth promotive hormones as a biological fertilizer. In this regard, due to a wide range of its host plants, variety of species, capability of alleviating stressful conditions and improving plant growth and productivity made this bacterium prevalent in agriculture. Using *Azospirillum in* inoculation along with fixing nitrogen by producing growth promotive materials improves the root growth which leads to increase water and minerals absorption [6].

There have been some successful reports related to applying this biological fertilizer to mitigate drought stress in plants [7]. Furthermore, as the global approaches to medicinal plants production are inclined towards improving both quality and quantity, it seems that proper plant nutrition by applying biological fertilizers is compatible with the goals of medicinal plants production.

Salicylic acid (SA) is a phenolic material which is considered as a hormone-like regulator and plays an important role in defensive mechanisms against biotic and abiotic stresses in plants. Flowering induction, plant growth and development, synthesis of ethylene, opening and closure of stomata and respiration are some of the important functions of SA in plants [8]. SA plays a defensive role in plants under environmental stresses. This hormone increases resistance to drought in some plants like tomato and bean [9]. SA protects plants from damages caused by oxidative reactions through increasing antioxidants enzymes activities. Considering the issues mentioned in the present study the role of SA and *Azospirillum* on basil plants under drought stress were evaluated.

MATERIAL AND METHODS

The current study was conducted in Ilam University to examine *Azospirillum* bacteria and SA effects on basil plant under drought stress in 2012. First, soil sterilization was carried out by oven in 120 ^{o}C within 4 hours during 3 days. Seeds of *Occimuum basilicum* L. (obtained from PakanBazr Esfahan Co) were disinfected in 1% (active ingredient) sodium hypochlorite solution for 10 min to eliminate possible seed-borne microorganisms, rinsed for 1 min under running water prior to drying for 30 min at room temperature. Then *Azospirillum* inoculated and non-inoculated seeds were cultured in plastic pots which were filled with 1:1:2 mixtures of fine sand, leaf mould and garden soil. In order to inoculate the seeds, first 100 ml of mitigated liquid media was obtained and then the seeds were moistened with them. Then, seeds were placed on a shaker for a hour so as to facilitate the infiltration of bacterium into the seeds.

When 4-6 true leaves of seedling were expanded, SA was sprayed in ratio 0 and 0.75 mM until both sides of the leaves were completely wet. 72 h after foliar spray, all plants were subjected to three levels of drought stress including stress-free conditions (irrigation within the field capacity (100% FC), mild stress (60% of FC) and severe stress (30% of FC) until end of experiment. Irrigation treatments were conducted with daily weighing of pots and adding required water as a result of evapotranspiration (the amount of pots' lost weight).

In order to measure growth parameters in each pot, 10 plants were randomly chosen and height, fresh and dry weight of roots and shoots, number of lateral branches and number of inflorescences per plant were measured and recorded.

Chlorophyll (chl) content was determined by taking fresh leaf samples (0.1 g) from young and full developed leaves. The samples were homogenized with 5ml of acetone (80% v/v) using pestle and mortar and centrifuged at 3,000 rpm. The absorbance was measured with a UV/visible spectrophotometer at 663 and 645nm and chlorophyll contents were calculated using the equations proposed by Strain and Svec [10] given below:

$$\text{Ch.a=mg/g F.W}=\{12.7(A663)-2.69(A645)\}$$

$$\text{Ch.b mg/g F.W} =\{22.9(A645)-4.68(A663)\}$$

$$\text{Cha+b mg/g F.W} =\{20.2(A645)+8.02(A663)\}$$

In order to assess membrane permeability, electrolyte leakage was determined according to Korkmaz et al (2010). Leaf discs (1cm in diameter) from randomly chosen plants per replicate were taken from the middle portion of fully developed leaf and washed with distilled water to remove surface contamination. The discs were placed in individual vials containing 10 ml of distilled water. After inoculating the samples at room temperature on a shaker (150 rpm) for 24 h, the electrical conductivity (EC) of the bathing solution (EC1) was determined. The same samples were then placed in an autoclave at 121 ºC for 20 min

and a second reading (EC2) was determined after cooling the solution to room temperature. The electrolyte leakage was calculated as EC1/EC2 and expressed as percent.

Proline content was determined according to the method described by Bates et al [28]. Fresh leaf material (0.5 g) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. 2 milliliter of the supernatant was mixed with 2ml of acid ninhydrin and 2ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4ml toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520nm with a UV/visible spectrophotometer. Appropriate proline standards were included for the calculation of proline in the samples.

Data were analyzed for significant differences using a factorial analysis of variance with drought stress levels, SA concentrations and *Azospirillum* as main factors. Statistical analysis was performed using SAS and MSTATC software programs and the means compared using the Duncan's Multiple Range Test at p=0.05.

RESULTS AND DISCUSSION

Plant growth parameters

The analysis of variance showed that the applying SA, inoculating seeds with *Azospirillum*, and imposing different levels of drought stress on basil plants significantly had effect on many plant growth parameters including plant height, root and shoot fresh and dry weight, the number of auxiliary branch and inflorescence per a plant. Statistical analysis revealed that reducing growth parameters is correlated with increasing the level of drought stress (table-1). Drought stress, as a deleterious factor in plant physiological processes, can also have an impact on plant growth parameters. So far, in some studies, the effect of drought stress on reducing growth and yield of wheat [11] and barely [12] has been reported being in agreement with our findings.

The first typical symptom of water deficit in plants is the reduction of turgor pressure leading to reduce growth and development of cells located in stem and leaves. The cells' growth is the main process primarily affected by water stress and, hence, on the deficit water condition, the size of organs and leaves will be weakened, associated with reducing cellular development. For this reason, it is postulated that a decline in the size of leaves or the height of plants can be taken into consideration as a sign of deficit water on plants.

On the other hand, the reduction of available water around plants not only leads to reduce nutrition uptake, but also diminishes the leaf growth and consequently the rate of plant transportations. The reduction of leaf growth, as a usual plant reaction to deficit water, leads to lessen photosynthesis rate, thereby reducing plants' dry matter production and yield [13]. Causing serious disturbance in some plant metabolic processes, such as photosynthesis, transportation, chlorophyll forming and cell division is considered as a result of reducing plant growth parameters due to exposing plants to drought stress conditions.

The results of this research demonstrated that inoculating plants with *Azospirillum* is resulted in increasing plant growth parameters in comparison to control plants. So far, the different *Azospirillum*-inoculated effects on growth parameters have been reported. In this regard, Zaady *et al* [14] revealed that corn plants inoculated with *Azospirillum* gained high yield, productivity, as compared to control plants. He continued that this bacterium has an effect on root expanding of host plants, thereby enhancing nutrition uptake resulted in augmenting plat growth parameters.

Fixation of molecular nitrogen paves the way for increasing the plant growth parameters. Besides nitrogen fixation, His attention also paid to the role of this bacterium on inducing the synthesis of different phytohormones in the inoculated plants [15]. In this regard, these plants are able to synthesis auxin around roots and improve their growth parameters [16]. The results of mean comparison of the interactional effect of drought stress and *Azospirillum* showed that the traits of root fresh, dry weight, foliage, and the number of inflorescence gained the highest on %100 F.C and while inoculating them with *Azospirillum* (fig- 1-2-3-4).

The application of SA only caused to increase significantly the dry and fresh weight of shoots in comparison to control plants and did not significantly influence on other growth parameters. The effect of SA on some plants under drought stress like barely [17], tomato and bean [9] have been reported, which their upshots are in agreement with our findings. By inducing a wide range of processes involved in plants' resistance to stresses, applying SA has been proven effective protecting plants from some abiotic stresses [18]. In this respect, the ways of using SA have been proposed include soaking seed in its solute before cultivating and adding it to the hydroponic environment, soil treatment and foliar spray).

Using 0.5 mM SA over improving wheat plants under drought stress, was found to be effective in increasing the activities of cell division on apical meristem, plant growth and yield [15]. External application of SA also increased chlorophyll content and photosynthesis rate and created stability of cellular membrane, reduction of electrolyte leakage in barely plants and finally resistance plants to drought stress [17]. A combination of SA and *Azospirillum* significantly influenced on root expanding.
Inoculating plants with *Azospirillum* without using SA was resulted in gaining the highest amount of root's fresh and dry weight (fig 5-6). The interaction effect of drought stress, *Azospirillum* inoculation and SA application significantly gained effective on root fresh weight. Using SA and drought stress at %100 F.C along with inoculated and un-inoculated plants to *Azospirillum* led to achieve the highest amount of root fresh weight.

Electrolyte leakage

By intensifying drought stress from %100 F.C to % 33 F.C, the rate of Electrolyte leakage has been increased and the highest rate Electrolyte leakage was achieved on %33 F.C stressful condition (table-1).The Electrolyte leakage is considered as an index for showing the extent of cellular membrane stability during tolerating drought stress, and measuring the range of damage to cellular membrane can be served for showing damage to plant cellular structure as well as its functions under drought stress. Some stresses like environmental adverse conditions can lead to emerge Reactive Oxygen Species (ROS) stress, as a result of closing stomata and reducing CO_2 into cells and consequently blocking photosynthesis activities. ROS stress damages cellular membrane due to lipid peroxidase [12]. As a consequence, the rate of ionic leakage will be increased in these cells. The amount of leakage can be served as benchmark for determining the extent of damage to plants due to drought stress. In this experiment, the rate of ionic leakage was increased as the available water around plant reduced. These findings are in agreement with the results of El-Tayeb et al [17] who found the similar upshot over effect of salt stress on barely plants.
In this experiment, inoculating plants with *Azospirillum* and using SA significantly reduced the rate of electrolyte leakage, as comparing to control plants (table-1). It is believed that their effect on the stability of cellular membrane causes the reduction of electrolyte leakage on plants under drought stress.

Photosynthesis pigments

According to the results of this experiment, the simple effect of drought stress, *Azospirillum* inoculation, and SA had a significant effect on the content of chlorophyll a, chlorophyll b and total chlorophyll. The reduction of chlorophyll is a sign of sensing environmental stresses which mainly depends on genotype of the plant [18]. In this experiment, the reduction of chlorophyll was occurred due to drought stress and drought stress at % 33 F.C resulted in gaining the lowest rate of chlorophyll a, chlorophyll b and total chlorophyll (table-1). In this respect, there are same reports over reducing chlorophyll due to exposing plants to drought stress. According to Schutz and Fangmeir [11], drought stress leads to increase the production of free radical oxygen in cells. These free radicals cause peroxidation and consequently decomposing the Photosynthesis pigments. Occurring these events, the growth of plant will be affected and reduced.
The statistical analysis displayed that inoculation plants with *Azospirillum* bring about the reduction of damage to some parts of cells like chlorophyll. The lowest destructive effect of drought stress on reduction chlorophyll content was observed in plants treated with *Azospirillum* inoculation (table-1). Inoculating crops with *Azospirillum* significantly leads to increase chlorophyll b. inoculating wheat and barley plants with *Azospirillum* caused to rise the amount of their chlorophyll content as much as %15 [20]. Regarding the role of nitrogen directly in chlorophyll structure, it seems that there is a positive and significant relationship between nitrogen and chlorophyll content in cells. Nitrogen plays a significant role in chlorophyll synthesis. In this respect, rubisco enzyme, served as catalyzer in physiological reactions, has a main effect on fixation Co_2 in plant cells so as to accomplish photosynthesis activities in plants. In general, the effect of total nitrogen on the photosynthesis processes can be regarded through its effects on rubisco enzyme and chlorophyll formation. In plants, ammonia is the initiative form of nitrogen, then it will be converted to glutamic acid by glutamine syntheses enzyme. Glutamic acid is taken into account as the basic material for synthesis amino acids, nucleic acids, and forming porphyria ring existing in chlorophyll structure [21]. The interaction effect of drought stress and *Azospirillum* inoculation on the amount of chlorophyll a, chlorophyll b and total chlorophyll was significant. During exposing plants to %100 F.C without inoculation them with *Azospirillum*, the highest amount of chlorophyll a and chlorophyll b was gained. Moreover, Exposing plants to %100 and %66 F.C without inoculating them with *Azospirillum* paved the way for gaining the highest amount of chlorophyll b (fig. 8, 9 and 10). Using SA did not have a significant effect on the amount of chlorophyll, though its interaction effect with drought stress had a significant impact on the amount of chlorophyll b and total chlorophyll content. The highest amount of chlorophyll b was gained while exposing plants to %66 F.C without applying SA (fig. 11) and the lowest

amount of total chlorophyll content was achieved while exposing plants to %33 F.C without applying SA (fig. 12). In this regard, Panchova et al [22] declared that a seven-day treatment of barely transplants with SA led to decrease the amount of chlorophyll content, whereas a two-day treatment did not have a significant effect on the amount of chlorophyll content.

The interaction effect of drought stress, *Azospirillum* inoculation, and SA application had a significant effect on chlorophyll b and total chlorophyll content. The highest amount of chlorophyll b was achieved while exposing plants to %66 F.C along with inoculating plants with *Azospirillum* and without using SA (fig-15). Facing plants to %100 F.C without *Azospirillum* inoculation and SA application led to achieve the highest amount of total chlorophyll content. SA regulates biochemical processes and can serve as plant growth regulators so as to improve plant resistance to environmental stresses, but its efficiency mainly depends on many different factors such as plant species, plant growth and development stage, its application method [23]. Therefore, the lack of appropriate impact of SA on the amount of chlorophyll content may be due to mentioned factors above.

Proline

Proline is one of the active amino acids involving in osmotic regulatory phenomenon by which the osmotic pressure in cells of plants will be maintained. Its accumulation within cells during exposing plants to stressful conditions is mounted up [24].

The rate of proline accumulation within cells increases as the level of drought stress rose in a way that the highest rate of proline production (3.12 micro moles per gram of fresh weight) was gained at %33 F.C (table-1).

The reasons of accumulation this amino acid in cells is various. Some factors such as preventing decomposing proline, preventing from converting proline to protein or increasing the decomposing of proteins have been reports as the reasons of accumulation of proline during experiencing stresses [25]. The rising proline accumulation in plants while exposing to environmental stresses is a kind of defendant mechanism by which the activities of enzymes forming hydroxyl radicals will be blocked and the capacity of plants for tolerating environmental stresses will be improved [26]. In this experiment, inoculating plants with *Azospirillum* significantly led to increase the rate of proline in comparison to the control plants (table-1). In order for establishing the equation between environmental osmosis and cellular protection, *Azospirillum* species can collect organic compound such as proline, also called osmotic pressure regulators in cells.

In this respect, all accumulated osmolytes in some strains of *Azospirillum brazilence* have been identified during exposing plants to salt stress. In species of *Azospirillum*, organic compounds like trehalose, glycine betaine, glutamate and proline have been recognized as osmolite. It has been proven that in the genus of *Azospirillum*, in resistance to salt and drought sresses, *Azospirillum amazonnes*, *Azospirillum lipofrom* and *Azospirillum brazilnes* have the most effect, respectively. This event is considered as the existence of a regulating osmotic mechanism in a result of increasing osmolite absorption from the environment and increasing osmolite biosynthesis or both of them [27].

The interaction effect of drought stress and *Azospirillum* inoculation had a significant impact on the rate of proline, and the highest rate of accumulated proline was achieved while plants inoculated with *Azospirillum* at the severe water stress (%33 F.C) (fig-13).

SA had a significant effect on the rate of proline, as compared to control plants. These findings are in agreement with those of Delavari et al [27] who investigated the effect of salt stress on basil plants. The analysis of mean comparison showed that the interaction effect of drought stress and SA application on the rate of proline under %33 F.C along with using SA led to gain the highest rate of proline (fig-14).

The accumulation of proline on plants under stressful conditions causes their resistance to such environmental conditions. Therefore, growing the rate of proline in basil plants under drought stress due to using SA and inoculating plants with *Azospirillum* represents the effectiveness of these materials so as to improve plant tolerance to drought stress.

According to the results of this research, it can be concluded that inoculating basil plants with *Azospirillum* can improve plants growth parameters and their resistance to drought stress. Although using SA also improved some growth parameters, inoculating plants with *Azospirillum* in comparison to using SA acted better from stands point of diminishing the destructive effects of drought stress and increasing the plants growth parameters. *Azospirillum* inoculation and SA application simultaneously have more effective rather than other treatments and hence, it is suggested as an appropriate treatment for improving plant growth and yield as well as alleviating the damage of stressful conditions.

Table 1. The effect of drought stress, *Azospirillum* and salicylic acid on some measured parameters of sweet basil plant

Treatments	Chlorophyll a (mg/g F.W)	Chlorophyll b (mg/g F.W)	Total chlorophyll (mg/g F.W)	Proline (µm/g FW)	Electrolyte Leakage (%)	Number of inflorescences per plant
S0	1.97 a	1.14 b	3.11 a	2.02 c	16.22 c	1.25 a
S1	1.81 ab	1.32 a	3.14 a	2.61 b	20.11 b	0.657 b
S2	1.67 b	1.05 b	2.73 b	3.12 a	22.65 a	0.127 c
B0	1.62 b	1.06 b	2.69 b	2.41 b	20.83 a	0.509 b
B1	2.01 a	1.28 a	3.29 a	2.76 a	18.49 b	0.846 a
, SA0	1.76 a	1.16 a	2.92 a	2.23 b	18.90 b	0.66 a
SA1	1.88 a	1.19 a	3.07 a	2.94 a	20.43 a	0.69 a

S0, S1 and S2: 100, 66 and 33% of field capacity moisture, respectively. B0: not inoculated, B1: inoculated with strain *Azospirillum*, SA0: salicylic acid no application, SA1: salicylic acid application. The means in each column having the same letter are not significantly different at 5% Level of probability based on Duncan test.

Continued Table 1

Treatments	Plant height (Cm)	Root fresh weight (g)	Root dry weight (g)	Shoot fresh weight (g)	Shoot dry weight (g)	Number of lateral branches per plant
S0	46.60 a	1.83 a	0.42 a	10.84 a	3.79 a	13.43 a
S1	40.78 b	0.90 b	0.23 b	8.46 b	2.23 b	11.38 b
S2	31 c	0.63 c	0.12 c	4.38 c	1.05 c	7.79 c
B0	37.50 b	0.93 b	0.21 b	7.22 b	1.67 b	10.03 b
B1	41.42 a	1.30 a	0.30 a	8.57 a	3.04 a	11.70 a
SA0	37.96 a	1.11 a	0.25 a	7.15 b	2.11 b	10.85 a
SA1	40.96 a	1.13 a	0.26 a	8.63 a	2.61 a	10.88 a

S0, S1 and S2: 100, 66 and 33% of field capacity moisture, respectively. B0: not inoculated, B1: inoculated with strain *Azospirillum*, SA0: SA no application, SA1: SA application. The means in each column having the same letter are not significantly different at 5% Level of probability based on Duncan test.

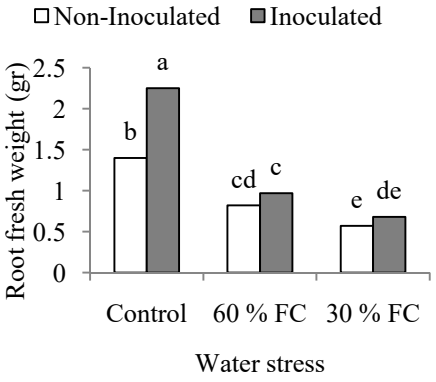

Fig.1. Effect of *Azospirillum* on root fresh weight of basil plant under drought stress condition.

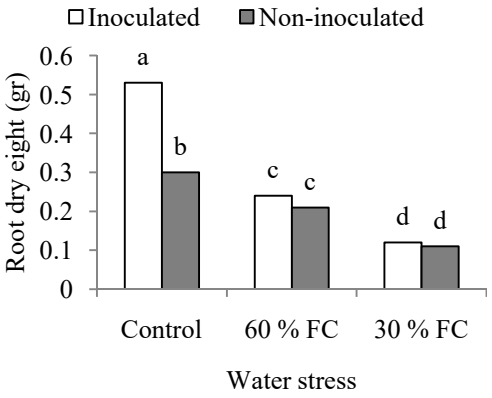

Fig.2. Effect of *Azospirillum* on root dry weight of basil plant under drought stress condition.

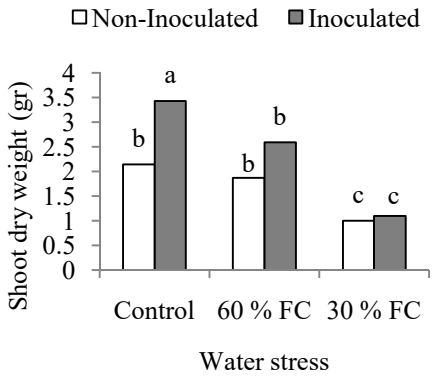

Fig.3. Effect of *Azospirillum* on shoot dry weight of basil plant under drought stress condition.

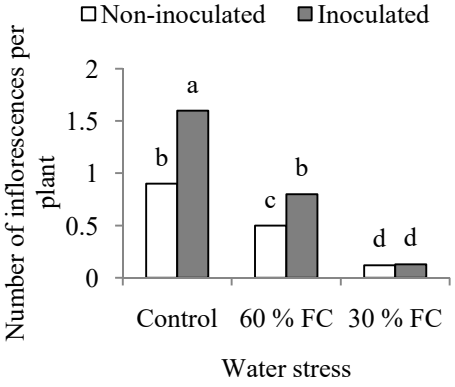

Fig.4. Effect of *Azospirillum* on number of inflorescences in basil plant under drought stress condition.

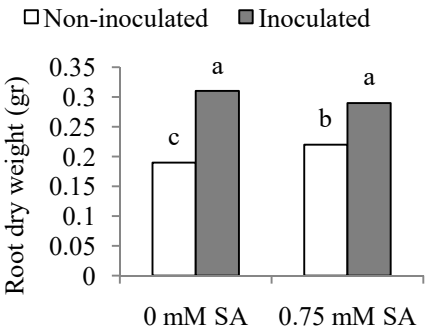

Fig.5. Effect of *Azospirillum* and SA on root fresh weight of basil plant under drought stress condition.

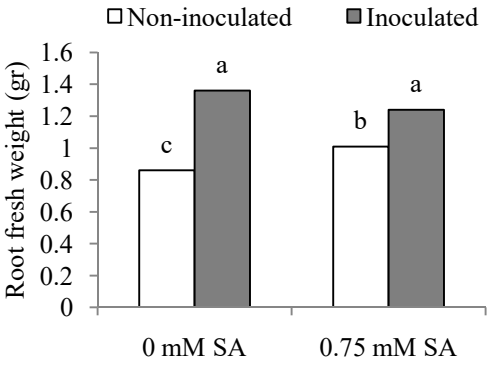

Fig.6. Effect of *Azospirillum* and SA on root dry weight of basil plant under drought stress condition.

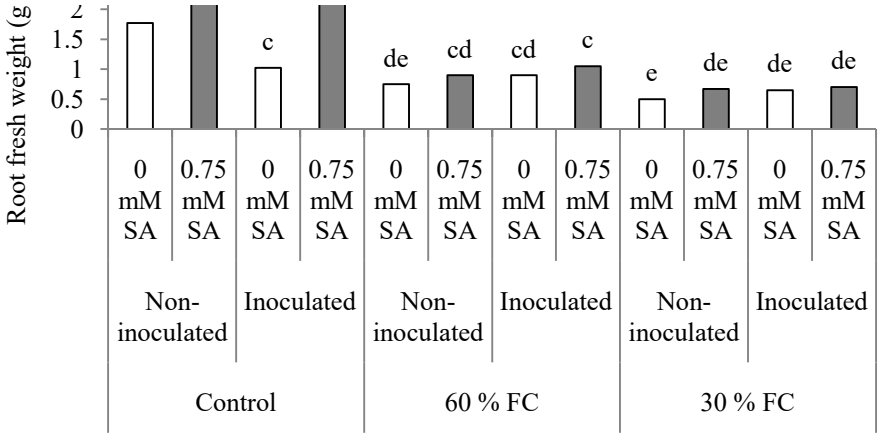

Fig.7. Effect of *Azospirillum* and SA on root fresh weight of basil plant under drought stress condition.

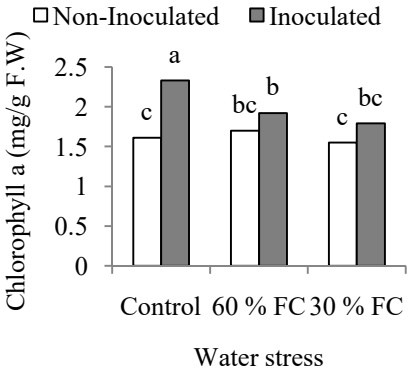

Fig.8. Effect of *Azospirillum* on chlorophyll a in basil plant under drought stress condition.

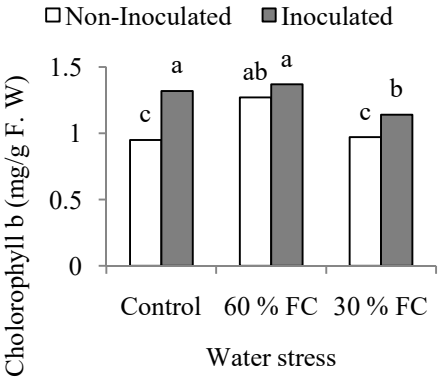

Fig.9. Effect of *Azospirillum* on chlorophyll b in basil plant under drought stress condition.

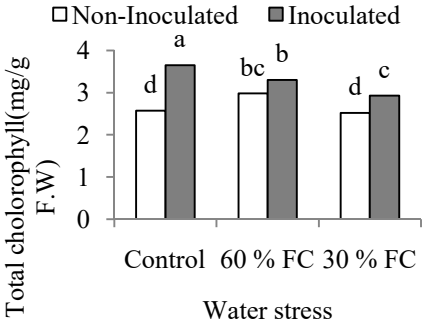

Fig.10. Effect of *Azospirillum* on total chlorophyll in basil plant under drought stress condition.

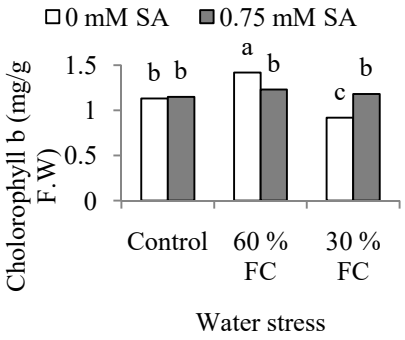

Fig.11. Effect of SA on chlorophyll b in basil plant under drought stress condition.

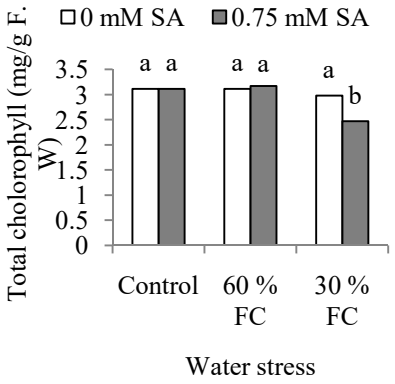

Fig.12. Effect of SA on total chlorophyll in basil plant under drought stress condition.

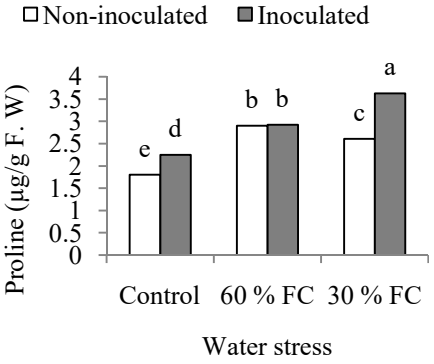

Fig.13. Effect of *Azospirillum* on proline content in basil plant under drought stress condition.

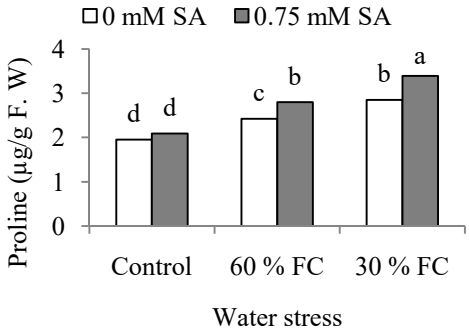

Fig.14. Effect of SA on proline content in basil plant under drought stress condition.

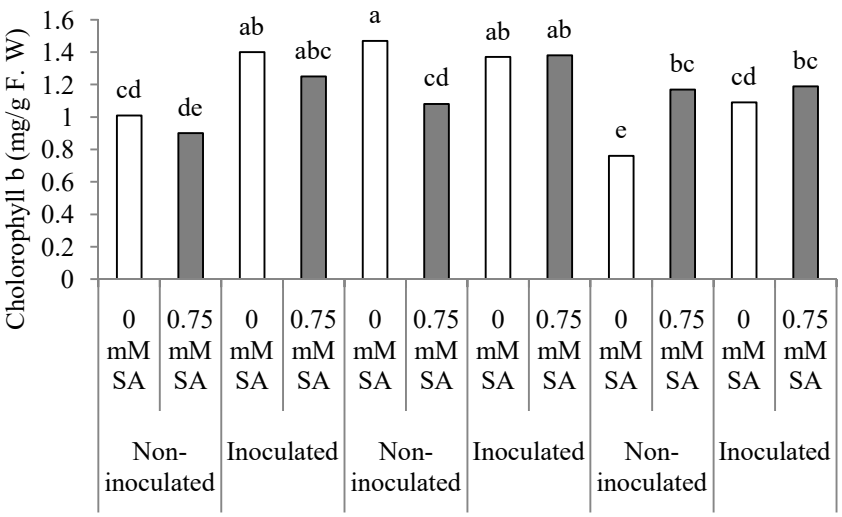

Fig.15. Effect of *Azospirillum* and *SA* on chlorophyll b in basil plant under drought stress condition.

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