Arbuscular mycorrhizal fungi alleviate salt stress in lupine (*Lupinus termis* Forsik) through modulation of antioxidant defense systems and physiological traits

Abeer Hashem1,2, Elsayed Fathi Abd_Allah3, Abdilaziz A. Alqarawi, Stephan Wirth1 and Dilfuza Egamberdieva1

Department of Plant Production, Faculty of Food & Agricultural Sciences, P.O. Box. 2460 Riyadh 11451, Saudi Arabia.  
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**ABSTRACT**

The present study was carried with the aim to demonstrate and examine the impact of arbuscular mycorrhizal fungi (AMF) on the growth, anti-oxidants metabolism and some key physio-biochemical attributes including the osmotic constituents in *Lupinus termis* exposed to salt stress. Salt stress (250 mM NaCl) reduced growth, AMF colonisation, relative water content and chlorophyll pigment content. However, AMF ameliorated the negative effect of salinity on these growth parameters. Salt stress increased the activities of key antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD). Inoculation of AMF enhanced the activities of these enzymes and caused an increase in the accumulation of osmotic components resulting in the maintainence of tissue water content. Proline, glycine betaine and sugars increased with salinity stress and AMF inoculation. Plants subjected to salt stress showed considerable variations in the endogenous levels of growth hormones. Reduced lipid peroxidation and increased membrane stability in AMF inoculated plants and enhanced activity of anti-oxidants enzymes confers the role of AMF in assuaging the salt stress induced deleterious effects.

**Key words:** AMF, Antioxidants, Lupine, Osmolytes, Phenol, Plant growth hormones, Salinity.

**INTRODUCTION**

Salinity is one of the important problems that cause considerable reductions in normal plant metabolism (Hameed et al., 2014; Iqbal et al., 2015). It has been estimated that 7% of the global land is having higher salt concentrations resulting in perturbations of plant growth and development (Ruiz-Lozano et al., 2012; Ahanger et al., 2014). High salt concentrations result in osmotic and ionic effects causing alterations in important physio-biochemical processes including photosynthesis, ion homeostasis, nitrogen and antioxidant metabolism (Abd_Allah et al., 2015a,b). Salinity induced alteration in phosynthetic attributes results in perturbed carbon assimilation and hence restricts supply of photoassimilates for plant development (Hameed et al., 2014; Iqbal et al., 2015).

Altered photosynthetic ability due to salt stress causes increased production of toxic free radicals or reactive oxygen species (ROS) by transferring electrons from highly energetic molecules to molecular oxygen (Ahanger et al., 2014; Wu et al., 2014). Oxidative damage to important cellular macromolecules like proteins, nucleic acids result in hindered metabolism and growth. Exposure to high salt concentrations triggers production of toxic reactive oxygen species (ROS) ultimately resulting in oxidative stress (Wu et al., 2014; Hashem et al., 2015; Iqbal et al., 2015). Excessive ROS accumulation in tissues like leaves triggers rapid oxidation of several important cellular molecules including lipids, proteins and chlorophylls. Oxidation of lipids and photosynthetic pigments results in enhanced membrane leakage and reduced photosynthesis respectively (Wu et al., 2014; Hashem et al., 2015). In order to ameliorate the damaging effects of ROS, plants have developed several defense mechanisms. These mechanisms include the antioxidant system, accumulation of compatible osmolytes and upregulation as well as down regulation of growth hormones so as to mediate proper signalling and elicitation of responses during stress (Hashem et al., 2014; Abd_Allah et al., 2015b; Khan et al., 2015). Antioxidants can be categorized into two types: enzymatic and non-enzymatic forms in the intracellular and extracellular environment that mediate scavenging of toxic radicals hence has an important role in cellular protection. In addition to this accumulation of compatible organic osmolytes including proline, glycine betaine and soluble sugars also contribute to enhanced growth under stressful conditions by favoring the maintainence of cell turgor (Wu et al., 2014; Ahanger et al., 2014). These intricate adaptive mechanisms help plants to avoid stress-induced negative changes in metabolism.

*Corresponding author’s e-mail: eabdallah@ksu.edu.sa. 1Botany and Microbiology Department, Faculty of Science, King Saud University, P.O. Box. 2460 Riyadh 11451, Saudi Arabia. 2Mycology and Plant Disease Survey Department, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt. 3Institute für Landscape Biogeochemistry, Leibniz Centre for Agricultural Landscape Research, 15374 Müncheberg, Germany.
Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with several plant species. AMF contributes to growth improvement of the host plant by enhancing the characteristics of rhizospheric soil as well as modifying and protecting the root architecture of the host plant (Ahanger et al., 2014; Abd_Allah et al., 2015a,b). Though its beneficiary effects on the host plant are well documented, it has been reported to enhance the stress tolerance of plants by protecting key physiological processes by mediating a quick scavenging of toxic ROS through upregulation of antioxidant enzymes. In *Helianthus annus*, AMF inoculation mitigated cadmium induced negative changes in growth by enhancing the mineral nutrition and antioxidant potential (Abd_Allah et al., 2015a). AMF improve growth of host plants by its active involvement in some basic processes like photosynthesis and mineral nutrition.

*Lupinus termis* is an important traditional legumes cultivated mostly in the mediterranean regions and falls within the family Fabaceae, a pubescent plant that normally attains a height of 30-120 cm and is widely cultivated in several mediterranean regions in addition to its occurrence in meadows, slopes and pastures. The present investigation was aimed to evaluate the impact of salt stress on growth, anti-oxidants, hormone and osmolytes accumulation in *Lupinus termis* and to assess the ameliorative role of AMF in mitigating the damaging effects of salt stress.

**MATERIALS AND METHODS**

**Seeds, soil and fungal inoculum:** Seeds of white lupine (*Lupinus termis* Forsik) cultivar Giza-1 were obtained from Agricultural Research Center, Giza, Egypt. Healthy seeds were surface sterilized using 0.05% NaOCl for 5 min, followed by thorough washing with distilled water. The seeds were incubated on blotter paper in petri dishes (five seeds/9 cm petri dish) in growth chamber at 25°C with a light/dark photoperiod of 16/8 hrs and light intensity of 1500 mol m⁻² S⁻¹. Germinated seedlings were transferred in plastic pots and were maintained in the growth chamber of the Plant Production Department, Faculty of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia. Salt stress was induced by supplementing Hoagland’s solution with NaCl to get concentration of 200 mM salinity. Pots receiving full strength Hoagland’s solution served as control. Pots were irrigated after every three days. The pots were maintained in the growth chamber of the Plant Production Department for each treatment and supplied with normal Hoagland’s nutrient solution. At the end of pot experiment, the plants were removed from the pots very carefully and washed with distilled water and fresh weight was taken thereafter by drying in oven at 105°C for 48hrs. Fresh leaves were used for estimation of photosynthetic pigments, growth regulators, antioxidant enzymes and acquired resistance attributes, however, sugars and elements accumulation were analysed in dried samples.

**Determination of arbuscular mycorrhizal colonization:** Mycorrhizal spores from the experimental soil of individual treatment were extracted by wet sieving and decanting method as described by Daniels and Skipper (1982) and modified by Utobo et al. (2011). Total population of mycorrhizal spores was calculated per hundred gram soil. The root system was carefully washed in ice-cold water (4°C) and subsequently cleaned using 10% KOH followed by staining with trypsin blue in lactophenol. Thereafter, stained root segments were examined under a light microscope at 400x magnification and the intensity of fungal infection (mycelium, vesicles and arbuscules) and its development within the infected roots were calculated.

**Determination of photosynthetic pigments:** Photosynthetic pigments were extracted from leaf samples in 80% acetone as described by Arnon (1949). The optical densities of the supernatant were recorded at 480, 645 and 663 nm against a blank of acetone (80%).

**Estimation of lipid peroxidation (malondialdehyde, MDA):** 0.1 g leaves were homogenized with 1 mL of 10% trichloroacetic acid and centrifuged at 1000 g for 10 minutes. To 1 ml supernatant, 20% trichloroacetic acid containing 0.25% thiobarbituric acid was added and the mixture was heated at 95°C for 30 min, quickly cooled in an ice bath and then centrifuged again at 10,000 ×g for 10 min in a refrigerated centrifuge at 4°C. The absorbance of the supernatant was read at 532 and 600 nm (Heath and Packer, 1968). Calculations were done using an extinction coefficient of 155 mM cm⁻¹.

**Estimation of hydrogen peroxide (H₂O₂) content:** H₂O₂ content of leaf samples was calorimetrically measured as described by Mukherjee and Choudhuri (1983). Leaf samples were extracted with cold acetone. 200 µL aliquot was mixed with 0.04 mL of 0.1% TiO₂ and 0.2 mL NH₄OH (20%). The pellet was decolored with acetone and resuspended in 0.8 mL H₂SO₄. The mixture was then centrifuged at 6,000 ×g for 15 min and the supernatant was read at 415 nm.

**Membrane stability index:** The membrane stability index (MSI) was determined according to the method described by Abd_Allah et al. 2015a. 100 mg of fresh leaf samples...
were taken in test tubes in two sets and 10 ml of double distilled water was added to each. One set was kept in a water bath for half an hour at 40°C and the electric conductivity was recorded \((C_1)\), while the other set was at boiling temperature (100°C) and its EC was also recorded \((C_2)\). Calculation of MSI (%) was done by the following formula: 
\[
\text{(MSI)} = \left[1 - \left(\frac{C_1}{C_2}\right)\right] \times 100
\]

Relative water content: For determination of RWC, samples of leaf discs were taken from each treatment and the fresh weight was determined. Subsequently, the same leaf disc samples were kept in distilled water for 4 hrs and turgid weight was recorded. The leaf samples were then oven dried at 85°C for dry weight (Smart and Bingham, 1974). Calculation of RWC (%) was done by the following formula:
\[
\text{(RWC)} = \left[\frac{\text{(turgid weight – dry weight)} \times 100}{\text{(fresh weight – dry weight)}}\right]
\]

Estimation of total phenolics: Total phenolics were extracted with 80% (v/v) acetone and estimated using Folin and Ciocalteau’s phenol reagent following Slinkard and Singleton (1977). Optical density of the mixture was read at 750 nm. Computation was done from a standard curve of pyrogallol.

Glycine betaine (GB) content: Dry powdered leaf material (0.5 g) was shaken in 10 mL toluene (0.5%) and was left over-night at 4°C. After centrifugation, one mL of the filtrate was added to 1.0 mL of sulfuric acid (2N) and from this 0.5 mL was taken in a test tube and potassium tri-iodide (200 \(\mu\)L) solution was added. The contents were cooled in a chiller. Thereafter, 2.8 mL of ice cooled de-ionized H\(_2\)O and 5 mL of 1-2 di-chloroethane were added and the absorbance of the organic layer (lower layer) was recorded at 365 nm spectrophotometrically (Grieve and Grattan, 1983). Concentrations of GB were calculated from a standard curve of GB.

Estimation of proline: Free proline was estimated following the method of Bates et al., (1973). 0.5 gm leaf was extracted in sulfosalicylic acid (3.0%) followed by centrifugation at 3000g for 30 minutes. 2.0 ml supernatant was mixed with equal volume of acid ninhydrin solution [1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml of 6 M phosphoric acid] and glacial acetic acid. The samples were then incubated at 100°C for 10 min and reaction was terminated by keeping the tubes in an ice container. After cooling, proline was separated with 4 ml toluene and optical density was measured at 520 nm.

Extraction and estimation of carbohydrate fractions: The extraction of soluble carbohydrate fractions was carried out according to the method of Said et al., (1964). In this method, known weights of powdered leaf samples were homogenized in a mixture of 5.0 mL of 20% (w/v) phenol and 10.0 mL of 30% (w/v) trichloroacetic acid (TCA) and the homogenate was left overnight in a refrigerator at 4°C followed by centrifugation at 5000g for 15 min. The supernatant was used for estimation of water-soluble carbohydrates (monosaccharides and disaccharides). The pellet left after the water-soluble carbohydrate extraction was oven dried (at 80°C for two successive constant weights) and used for estimation of polysaccharides. The content of soluble sugars (monosaccharides and disaccharides) and insoluble sugars (polysaccharides) was estimated in accordance with the method of Nelson (1944) as modified by Naguib (1964).

Extraction and quantification of plant growth regulators: Plant growth regulators were extracted in 80% aqueous acetone (4:1, v/v) supplemented with 10 mg/l butylated hydroxytoluene and purified using EtOAc and NaHCO\(_3\), as described by Kusaba et al., (1998). Quantitative estimation of indole acetic acid (IAA) and indole butyric acid (IBA) was carried by subjecting the purified extract residue to HPLC on a column of PEGASIL ODS (6 mm i.d.×150 mm, Senshu Kagaku, Tokyo, Japan) according to the method of Kelen et al. (2004). Standard curves of IBA ranging from 10 to 200 ng/ml were used as references for quantification. Concentration of SA was determined with an HPLC system equipped with a fluorescence detector (Senshu Kagaku, Tokyo, Japan) following the method of Siegrist et al. (2000) and Metwally et al., (2003).

Extraction and determination of anti-oxidant enzymes: The antioxidant enzymes were extracted by homogenizing fresh leaf tissue (250 mg) in 50 mM potassium phosphate buffer (pH7.8) containing 1 mM EDTA, 1 mM dithiothreitol, and 2% (w/v) polyvinyl pyrrolidone (PVP) as described by Malik and Singh (1980). Extraction buffer for ascorbate peroxidase (APX) was supplemented with 2.0 mM ascorbate in addition to other ingredients. The homogenate was centrifuged at 15,000 g at 4°C for 30 min and the supernatant was used as enzyme source. Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Van-Rossum et al., (1997) monitoring the photoreduction of nitroblue-tetrazolium at 560 nm. The reaction mixture contained 500 \(\mu\)L phosphate buffer (pH7.8), 0.5 mL distilled H\(_2\)O, 100 \(\mu\)L methionine, 50 \(\mu\)L NBT and 50 \(\mu\)L enzyme extract. One unit of SOD was defined as the amount of protein causing a 50% decrease of the SOD-inhibitable NBT reduction and activity was expressed as Unit mg\(^{-1}\) protein. Catalase (CAT, EC 1.11.1.6) activity determined by observing decrease in absorbance at 240 nm for 2 min and activity was expressed as EU mg\(^{-1}\) protein. The reaction mixture contained 1.9 mL phosphate buffer (50 mM; pH7.0) and 1 mL H\(_2\)O\(_2\) (5.9 mM) and the reaction was initiated by adding 100 \(\mu\)L of the enzyme extract (Luck, 1974). The method of Chance and Maehly (1955) was used for the determination of peroxidase (POD; EC 1.11.1.7) activity, the reaction mixture contained 50 mM potassium phosphate buffer (pH5.0), 100 \(\mu\)L guaiacol (20 mM), 100 \(\mu\)L H\(_2\)O\(_2\) (40 mM) and 100 \(\mu\)L enzyme extract in a final volume of 1 mL.
The increase in OD at 470 nm was monitored for 2 min and activity was expressed as EU mg⁻¹ protein. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined following decrease in absorbance at 265 nm and activity was expressed as EU mg⁻¹ protein (Nakano and Asada, 1981).

**Determination of protein:** Protein content was estimated according to the method of Bradford (1976) and absorbance was recorded spectrophotometrically at 595 nm (Beckman 640 D, USA) using bovine serum albumin as a standard (10-100 µg ml⁻¹).

**Estimation of ion accumulation:** Na⁺, K⁺ and Ca²⁺ were estimated using an atomic absorption spectrophotometer (Analyst 300, Perkin-Elmer, Germany) following Wolf (1982). Powdered shoot (0.1 g) was digested in a H₂SO₄/HNO₃ mixture (1/5, v/v) for 24 h, followed by treatment with a HNO₃/HClO₄ mixture (5/1, v/v). Calculation was done from the standard curve (10-100 µg/ml) of each mineral. A chloride analyzer (Model 926, Sherwood Scientific Ltd., Cambridge, UK) was used to determine Cl⁻ concentration in the extracts.

**Statistical analysis:** Two-way analysis (ANOVA) was used for statistical analysis followed by Duncan’s Multiple Range Test (DMRT). The values obtained were the mean ±SE for five replicates in each group. P-value at 0.05 was considered as significant.

### Table 1: Effect of salinity stress (250 mM NaCl) on structural colonization of arbuscular mycorrhizal fungi (AMF) in roots of *Lupinus termis*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mycelium</th>
<th>Vesicles</th>
<th>Arbuscules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>73.33</td>
<td>27.34</td>
<td>58.00</td>
</tr>
<tr>
<td>Salt stress (250mM)</td>
<td>17.67</td>
<td>24.62</td>
<td>39.30</td>
</tr>
<tr>
<td>LSD at 0.05</td>
<td>11.522</td>
<td>13.088</td>
<td>11.144</td>
</tr>
</tbody>
</table>

### Table 2: Effect of salinity stress (250 mM NaCl) on photosynthetic pigments (mg/g fresh wt) in presence and absence of arbuscular mycorrhizal fungi (AMF) in *Lupinus termis*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chlorophyll- a</th>
<th>Chlorophyll- b</th>
<th>a/b</th>
<th>a + b</th>
<th>Carotenoids</th>
<th>Total pigments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.46</td>
<td>0.816</td>
<td>1.790</td>
<td>2.27</td>
<td>0.28</td>
<td>2.55</td>
</tr>
<tr>
<td>Salt stress (250mM)</td>
<td>0.473</td>
<td>0.306</td>
<td>1.553</td>
<td>0.78</td>
<td>0.07</td>
<td>0.85</td>
</tr>
<tr>
<td>Salt stress + AMF</td>
<td>0.966</td>
<td>0.561</td>
<td>1.671</td>
<td>1.52</td>
<td>0.17</td>
<td>1.70</td>
</tr>
<tr>
<td>AMF</td>
<td>1.723</td>
<td>1.016</td>
<td>1.711</td>
<td>2.74</td>
<td>0.53</td>
<td>3.27</td>
</tr>
<tr>
<td>LSD at 0.05</td>
<td>0.081</td>
<td>0.1018</td>
<td>0.2201</td>
<td>0.1541</td>
<td>0.0417</td>
<td>0.1661</td>
</tr>
</tbody>
</table>

### Table 3: Effect of salinity stress (250 mM NaCl) on hydrogen peroxide (H₂O₂, µmole/g fresh wt), relative water content (RWC, %); membrane stability index (MSI, %) malondialdehyde content (MDA, µg/g fresh wt) in presence and absence of arbuscular mycorrhizal fungi (AMF) in *Lupinus termis*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H₂O₂ (µmole/g fresh wt)</th>
<th>MSI (%)</th>
<th>MDA (µg/g fresh wt)</th>
<th>RWC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.686</td>
<td>87.90</td>
<td>6.43</td>
<td>81.48</td>
</tr>
<tr>
<td>Salt stress (250mM)</td>
<td>1.473</td>
<td>41.07</td>
<td>10.77</td>
<td>45.52</td>
</tr>
<tr>
<td>Salt stress + AMF</td>
<td>1.030</td>
<td>68.14</td>
<td>7.80</td>
<td>69.98</td>
</tr>
<tr>
<td>AMF</td>
<td>0.560</td>
<td>93.91</td>
<td>5.00</td>
<td>86.47</td>
</tr>
<tr>
<td>LSD at 0.05</td>
<td>0.0814</td>
<td>0.5784</td>
<td>0.3074</td>
<td>1.91</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

**AMF colonization:** Salinity stress drastically affected the spore production and total root colonization (Table 1). Compared to their respective AMF counterparts, salt stress reduced mycelium, and number of vesicles and arbuscules by 75.9%, 9.7% and 32%, respectively.

**Pigments system:** *Lupinus termis* subjected to salinity treatment showed reduced chlorophyll a, chlorophyll b, carotenoid and total pigment contents. Percent decrease for chlorophyll a, chlorophyll b, carotenoid and total pigment content was 69.02%, 62.5%, 75% and 66.66%, respectively (Table 2). However, AMF inoculated plants showed an increase in chlorophyll a, chlorophyll b, carotenoid and total pigment content by 18.01%, 24.5%, 89.28% and 28.23%, respectively. Deleterious effects of salinity were ameliorated by AMF where the reduction as compared to the control was only 31.25%, 39.28% and 33.33%, respectively (Table 2).

**Leaf water content:** Salt stress declined relative leaf water content (RWC) by 44.13%, while AMF inoculated salt stressed plants showed only 14.11% reduction in RWC (Table 3). However, AMF alone caused an increase of 6.12% in RWC (Table 3).

**Hydrogen peroxide, membrane stability index and malonaldehyde (MDA):** Results for hydrogen peroxide, membrane stability index (MSI) and malonaldehyde (MDA)
are depicted in Table 3. Salinity stress caused a reduction in MSI by 53.27% while inoculation of AMF enhanced MSI by 6.83%. AMF inoculated salt stressed (250mM NaCl +AMF) plants showed only 14.11% reduction in MSI (Table 3). Hydrogen peroxide was increased by 114.7% and MDA content by 67.49% in salt stressed plants (Table 3).

Proline, glycine betaine and phenols: Salt stressed plants showed an increase in proline and glycine betaine accumulation as compared to the control. Relative to the control, salt stress increased proline and glycine betaine by 62.13% and 84.30%, respectively. AMF inoculated plants showed 17.15% and 34.14% increase in proline and glycine betaine (Table 4). AMF inoculated plants at salinity stress (200 mM +AMF) showed an increment of 12.2% and 34.14% in proline and glycine betaine respectively. Salt stressed *Lupinus termis* plants showed 134.95% increase in total phenol content and inoculation of AMF caused an increase of 246.27% (Table 4). Inoculation of AMF to salinity stressed plants further increased phenol content by 58.25%.

Anti-oxidant enzymes: Activity of antioxidant enzymes studied are depicted in Figure 1a-d. CAT, POD and SOD showed an increase of 353.15%, 62.17% and 0.63%, respectively, but APX was found to decrease by 2.24% in salt stressed plants (Fig. 1A-D). In salt stressed and AMF inoculated (250mM +AMF) plants the percent increase in activity of CAT and POD was 190.62% and 41.99%, respectively (Figure 1a, b). AMF inoculation alone increased CAT and POD by 73.66% and 23.24%, respectively, however a decrease of 30.66% and 22.51% was observed in APX and SOD, respectively (Fig. 1A-D).

Growth hormones: Salt stress increased salicylic acid by 199.1% while decreased indole acetic acid (IAA) and indole butyric acid (IBA) by 95.40% and 29.79%, respectively (Fig. 2A-C). AMF alone caused an increase of 19.17%, 5.59% and 18.98% in the endogenous levels of salicylic acid, acetic acid and indole butyric acid, respectively (Fig. 2A-A).

Soluble sugars: Salt stressed plants showed increased accumulation of sugars, however AMF alone reduced the accumulation of sugars in *Lupinus termis* (Table 5). Relative to the control, in salt stressed plants total monosaccharides and disaccharides increased by 44.66% and 44.09%, respectively. Individually, in salt stress plants glucose, fructose and sucrose increased by 45.08%, 43.96% and 44.09% respectively while in salt stressed and AMF inoculated plants (250mM + AMF) an increase of 26.20%, 19.37% and 21.05% for glucose, fructose and sucrose was observed (Table 5). AMF alone reduced total monosaccharides and disaccharides by 13.58% and 4.59%, respectively.

**Table 4: Influence of salinity stress (250 mM NaCl) on proline content (mg/ g dry wt), protein (mg/g fresh wt), glycine betaine (GB; µg/ g fresh weight) and total phenol (mg /g dry weight) in presence and absence of arbuscular mycorrhizal fungi (AMF) in *Lupinus termis*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proline</th>
<th>Protein</th>
<th>Glycine betaine</th>
<th>Total Phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.96</td>
<td>1.630</td>
<td>60.07</td>
<td>3.09</td>
</tr>
<tr>
<td>Salt stress (250mM)</td>
<td>79.38</td>
<td>0.676</td>
<td>110.71</td>
<td>7.26</td>
</tr>
<tr>
<td>Salt stress + AMF</td>
<td>54.97</td>
<td>1.206</td>
<td>80.58</td>
<td>10.07</td>
</tr>
<tr>
<td>AMF</td>
<td>57.36</td>
<td>2.043</td>
<td>64.93</td>
<td>4.89</td>
</tr>
<tr>
<td>LSD at 0.05:</td>
<td>10.483</td>
<td>0.092</td>
<td>2.2892</td>
<td>0.2736</td>
</tr>
</tbody>
</table>

![Fig 1: A-C: Effect of salinity stress (250 mM NaCl) on plant growth hormones. A, salicylic acid; B, indole acetic acid, and C, indole butyric acid in presence and absence of mycorrhizal fungi in *Lupinus termis*.](image-url)
In this study, an increased salinity causes a serious decline in the resource supply required for the optimal survival of the inhabiting AMF. Our observation of reduced colonization in salinity stressed *Lupinus termis* plants corroborate with the findings of Aroca *et al.*, (2013) for lettuce and Hashem *et al.*, (2014) for *Vicia faba*. Recently, Abd_Allah *et al.*, (2015b) observed drastic decline in the colonization potential and growth of AMF in salt stressed *Sesbania sesban*.

Salt stress caused decline in the synthesis of photosynthetic pigments resulting in hampered photosynthetic
potential. Reduced photosynthesis in salt stressed plants results in down regulation of photoassimilate supply to growing tissue. However, AMF not only enhanced the synthesis of chlorophyll pigments but also mitigated the salinity induced decline to considerable extent. Our results of reduced chlorophyll synthesis due to salt stress are in corroborations with the findings of Rasool et al., (2013) and Alqarawi et al., (2014) for Cicer arietinum and Ephedra alata respectively. Enhanced chlorophyllase activity causes degradation of photosynthetic pigments and associated proteins of photosynthetic pigment complex (Ahanger et al., 2014; Abd_Allah et al., 2015a). AMF inoculation causes an increase in the uptake of minerals like magnesium resulting in maintained chlorophyll biosynthesis. Our observation of positive effects of AMF on the chlorophyll synthesis as well as the mitigation of salt induced deleterious changes support the findings of Vicia faba (Hashem et al., 2014); Ephedra aphylla (Alqarawi et al., 2014).

Lupinus termis plants subjected to salinity showed reduced membrane stability index due to enhanced production of radicals like hydrogen peroxide causing peroxidation of membrane lipids. Earlier Alqarawi et al., (2014) have also observed increased production of malondialdehyde (MDA), a product of lipid peroxidation, in salt stressed Ephedra alata plants. Salt stress causes increase in peroxidation of lipids resulting in decrease in the ratio of polyunsaturated fatty acids. Reduced presence of unsaturated fatty acids in membrane lipids causes hindrances in the membrane integrity and hence affects their biological activity (Alqarawi et al., 2014). This enhanced peroxidation and damage to membrane lipids results in leakage of cellular components and hence disturbing the cellular homeostasis. An obvious reduction in the production of H$_2$O$_2$ was observed in AMF inoculated plants and it also reduced the adverse effects of increased salinity. Reduced MDA production in AMF inoculated plants may be ascribed to increased activities of antioxidant enzymes resulting in quick scavenging of free radicals and hence averting the oxidative damage (Estrada et al., 2013; Alqarawi et al., 2014).

Results relating to the impact of salinity on the total phenols content show an increase due to salt stress and AMF inoculation caused further increase in the accumulation of phenols. Our results of increased synthesis of phenols due to salt stress are in agreement with the findings of Lim et al., (2012) for Fagopyrum esculentum and Baatour et al., (2012) for Origanum majorana. Phenols including flavonoids, tannins are accepted to perform the radical scavenging functioning to protect cells from the oxidative stress induced damage (Hameed et al., 2014; Wu et al., 2014; Abd_Allah et al., 2015a). Phenols mediate growth maintainance under normal and stress conditions by preventing the interactions of free radicals with membrane fatty acids thereby impart stability to membranes (Khattab, 2007). Plants maintaining higher contents of polyphenols are able to grow well as compared to the less accumulating ones (Alqarawi et al., 2014; Hashem et al., 2015).

Increased accumulation of organic osmolytes contributes to enhance the stress tolerance of plants (Ahanger et al., 2014). Lupinus termis plants subjected to salinity showed increased accumulation of osmolytes like proline and glycine betaine. Increased accumulation of osmolytes directly affects the water balance of plants by maintaining the solute potential and thereby causing direct positive effect on the photosynthetic efficiency. Under salt stress, increased accumulation of glycine betaine has been reported by Moghaieb et al., (2004) and Khan et al., (2014). AMF significantly enhanced the accumulation of proline and glycine which strongly supports its role in protecting from salt stress induced cellular damage to plants. In addition to this increased accumulation of soluble sugars including monosaccharides and disaccharides was also evident in salt stressed plants and the accumulation was also enhanced by AMF inoculation. In Thymus vulgaris, Hoseini (2010) have observed that increased accumulation of carbohydrates and proline impart tolerance to increasing salt concentrations and hence protect metabolism under such conditions. Furthermore, the accumulation of carbohydrates during stressed conditions enhances stress tolerance by maintaining the membrane structure and reducing the chances of ROS formation (Masood et al., 2013; Ahanger et al., 2015).

In order to avoid the ROS induced oxidative damage to sensitive macromolecules like proteins, plants up-regulate the activities of antioxidant enzymes mediating quick scavenging of the toxic ROS. Higher activities of antioxidants maintain the levels of ROS within non toxic levels (Wu et al., 2014; Hashem et al., 2015; Ahanger et al., 2015). In our present study, activities of antioxidant enzymes CAT, POD, APX and SOD increased when plants were subjected to salinity and our results are in confirmation with the results of several studies like Rasool et al., (2013) for Cicer arietinum L; Hashem et al., (2014) for Vicia faba and Abd_Allah et al. (2015b) for Sesbania sesban. Antioxidant enzymes SOD, CAT APX work in close coordination for removal of radicals. SOD converts superoxide to hydrogen peroxide that is acted upon by either CAT or APX resulting in the conversion of hydrogen peroxide to water therefore prevent the further oxidative damage to cellular structures. APX is also the important component of important radical
scavenging pathway, the ascorbate-glutathione cycle. The ascorbate-glutathione cycle contributes to maintained photosynthesis by mediating quick scavenging of the free radical, hydrogen peroxide, so that flow of electrons to molecular oxygen remains under control and the formation of superoxide radicals is reduced (Norton and Foyer, 1998). AMF inoculation induced enhancement in activities of antioxidant enzymes demonstrated in the present study support the findings of Abdel Latef and Chaoxing (2011) for tomato and Hashem et al., (2014) for Vicia faba. Increased activity of defensive enzymes in AMF inoculated plants may be caused by direct implications on the uptake of important trace elements that serve as enzyme cofactors and increase the synthesis of molecules that act as reductants.

Salt stressed plants showed considerable variation in the endogenous levels of growth hormones with salicylic acid (SA) showing an increase with the salt stress and indole butyric acid (IBA) and indole acetic acid (IAA). Maintaining the levels of growth hormones directly affects growth rate and signalling mechanisms in plants. Hormones like SA and ABA have been recognised for their role under stressful environments (Ahanger et al., 2014; Iqbal et al., 2014), when levels of SA and ABA are upregulated resulting in elicitation of effective stress tolerance mechanisms. Several reports are available suggesting the positive role of growth hormones in mitigating stress induced negative changes (Khan et al., 2015). Auxins are important in several crucial physiological processes like the stimulation of cell elongation in responses to light. The increase in the synthesis of growth hormones in AMF colonized plants induces a redistribution and accumulation of hormones leading to ion flux and turgor loss thereby inducing stomatal movements (Abd_Allah et al., 2015b). It has been proposed that plants tend to acquire greater tolerance to stress by up-regulating synthesis of certain growth hormones while certain others are downregulated (Ahanger et al., 2014; Hameed et al., 2014; Wu et al., 2014). In our results salt stress caused detectable alteration in metabolism of plant growth hormones of Lupinus termis during alleviation of salt stress by AMF can provide a sensitive monitor of the physiology of plant-AMF interactions under salt stress.

CONCLUSION

Lupinus termis plants subjected to salinity stress showed an obvious reduction in growth, AMF infection, and impacts on plant physiology and biochemistry. Inoculation of AMF not only enhanced the parameters studied but also alleviated several negative effects of salinity to some extent. Thus, AMF enhanced the accumulation of compatible osmolytes leading to maintained cell turgor and growth rate. Increased activity of antioxidant enzymes in AMF inoculated plants was evident resulting in a quick scavenging of toxic ROS and maintaining membrane structure and integrity. Increases in the levels of growth hormones in AMF inoculated plants confer the protective role of AMF in maintaining growth and metabolism under salinity stress.

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