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IMPROVED TOLERANCE OF *WITHANIA SOMNIFERA* L. (DUNAL) TO SALT STRESS BY THE APPLICATION OF ARBUSCULAR MYCORRHIZA (*GLOMUS FASCICULATUM)*

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Abstract: Present investigation was conducted to assess the effect of arbuscular mycorrhizal fungi (AMF) in mitigating salinity-induced variations during physiological and biochemical activities of *Withania somnifera* L. (Dunal). Allevated level of NaCl stress was effective in decreasing the total chlorophyll content, while proline accumulation increased. However, AMF-inoculated plants showed enhanced chlorophyll content with decrease in proline than non AMF plants. The presence of Mycorrhiza significantly control the reduction of chlorophyll a and b content and synthesis of proline in high amount under saline condition, thus can ameliorate the salinity effect. The amount of chlorophyll a got reduced by 27% while chlorophyll b by 23% at 100mM salt treatment than control. The level of proline also increased steadily with the simultaneous increase in salt (25 mM to 50 mM NaCl). Proline, an osmoprotector amino acid is reported to be increased in plants as a stress response, thus increasing the stress tolerance to the plants. However, proline accumulation was lower in plants with mycorrhiza (1.64 µmol/g fw) which may indicate a lower stress level in the plants as compared to non AMF plants Our results also demonstrated that inhibitory effect of NaCl on *Withania somnifera* (L.) Dunal seedlings could be convalesced by AMF association.

Keywords: AMF, *Withania somnifera* L. (Dunal), NaCl stress, osmolytes, chlorophyll, proline.

Introduction

Evolution and adaptation of living organisms on the earth is highly influenced by environmental stresses such as drought, extreme cold or heat, high wind, pH, salt, toxic compound and infection by insects or parasites. Such stresses cause an extreme reduction in cultivated land area, reproductive output and quality and when persist, the conditions may lead to permanent damage[1,2]. Salinity arises in the terrestrial land through natural process or human-induced progressions, resulting in the accumulation of dissolved salts in the soil water which may affect plant growth. Salinity in the soil is one of the major limiting factors which restrain the productivity of crop, horticulture and forage production mainly in arid and semi-

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arid regions. According to the Food and Agriculture Organization of the United Nations (FAO) Land and Plant Nutrition Management Service, more than 6% of the world's terrestrial area is affected by salinity. Jamil et al.[3] & Shrivastava & Kumar[4] reported that worldwide around 20% land is under cultivation, in which 33% of irrigated agricultural land facing problem of severe salinity. Furthermore, salinization in cultivated land is found to be increasing at a rate of 10% annually for various reasons including weathering of rocks, low precipitation, irrigation with saline water, poor cultural practices and high surface evaporation. It has been predicted that more than 50% of the cultivated land would be salinized by the year 2050.

Salts in the soil water may reduce or inhibit plant growth due to inability of the plant to take up water, and thus reductions in the growth rate[5]. This is referred to as the osmotic or water-deficit effect of salinity. If excess amount of salt enter the plant during transpiration stream, it can injure the cells in the leaves and this may further cause reductions in growth known as salt-specific or ion-excess effect of salinity[6]. Under salt stress, accumulation of Na^+ and Cl^- and reduction in K^+ and Ca^+ concentration leads to major problems like increase in osmotic stress and decrease in water potential. Muthulakshmi et al.[7] found that the damage caused by salinity affects the germination, development of plant, protein synthesis, photosynthesis, leaf chlorosis lipid metabolism and senescence etc. Furthermore, it adversely effects on germination, growth, vigor and yield of the plant, ion toxicity, nutritional disorders, oxidative stress, genotoxicity, variation in metabolic processes, membrane disorganization, reduction of cell division and expansion which ultimately reduce plant growth, development and survival[8, 9, 10 & 11].

Medicinal plants are rich in secondary metabolites like alkaloids, glycosides, steroids and flavonoids. Biosynthesis of secondary metabolites is strongly affected by salt stress resulting in considerable fluctuations in their quality and quantity[12]. Higher amounts of salty substances in soil hinder water uptake and destroy soil structure[13]. Several metabolic alterations in response to water deficit can occur in plant tissue at one of the following three levels: i) disturbance of metabolic pathways leading to an accumulation or loss of metabolites; ii) alterations in enzyme activities and iii) changes in the patterns of protein synthesis[14]. Gupta & Huang[15] identified proline as an enzyme protector involved in antioxidant defence system and serves as an organic nitrogen reserve during stress recovery.

To discourse the above aforesaid impacts on plant growth and agricultural losses, innovative methods are being investigated and introduced to overcome salinity which may include using

chemicals to leach excessive salts from soil, and the use of desalination machines to remove salts from irrigation water. However such methods are very expensive and cannot afford by the farmers of developing countries. Recent studies have recommended using Arbuscular Mycorrhizal species as a biological tool and financially cost effective method to combat soil salinity and enhance plant production[16,17]. Mycorrhizal association reduces the stress by improving the physio-biochemical features that could be primarily attributed to increase in photosynthetic efficiency, enhanced nutrient acquisition, root hydraulic conductivity and stabilization of osmotic balance under salt stress conditions.

The objective of this study is to determine the salt tolerance of Ashwagandha with and without mycorrhiza through proline accumulation and chlorophyll content.

Materials and Methods

In the present investigation, plantlets were raised from the seed material of the genotype JA 20 (Jawahar Asgandh 20), a cultivated variety of Ashwagandha developed by Jawahar Lal Nehru Krishi Vishwa Vidhalaya Mandsore, Madhya Pradesh and mycorrhizal spores used were of *Glomus mosseae*, procured from "The Energy and Resource Institute (TERI), New Delhi".

Seeds were surface sterilized by 0.2 % $HgCl_2$ solution for 5 minutes with recurrent shaking and then thoroughly washed with autoclaved deionized water. Sterilized seeds were first germinated on petri plates and were later transferred in plastic pots filled with autoclaved sand and were divided in two sets: control and inoculated with mycorrhiza.

Both the sets were established for five weeks-old seedlings before being subjected to various levels of NaCl (0, 25, 50 and 100mM) by watering with salt solution and once in two weeks with Murashige and Skoog medium[18].

Inducing saline condition in Ashwagandha plants (with and without mycorrhiza)

To establish Ashwagandha seedlings and the candidate AM fungus to high NaCl concentrations, salinity stress was imposed on the seedling by irrigating with increasing concentration of salt. Each treatment was watered first with the lowest NaCl concentration, then with the next higher concentration until each treatment reached its designed salt concentration. The acclimatization took 1.5 weeks to get to 0.25 mM treatment. The treatment was given twice a week with appropriate salt concentration. To maintain NaCl salination at the correct level, before applying each subsequent saline irrigation, all the pots were percolated with distil water (approx.700 ml/pot) to prevent salt accumulation beyond the experimental concentration (Fig.1 & 2). The experiment was terminated after 9 weeks of

salt treatment. Leaf chlorophyll content was estimated by Arnon[19] and Proline was estimated by ninhydrin method[20]. A blank was maintained with all the reactants except the leaf extract. The proline concentration (µmol/g FW) was determined from a standard curve.

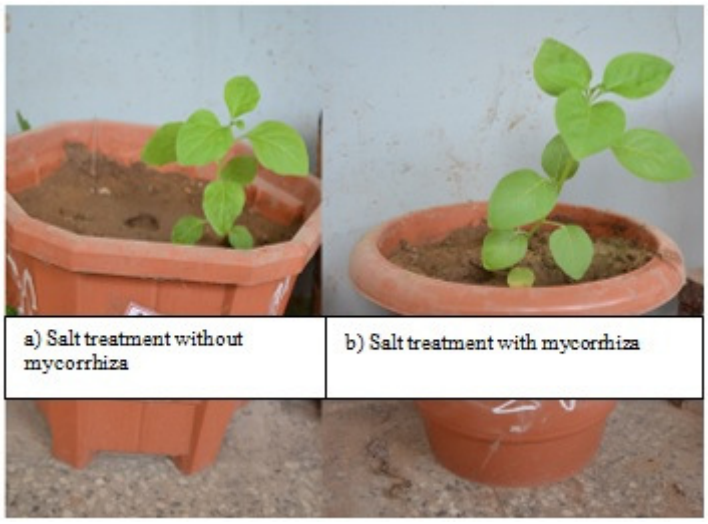

Fig. 1. Effect of salt stress and mycorrhiza on 40 days old plant

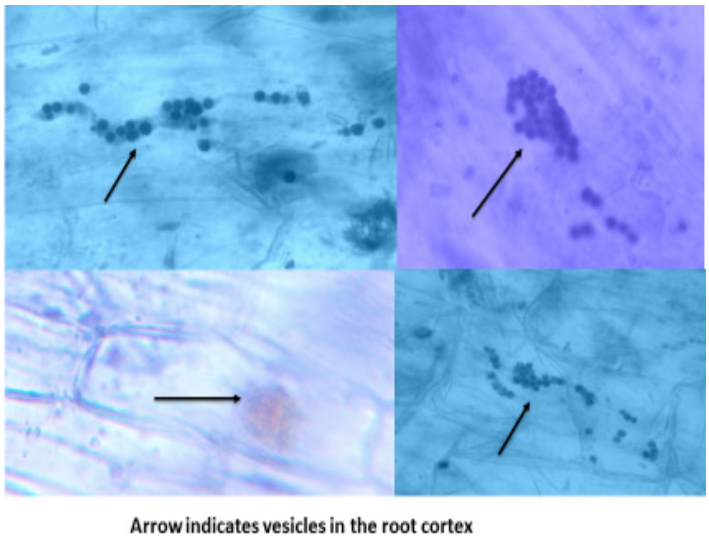

Fig.2. Trypan Blue stained roots of Ashwagandha under compound microscope (40X magnification)

Statistical analysis

Analysis of Variance (ANOVA) was used for determining the degree of variation or similarity between two or more groups of data and significance between the variables i.e. the effect of treatment on the Ashwagandha variety. All the experiment were repeated five times manipulating 10 explants in every treatments. Two-way analysis of variance (ANOVA) was

performed on Ashwagandha with the AMF and different salinity level as the two factors. A two factor CRD individual analysis was performed to determine the significant differences between the mean of each parameter tested in the study. The statistical analysis was performed by using SPSS software (Version 15.0, SPSS Inc., USA).The significance was tested at 5% level.

Results and discussion

Chlorophyll content

Salt stress induces physiological drought in plants by decreasing the osmotic potential which affects plant growth and trim down the yield. Salinity stress had strong effects on leaf chlorophyll content. It was observed that salinity induces a significant decrease in the contents of pigment fractions in both chlorophyll a and b and was found directly correlated with the concentration of salt**.** The result showed extreme and significant ($p \leq 0.05$) decrease in the content of chlorophyll a and chlorophyll b with increasing concentrations of salt. With maximum concentration of salt used (100uM) chlorophyll a got reduced to 0.134 mg/g fw from 0.514 mg/g fw and chlorophyll b reduced to 0.084 mg/g fw from 0.396 mg/g fw.

Our results of reduced chlorophyll content under salt (NaCl) stress corroborate with the findings of Zuccarin [21], Doganlar et al.[22], Rasool [23], Alqarawi et al.[24], Hassan & Ali[25] and Sarwat et al.[26] for *Solanum lycopersicon*, *Lycopersicon esculentum*, *Cicer arietinum*, *Ephedra alata* , *Simmondsia chinensis* and *Brassica juncea* respectively. This reduction can be attributed to either suppression of specific enzymes involved in the synthesis of photosynthetic pigments [27]or due to enhanced chlorophyllase activity which causes pigment degeneration[28]. In fact, salt stress opens porphyrin rings resulting in transfer of harmful matters to vacuole. Presence of these compositions demolish green colour of leaf and ultimately reduces the chlorophyll concentration in the leaf[29]. Ramakrishnan and Bhuvaneswari[30] clearly indicated the antagonistic effect of NaCl on N absorption, an essential component of the structure of chlorophyll molecule. A reduction in the uptake of minerals (e.g. Mg) needed for chlorophyll biosynthesis in presence of salt also reduces the chlorophyll concentration in the leaf [31].

However, presence of Mycorrhiza significantly controlled the reduction of chlorophyll a and b content under saline condition. It was found that chlorophyll a got increased from 0.134mg/gfw to 0.383mg/g fw while chlorophyll b got increased from 0.084 mg/g fw to 0.296 mg/g fw at 100mM salt treatment thus confirms the effect of mycorrhiza in combating the salt stress in plants (Table 1).

Present findings convincingly supports that mycorrhiza under saline condition had a recovery effect. The soil salinity drastically lowered the amount of leaf chlorophyll a and chlorophyll b in non-mycorrhizal plants but with mycorrhizal inoculation it lowered the effect of stress considerably. The progression in plant growth demonstrated by AMF-inoculated plant in our study can be attributed to improved absorption of water by plant roots by increasing its surface area. Earlier reports indicates improvement in root hydraulic conductivity, osmotic balance, uptake of phosphorus and composition of carbohydrates in AMF-colonized plants further contributing to increased water potential[32]. The increased water potential also dilutes the toxicity of the sodium ions [33]. In the presence of mycorrhiza, the antagonistic effect of Na^+ on Mg^{2+} uptake is counterbalanced and suppressed[34]. Zhu et al.[35] showed an increase in photosynthesis rate, transpiration and chlorophyll a, b content under cold stress in maize plant inoculated with *Glomus etunicatum*.

Proline content

The capacity of the plant to accumulate proline under saline conditions can be directly correlated to the concentration of salt in the irrigation water. In present study, the level of free proline in Ashwagandha plants were measured after a period of 8 days, revealed that under non-stress conditions, the level of proline was found to be low which increased steadily with the increasing concentration (25 mM to 50 mM NaCl) of salt. Proline content recorded highest (2.96 µmol/g fw) in plants with 100mM NaCl (Table 1).

In salt stressed plants, proline accumulation is a primary defense response required in adjusting osmotic pressure [36]. Elevated level of proline was reported as an adaptive response to salinity in various plants such as sugar beet[37], tobacco[38], *Brassica juncea*[26]. Stewart[39] reported that an increase in proline could be due to the induction of proline biosynthesis enzymes and/or to the reduction of oxidation to glutamate. Proline has a key role in scavenging Reactive Oxygen Species[40], osmotic adjustment[41] and facilitating water uptake[42]. The accumulation of proline in the plants and its role in osmotic adjustment is well established. The proline accumulation helps in maintenance of cell metabolism with decreasing water status, thus enabling plant survival under extremely severe conditions.

However with mycorrhizal inoculation the stress level of plant reduced by a decrease in proline content (1.64 µmol/g fw) as compared to plants without mycorrhiza (Table 1). Our results are in agreement with the earlier studies for AM plants under salt stress [43]. Alqarawi[24] observed an increase in proline, phenol, and lipid peroxidation in *Ephedra phylla* with

increasing concentration of NaCl, but it could be lowered in presence of mycorrhizal association.

Bañuelos et al.[44] discussed in his findings that the reduction in proline content in mycorrhizal plants, even in the presence of the pathogen (nematodes), may be associated with qualitative and quantitative influence on flavonoid content and metabolism in presence of mycorrhiza, thereby reducing proline synthesis. Plants inoculated with the AMF revealed a lower concentration of proline, which may specify a lower stress level in the plant under normal conditions, as observed by Hare and Cress[45]. Reduced stress condition may be related to an enhanced nutritional status as discussed by Cantrell and Linderman[46].

From the present findings, thus it is concluded that mycorrhizal colonization in Ashwagandha is showing a significant increase in chlorophyll content and reduction in free proline content under salt stress which gives a clear indication that these plants were less stressed, as compared to plants without mycorrhiza.

Table 1: Analysis of variance for chlorophyll a & b and proline content without and with mycorrhiza in Ashwagandha

	Chlorophyll a		Chlorophyll b		Proline	
	NM	AM	NM	AM	NM	AM
0 mM salt	0.51	0.52	0.39	0.40	1.13	1.13
25 mM salt	0.49	0.51	0.35	0.39	1.69	1.39
50 mM salt	0.37	0.41	0.24	0.31	2.08	1.56
100 mM salt	0.13	0.38	0.08	0.29	2.96	1.64
D F	1		1		1	
C V%	8.03		9.23		5.47	
SE	0.08	0.11	0.006	0.009	0.021	0.029
Signifinace test	*	*	*	*	*	*

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