Materials and Methods

This section gives information about the materials used during the course of the whole study. It also gives an account of different methods employed during the research work with suitable references.

2.1. MATERIALS:

2.1.1. Chemicals:

All the chemicals used in the whole study were of Analytical Reagent grade and were purchased from Hi-media, Mumbai, India.

2.1.2. Seeds for sowing:

Seeds of *A. hypogaeae* (cv. TG-26), *C. tetragonoloba* (cv. Kasturi), *Zea mays* (cv. Karveer), *Trigonella foenum graecum* (cv. Amar Harita) were purchased from Amar Seeds Pvt. Ltd., Pune and the seeds of *G. max*, *Sorghum bicolor*, *A. arabica* were purchased from Naik Seeds Pvt. Ltd., Pune (Fig. 2.1).

![Seeds used for efficacy study](Image)

Fig. 2.1: Seeds used for efficacy study [*A. hypogaeae* (up, left), *A. arabica* (up, right), *G. max* (down, left), *C. tetragonoloba* (down, right)]

2.1.3. Water:

At pot level study, tap water (sieved through 105μ sieve) was used for irrigation purpose (with composition: [cations (meq/L): Ca 2.8, Mg 2.0, Na 8.2; anions (meq/L): bicarbonate 6.2, carbonate 0.2, chloride 1.7, sulfate traces; K₂O: traces, pH 7.8, SAR 5.29]).
2.1.4. **Soil:**

Soil used for several pot study were of slity loam type with composition: sand 35%, slit 57% and clay 8%, pH 6.0, organic matter 1.3%, available phosphorus 0.75mg/100g, available nitrogen 0.4% and available potassium 4.5mg/100g (www.pedosphere.com).

2.1.5. **Sand:**

During formulation of plant growth medium, river sand with particle size of <0.3mm was used.

2.2. **METHODS:**

2.2.1. **AM diversity in soil rhizospheres and its relation with soil properties:**

In this section, diversity along with distribution of AM fungi in the rhizospheres of *Saccharum officinarum* (Sugarcane), *Arachis hypogaea* (Groundnut), *Glycine max* (Soybean), *Cyamopsis tetragonoloba* (Cluster bean) and *Acacia arabica* (Babool) were examined and the relation between AM spore population and extent of root colonization with soil chemical properties were investigated.

2.2.1.1. **Study site:**

The survey was conducted during August 2009 to December 2010. Rhizospheric soil samples were collected from one hundred and twenty eight tehsil places belonging to twenty one districts of Maharashtra. Geographical coordinates of each tehsil places and surveyed host plant species are presented in Table 2.1.

As sugarcane is mainly cultivated in Western part of Maharashtra, hence rhizospheric soil samples were collected from the districts, Ahmadnagar, Satara, Kolhapur. Also, soil samples were collected from some sugarcane producing districts (Dhule, Aurangabad, Beed, Pune, Sangli, Jalgaon and Osmanabad).
### Table 2.1: Study sites with geographical coordinates and host plant

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*Materials and Methods*

*Studies on the effect of arbuscular mycorrhizal fungi on some plants under salinity stress*

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Materials and Methods

Studies on the effect of arbuscular mycorrhizal fungi on some plants under salinity stress

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Nandurbar, Dhule, Jalna and Aurangabad.

Soybean rhizospheric soils were sampled from major soybean producing districts viz. Akola, Amravati, Wardha, Yavatmal and Nanded.

Various arid and semi arid regions of Maharashtra were considered for collection of cluster bean rhizospheric soil samples and these include the districts of Jalgaon, Beed and Solapur.

Like cluster bean, A. arabica rhizospheric soil samples were taken from different dry land areas of Maharashtra including district Buldhana, Ahmadnagar, Pune and Sangli.

2.2.1.2. Collection of rhizospheric soil samples:

Soil samples including roots of host plants and stump (in case of sugarcane) were collected from ten different fields (or areas for A. arabica) of each tehsil place. For this purpose, from each field, a total of eight to ten sub-samples
Studies on the effect of arbuscular mycorrhizal fungi on some plants under salinity stress


2.2.1.3. Extraction of spore:

2.2.1.3.1. AM spore density:

One-third part of each collected rhizospheric soil sample was used for extraction of AM spore by wet-sieving and decanting method described by Gerdemann and Nicolson (1963). AM spores were extracted from 50g air dried soil sample in two triplicates for individual soil and were mixed with 500ml deionized water followed by thorough stirring for 1 to 2 min. Then soil suspension was passed through a series of sieves (37 to 500µ) (Jayant, Mumbai). Each sieving contained AM spores, while the debris was collected separately and was subjected to sucrose centrifugation (Daniels and Skipper, 1982). Spores were collected on grid-lined filter paper placed in a 9cm Petri dish and washed with deionized water for the even distribution of spores on entire grid-lined paper. Spores were examined and counted under trinocular compound microscope (Primo Star, Zeiss, Germany) with objectives of X10 and X40 magnification and X10 eye-piece. During the counting, care was taken to count only healthy intact spores and spores in cluster as well as sporocarp were considered as single unit. Mean spore density was calculated from spore number observed in two triplicates and data was expressed in terms of number of AM spores per 100g of air dried soil sample.
2.2.1.3.2. Relative abundance and frequency of AM spores:

Relative abundance (RA in %) and frequency (F in %) of AM spore at genus and species level was calculated using the following formulae:

\[
RA (\%) = \left( \frac{\text{Number of AM spores of a genus or a species}}{\text{Total number of spores}} \right) \times 100
\]
(Kumar and Ghose, 2008).

\[
F (\%) = \left( \frac{\text{Number of samples in which genus or species of AM fungi was observed}}{\text{Total number of samples}} \right) \times 100
\]
(Kumar and Ghose, 2008).

2.2.1.3.3. AM spore similarity index:

Between the two districts, similarity of AM spore population (at species level) in rhizospheric soils for individual plant was expressed in terms of Jaccard Index and this was calculated by the following formula:

\[
IS_J = \frac{C}{A+B+C}
\]
(Danesh et al., 2006).

[where, IS_J: Jaccard similarity index of AM species between two districts (a and b).
A: Number of the species only from district “a”
B: Number of the species only from district “b”
C: Number of the species common from district “a” and “b”]

2.2.1.4. Morphological identification of AM fungi:

Healthy, viable AM spores with prominent structural appearances are essential for morphological identification of AM fungi at species level. But, many times, spores from field collected samples do not fulfill these properties and it becomes difficult for morphological identification and hence to avoid this, trap culturing becomes essential.

**Trap culture:** Sporulation of field collected spores can sometimes be highly encouraged under controlled environment and hence, remaining one-third part of each collected soil sample was used to establish trap culture in 3kg nursery bag following the method of Eom *et al.* (2000) with slight modification. The trap was developed by using *Zea mays* (cv. Karveer) as host plant and the plants were allowed to grow for six months (two successive growth periods, each of three months) under green house conditions (temperature: 30/20°C day/night, a relative
humidity of 60-65% and at a photon flux intensity of around 280-350µmol/m²/S). After growth periods of third and sixth months, AM spores were extracted from all the one hundred twenty eight traps and counted as described above. Increase in spore density (fold) after third and sixth months was calculated using the following formula:

\[
\text{Increase in spore density (fold) after third month} = \frac{\text{Spore density}_{\text{after third month of trap}} - \text{Spore density}_{\text{in filed collected sample}}}{\text{Spore density}_{\text{in filed collected sample}}} \\
\text{Increase in spore density (fold) after sixth month} = \frac{\text{Spore density}_{\text{after sixth month of trap}} - \text{Spore density}_{\text{after third month of trap}}}{\text{Spore density}_{\text{after third month of trap}}}
\]

Trap was designated as positive, when spore counts of AM fungi (per 100g air dried soil) were increased either after third, sixth months or successive increment after both the periods were noticed and also new fresh spores were detected, which remained undetected in field collected sample. For each type, healthy, fresh, viable, morphologically similar AM spores (around 25 to 30) were separated from positive trap culture and fixed on glass slide in PVLG and PVLG with Melzer’s reagent.

Each type of AM spore was identified on the basis of parameters like, color, shape, size, surface ornamentation, spore wall structure and type of hyphal attachment as per the description provided in the databases (www.amf-phylogeny.com, http://invam.caf.wvu.edu) and as per the key features given in the manual (Walker and Trappe, 1981; Morton, 1988; Almeida and Schenck, 1990; Morton and Benny, 1990; Schenck and Perez, 1990; Morton and Redecker, 2001). Voucher specimens of AM species identified from the trap culture were deposited at Division of Biochemistry, Department of Chemistry, University of Pune. The most abundant AM spore type found in each positive trap culture was selected for further propagation study.

2.2.1.5. Estimation of AM root colonization:

To estimate extent of AM root colonization (%), the root samples were removed from fixative and washed gently with deionized water. Then, the root
samples were cut into 1 cm pieces and were thoroughly mixed. Roots (~50 pieces) were boiled in hot KOH (10%, w/v at 90°C) for 1 h and then cooled to room temperature. After cooling, root samples were washed with deionized water for several times and acidified with HCl (10%, v/v) for 3 min and then stained with either Trypan Blue or Lacto-glycerol acid fuchsin (0.05%, w/v) for 15 min at 90°C (Phillips and Hayman, 1970; Kormanik and McGraw, 1982). The percentage root colonization was quantified using the following formula:

\[ \text{AM root colonization (\%) = (Total number of root colonized / Total number of root observed) \times 100} \] (Borde et al., 2010).

2.2.1.6. Physico-chemical analysis of rhizospheric soil:

2.2.1.6.1. Soil pH and Electrical Conductivity (EC):

Rhizospheric soil samples were mixed with water (soil:water, 1:5 v/v) and the soil-water suspension was used to determine soil pH using pH meter (Hanna, 8732) and soil EC value using conductivity meter (Hanna, HI 8733) at 25°C.

2.2.1.6.2. Soil organic carbon (OC):

Soil organic carbon content was measured by rapid titration method (Walkley and Black, 1934). 0.2 mm sieved dry soil (1 g) mixing with a mixture of potassium dichromate (0.1667 M, 10 ml) and concentrated H₂SO₄ (20 ml) and was allowed to stand for 30 min at room temperature for oxidation. 200 ml distilled water, o-phosphoric acid (85% v/v, 10 ml) and diphenyl amine indicator (1 ml) were added to this mixture. Excess potassium dichromate solution leftover was titrated with ferrous ammonium sulphate (0.5 M) till the color changed from blue violet to green.

2.2.1.6.3. Soil available nitrogen:

Available nitrogen content in soil was determined titrimetrically (Jackson, 1973). 0.2 mm sieved dry soil (20 g) was mixed with distilled water
(20ml) and liquid parafin (1ml) was added. To prevent bumping and frothing during distillation, few glass beads were also added followed by addition of potassium permanganate (0.32% w/v, 100ml) and NaOH (2.5% w/v, 100ml). The content was allowed to distill in Kjeldahl assembly at a steady rate. Liberated ammonia was absorbed in the flask containing boric acid (2% w/v) and mixed indicator (methyl red with bromocresol green in ethanol). With absorption of ammonia, pink color solution turned to green and the content was titrated with H$_2$SO$_4$ solution (0.02N v/v), until pink color reappeared.

2.2.1.6.4. Soil exchangeable phosphorus:

Amount of exchangeable phosphorus presented in soil samples was determined by Olsen Method (Kalra and Maynard, 1991). 0.2mm sieved dry soil (2.5g) was mixed with sodium hydrogen carbonate solution (NaHCO$_3$) (0.5M, 50ml) at 1:20 ratio, shaked for 30min at a constant rate and filtered. Filtrate (10ml) was added with H$_2$SO$_4$ (2.5M, 1ml), distilled water (15.5ml), 8ml ascorbic acid solution in acid molybdate reagent containing antimony potassium tartarate. Then, 15.5ml distilled water was added and the solution was mixed thoroughly and incubated at room temperature for 10min. Absorbance of bluish purple colored solution was measured at 882nm. Standard KH$_2$PO$_4$ (1mg/l) solution was used for preparation of calibration curve.

2.2.1.6.5. Soil exchangeable potassium:

Concentration of exchangeable potassium in soil was determined by subtracting the amount of water-soluble potassium from extractable potassium following the method of Jackson (1958). Extractable potassium concentration was measured in ammonium acetate solution. 10g of air dried soil (<0.2mm) was mixed with 25ml ammonium acetate solution (1N, pH 7.0, glacial acetic acid in ammonium hydroxide) and centrifuged for 10min until supernatant of the solution became clear. Supernatant was filtered and volume was made up to 100ml with ammonium acetate solution (1N). Standard KCl solution (200ppm in ammonium acetate solution (1N), pH 7.0) was used to prepare the calibration curve. The photo
emission of soil extracts and standard solutions were measured on a Flame Photometer (Flame Photometer 128, Systronics) at 767nm.

Water-soluble potassium was determined on a Flame Photometer (Flame Photometer 128, Systronics) at 767nm through the process of shaking for 1h of 5g air dried soil (<0.2mm) mixing with 100ml deionized water and filtering.

2.2.1.6.6. Soil extractable sodium:

Sodium was extracted in ammonium acetate solution as per the extraction method (for exchangeable potassium) given in the above section (2.2.1.6.5). Standard NaCl (200ppm in 25ppm LiCl) solution was used for preparation of calibration curve. Concentrations of Na⁺ in soil extract and standard solutions were measured on a Flame Photometer (Flame Photometer 128, Systronics) at 589nm.

2.2.1.6.7. Soil exchangeable calcium and magnesium:

Soil exchangeable calcium and magnesium contents were determined in ammonium acetate extract of soil as per the extraction method (for exchangeable potassium) given in above section (2.2.1.6.5) followed by direct titration with EDTA (Hesse, 1971).

- **Determination of Calcium:**
  Ammonium acetate extract of soil was added with 10 drops each of hydroxylamine-hydrochloride (5%, w/v) and triethanolamine solution. To this mixture, NaOH (10%, w/v, 2.5ml) was added followed by addition with Calcon solution (1.0ml). The solution was titrated with EDTA until blue color appeared. Standard CaCl₂.2H₂O (0.01N) solution was used for preparing the calibration curve (Hesse, 1971).

- **Determination of Calcium plus Magnesium:**
  Ammonium acetate extract of soil was added with buffer solution (ammonium chloride-hydroxide, 15ml) and 10 drops each of hydroxylamine-hydrochloride (5%, w/v), potassium hexaferrocyanate (4%, w/v) and triethanolamine solution by keeping ammonium acetate soil extract (slightly warm) in magnetic stirrer. The
mixture was allowed to warm for another 3min and cooled to room temperature. Erichrome Black T (10 drops) was added to this and titrated with EDTA (Hesse, 1971).

- **Determination of Magnesium:**
  Magnesium content in soil was calculated by subtracting the concentration of calcium from the concentration of calcium plus magnesium.

### 2.2.1.6.8. Soil Copper, Zinc and Iron contents:

DTPA method was used to determine soil copper, zinc and iron contents (Lindsay and Norvell, 1978). 10g air dried soil (<0.2mm) was mixed with 20ml Diethylenetriaminepentacetic acid (DTPA) extractant (0.005M DTPA, 0.1M triethanolamine (TEA), 0.01M CaCl₂, pH adjusted at 7.30 with HCl). The mixture was kept shaking for 2hrs and filtered. The filtrate was subjected to AAS (Perkin Elmer 603) for determination of copper, zinc and iron contents. Standard solutions of copper (4ppm), zinc (1ppm) and iron (5ppm) were prepared in DTPA extractant solution.

### 2.2.1.7. Statistical analysis:

- **AM diversity and soil properties:**
  To normalize the experimental data, the values obtained for spore density and percentage root colonization, were subjected to $\log_e(x+1)$ transformation and arcsine square root transformation respectively (Zar, 1984; St. John and Koske, 1988). Soil chemical properties were checked in triplicates for individual sample and the mean value was mentioned.

  Pearson’s correlation coefficient was calculated to determine the relationship between spore density, percent root colonization and soil chemical properties. A statistical significant difference was set at $P<0.01$ level. Agglomerative hierarchical cluster analysis was performed using average linkage within groups and the result of complex multivariate relationship among variables was expressed as dendrogram (Chatfield and Collins, 1980; Romesburg, 1984). For all the statistical analysis, SPSS V. 9.0 was utilized.
Materials and Methods

Studies on the effect of arbuscular mycorrhizal fungi on some plants under salinity stress

2.2.2. Screening and multiplication of salt (NaCl) adapted, indigenous AMF spores / sporocarps:

Most abundant AM species detected in each positive trap cultures were only allowed to multiply.

2.2.2.1. Collection of AM spores / sporocarps from trap culture:

Individual dominant AM species were isolated from each positive trap culture by previously mentioned method (2.2.1.3.1). Following sucrose (30%, w/v) centrifugation, spores (or sporocarps) were extracted and most dominant, morphologically similar AM species were collected manually with a pasteur pipette. Collected spores (or sporocarps) were washed with sterile deionized water until complete elimination of fine debris was achieved. Spores (or sporocarps) were stored at 4°C in sealed petri dish for one week (Fortin et al., 2002).

2.2.2.2. Disinfection of AM spores / sporocarps:

Spores (or sporocarps) were transferred on filter paper and rinsed with sterile deionized water. Then those were treated with Chloramine T (2% solution with 2 to 3 drops of Tween 20) for 8 to 10min, followed by thorough washing with sterile deionized water for 2 to 3 times. Then, spores (or sporocarps) were treated with antibiotic solutions (Streptomycin sulphate 0.02% and Gentamicin sulphate 0.01%) for 8 to 10min and agitated with pasteur pipette gently. After 10min, spores (or sporocarps) were washed three times with sterile deionized water. Each AM spore type were removed from filter paper while observing under trinocular compound microscope (Primo Star, Zeiss, Germany) and placed in tube contained sterile Minimal medium (free from gelling agent) (Cravenbrock et al., 2005).

2.2.2.3. Propagation of AM spores / sporocarps:

Sterilized AM spores (sporocarps) were propagated individually in sterile funnel and in microtip, consisting of equal parts of sterile soil and river sand.
uniform layers and placed alternatively. Individual spores (or sporocarps) were placed 1cm below the surface sterilized seeds (with NaOCl 0.5% w/v) of *Sorghum bicolor* before seed germination. Seed sowing and inoculation of AM spores (or sporocarps) was done in sterile condition and funnel and microtip were incubated at 27°C in dark for 15 days. Here, AM spore cultures were developed from single spore (or sporocarp) and in two triplicates.

2.2.2.4. Continuous culture development:

After 15 days of incubation period, plant shoots were removed from soil-sand surface. The inoculum (containing mycorrhizal roots, AM spores or sporocarps) was removed from funnel and microtip separately and placed at the centre of a 3kg nursery bag containing fresh medium of equal parts (1:1 by volume) of same sterile soil and river sand. Sterile soil-sand mixture was selected to develop culture, as it is considered conventionally as the best substrate for multiplication of AM fungi (Singh, 2002). Seeds of *S. bicolor* were sown and allowed to grow under green house conditions (temperature of 30/20°C day/night, a relative humidity of 60 to 65% and a photon flux intensity of around 280 to 350 µmol/m²/s) for two months and after two months, shoots were removed and bags were reseeded and grown again for two months. After this continuous culture development, shoots were removed from soil-sand surface and roots were chopped and mixed with the whole inoculum for further study. For confirmation of AM colonization, representative roots were removed from nursery bags and colonization was checked qualitatively as per methods described previously (2.2.1.5).

2.2.2.5. Selection of efficient AM inoculum under NaCl stress:

This study was conducted to screen the most effective AM inoculum in adapting provided NaCl stress gradient. Before carrying out this study, a preliminary study was conducted in which NaCl solutions (25 to 250mM) were added to pots, each containing 3kg dry soil to bring them to the water-saturated
level. After 24h, EC values were measured in soil saturated extracts and measured EC values were plotted against NaCl concentration of the solutions added to the soil. A linear regression was fitted. Soil based inoculum from each nursery bag was transplanted in sterile nursery bag and inoculum was reseeded with surface sterilized *Trigonella foenum graecum* (cv. Amar Harita). Bags were kept in greenhouse and were irrigated everyday with sterile tap water. NaCl was added to sterile tap water and was supplied. It was decided to apply four levels of salinity including control (original soil EC of 1.04dS/m), first level (EC of 2.10dS/m), second level (EC of 5.94dS/m) and third level (EC of 8.26dS/m). First and second levels of salinity treatments were decided by considering the range of soil EC values observed in field collected soil samples and the third level was provided, which had higher EC value than the EC values obtained in field collected soil samples. To avoid salinity shock, NaCl solution was provided with gradual increase in strength. After 15 days of seed sowing NaCl solution (in increments of 25mM per day) began to provide first level of salinity stress and once soil EC value became 2.10dS/m, supply of salt solution was stopped. Again (on 30th day of seed sowing), salt solution supply was initiated to supply gradually to the pots, until it reached second level of stress. Then, to achieve soil EC at third level, NaCl solution was supplied (on 45th day of seed sowing) gradually. Just before addition of salinity stress (on 14th day of seed sowing), root colonization was checked. During this stress tolerance study, total phosphorus content was checked four times i.e, just before beginning of the first, second and third level of NaCl stress treatments (on 14th, 29th and 44th day of seed sowing respectively) and lastly after completion of stress study (on 59th day of seed sowing). Plants were irrigated with Hoagland solution, once in a week. After two months of seed sowing, shoots were removed from soil surface. Level of salt adaptation (in terms of soil dS/m) of AM inoculum was designated by considering positive utilization (fold) in P content. Fold increase was calculated by using the following formula:

\[
\text{Mycorrhizal P utilization at each NaCl stress level (fold)} = \frac{\text{(mycorrhizal P uptake at each level of NaCl stress)} - \text{(non mycorrhizal P uptake at each level of NaCl stress)}}{\text{(non mycorrhizal P uptake at each level of NaCl stress)}}.
\]
The most and second most salt stress adapted AM inoculum was identified morphologically and used in further pot study. Representative roots from the most and second most salt adapted AM inocula, were checked for root colonization as per previously described method. Before carrying out pot trials, the amount of each AM inocula to be used was determined.

**Determination of AM inoculum density required during pot trials:**

This study was conducted to find out the amount of inocula (the most and second most salt adapted AM inocula) required to form almost same AM spore count in 3kg growth medium. An increasing amount of these inocula (25, 50, 75 and 100g) was mixed with sterile sand-soil mixture to a final volume of 3kg in nursery bag and surface sterilized seeds of *T. foenum* allowed to grow for thirty days. After thirty days of seed sowing, spore counts were checked. Four replications were used at each treatment, for a total of 16 plants per fungal isolate. Presence of AM spore/10g air dried soil was calculated from linear regression analysis.

**Mass culture:**

Mass cultures of selected AM spores were developed in presence of *Zea mays* and *S. bicolor* as a host plant.

**2.2.3. Efficacy testing of AMF isolates on different host plants under different NaCl stress levels: Pot trials**

The purpose of this experiment was to check and compare the individual and combined effect of two AMF isolates on four host legume plants (*Arachis hypogaea*, *Cyamopsis tetragonoloba*, *Glycine max* and *Acacia arabica*) exposed to various levels of soil salinity stresses. Three crop legumes (*A. hypogaea*, *C. tetragonoloba*, *G. max*) and one tree legume (*A. arabica*) were chosen as test plants for this study because, during survey work, AM fungi were recovered from rhizospheric soils of these leguminous plants.
2.2.3.1. Experimental design:

The experiment was conducted separately for four legume plants namely, *A. hypogaea*, *C. tetragonoloba*, *G. max* and *A. arabica* and was designed in randomized manner, comprised of four mycorrhizal treatments [NM: non mycorrhizal (uninoculated), *Gm*: *G. mosseae* inoculated, *Gf*: *G. fasciculatum* inoculated, and *Gm+Gf* (mixed): both *G. mosseae* and *G. fasciculatum* inoculated] and five different soil salinity levels [EC of 1.04dS/m (control), 2.10, 3.78, 5.94 and 8.26dS/m], with four replicates (three plants per replicate). 50g of *G. mosseae* and 75g of *G. fasciculatum* soil based inoculum was used in respective *Gm* and *Gf* treatment. But, in case of mixed treatment, 25g of *G. mosseae* inoculum and 40g of *G. fasciculatum* inoculum were used to achieve almost equal spore numbers (~800spores/pot). In all the cases, respective soil based inoculum was placed 3cm below the seedling, just prior to seedling transplantation in nursery bags. Whereas, non-mycorrhizal plants did not receive any mycorrhizal inoculum and in all the mycorrhizal and non-mycorrhizal treatments, a constant sand : soil volume (a total of 3kg) was maintained. The amount of soil based inoculum used in the three mycorrhizal treatments (*Gm*, *Gf* and mixed) was based on the results, obtained in the previous experiment using host *T. foenum*, a legume plant, where almost equal number of AM spores were developed after thirty days of mycorrhizal inoculation. Plants were irrigated with sterile tap water (sieved through 105µ sieve), every alternate day and with P-free Hoagland solution (X/10) twice in a month (Hoagland and Arnon, 1940). Following irrigation, bags were weighed to maintain constant water content (sand : soil at 70% field capacity). Salinity stress was provided by applying NaCl solutions after one month of seedling transplantation and during this one month period, almost equal number of AM spores was generated and roots of host plants were allowed to become colonized with AM fungi. Gradient of NaCl solutions (50, 100, 150 and 200mM) was supplied in 3kg bags containing sand : soil mixture, to raise electrical conductivity to 2.10, 3.78, 5.94 and 8.26dS/m respectively. NaCl solution was not supplied in the control pot which conferred electrical conductivity of 1.04dS/m. Salinity stress was increased gradually to prevent shock and was supplied until the target salinity level (in terms
of EC value) was achieved. Irrigation was done in such a way to prevent leaching of nutrient solution as well as water. The whole study was conducted in the greenhouse conditions (temperature of 30/20°C day/night, a relative humidity of 60 to 65% and a photon flux intensity of around 280 to 350 µmol/m²/s).

2.2.3.2. Plant material:

Seeds of *A. hypogaea*, *C. tetragonoloba* and *G. max* were surface sterilized using sodium hypochlorite solution (0.5% w/v): reverse-osmosis water (1:3) and washed thoroughly with sterile distilled water for at least three times. Surface sterilized seeds were kept on sterile moist filter paper in dark for germination. Whereas, *A. arabica* seeds were first treated with dilute sulfuric acid (1% v/v) for 15 min and then washed thoroughly with sterile distilled water for three times. Then, the seeds were soaked in sterile distilled water for 24h and kept in dark on sterilized fine sand (autoclaved at 110°C for 1h) moistened with sterile distilled water, for germination. At first true leaf stage, seedlings of almost equal length were selected from individual plant species, for the transplantation in pot.

2.2.3.3. Soil preparation:

The soil (slity loam) used for greenhouse study was same as used in the previous study. This soil was mixed with river sand in 1:1 (v/v) and the sand : soil mixture was autoclaved (at 110°C for 1h, twice at an interval of two days) to remove indigenous AM propagules.

2.2.3.4. Mycorrhizal treatment:

Soil based AM fungal inoculum of *G. mosseae* (Nicol. & Gerd.) and *G. fasciculatum* (Thaxt.) Gerd. & Trappe. were collected from previous experiment (section 2.2.2.5).
2.2.3.5. Plant growth parameters:

After the growth period of sixty days, plants from each treatment were harvested and total height (cm) of the plants was measured. Shoots and roots, after individual treatments were washed separately in distilled water and kept in oven at 60°C. After 24h, dry weight of the shoot and root tissues were recorded. Relative mycorrhizal dependency (MD) was calculated from the following formula:

\[
MD(%)_{\text{at given salinity stress level}} = \frac{[\text{total dry weight of mycorrhizal plant} - \text{Total dry weight of non-mycorrhizal plant}]}{\text{Total dry weight of non-mycorrhizal plant}} \times 100
\]

(Plenchette et al., 1983).

2.2.3.6. Total chlorophyll content:

The younger and fully expanded fresh leaves (1g) from each treatment were collected and were extracted in 80% (v/v) acetone (20ml) and centrifuged for 5min. Supernatant was collected and residue was further extracted with 80% acetone, followed by centrifugation. Extraction procedure was repeated until residue was colorless. The volume of supernatant was made up to 100ml with 80% acetone. Absorption of the supernatant was measured at 645, 663 and 750nm using UV/Vis spectrophotometer (Shimadzu UV 1601). Absorbance at 750nm was subtracted from the absorbance at the other two wavelengths to correct for any turbidity in the extract, before calculating chlorophyll concentration. Concentration of chlorophyll “a” and chlorophyll “b” was calculated using the following formula (Strain and Svec, 1966):

Chlorophyll “a” (mg/g) = 11.64 X (absorbance at 663nm) – 2.16 X (absorbance at 645nm)

Chlorophyll “b” (mg/g) = 20.97 X (absorbance at 645nm) – 3.94 X (absorbance at 663nm)

The amount of total chlorophyll was calculated by addition of chlorophyll “a” and chlorophyll “b” contents and the value was expressed in terms of mg/g of fresh leaf.
2.2.3.7. Total soluble protein content:

For the determination of soluble protein content by the dye binding assay, the fresh shoot (including leaf) and root tissue (0.5g) was extracted in phosphate-buffered saline (PBS) (10ml) and filtered (Bradford, 1976). The filtrate was mixed thoroughly with dilute Commassie Brilliant Blue G-250 dye (5ml) and incubated at room temperature for 5min. The red dye turned into blue after binding with soluble proteins in the sample and absorbance was measured at 595nm using UV/Vis spectrophotometer (Shimadzu UV 1601). Bovine Serum Albumin (Fraction V) was used as the standard for preparation of calibration curve (1mg/ml).

2.2.3.8. Total soluble carbohydrate content:

The fresh shoot (including leaf) and root tissue (100mg) from each treatment were subjected to acid hydrolysis (2.5N HCl, 5ml) to convert complex carbohydrates into simple one and the soluble carbohydrate content was measured (Hedge and Hofreiter, 1962). After hydrolysis, the solution was neutralized with solid sodium carbonate and the volume was made up to 100ml with distilled water and centrifuged. Supernatant was added to anthrone reagent (0.2mg/ml, 4ml) and was allowed to heat for 8min in boiling water bath. Reaction mixture was allowed to cool to room temperature and absorbance of dark green colored solution was measured at 630nm using UV/Vis spectrophotometer (Shimadzu UV 1601). Soluble sugar content in sample was calculated from glucose (0.1mg/ml) standard curve.

2.2.3.9. Total reducing sugar content:

The fresh root and shoot (including leaf) tissue was used for the determination of total reducing sugar content using 3,5-dinitrosalicylic acid method (Miller, 1972). Fresh tissues (100mg) were extracted in hot ethanol (80%, 5ml) for two successive times. Supernatant was collected and allowed to evaporate on water bath. To dissolve sugars, distilled water (10ml) was added and aliquot of sample (0.5ml) was mixed with DNSA (1.0ml). Mixture was then heated for 5min.
Materials and Methods

Studies on the effect of arbuscular mycorrhizal fungi on some plants under salinity stress

in boiling water bath and cooled to room temperature and the absorbance was measured at 510nm. Standard glucose solution (500µg/ml) was used for preparation of calibration curve.

2.2.3.10. Free proline content:

Free proline content in fresh shoot (including leaf) and root tissue was determined using Ninhydrin acid reagent (Bates et al., 1973). The fresh tissues (0.5g) from each treatment were extracted in aqueous sulphosalicylic acid (3% w/v, 10ml) and filtered. Filtrate (2ml) was treated with glacial acetic acid (2ml) and acid ninhydrin reagent (2ml). The reaction mixture was kept in boiling water bath for 1h and exactly after 1h, the reaction was terminated by placing the tubes (containing reaction mixture) in ice bath. Toluene (4ml) was added to the tube and vortexed for 20 to 30sec. Toluene layer was separated and absorbance of pink color solution was determined at 520nm. Standard proline (0.4µg/ml) was used to prepare calibration curve. Proline content in fresh tissue is expressed as µmol/g and was calculated as,

\[ \mu \text{mol/g tissue} = \frac{[\mu \text{g proline/ml} \times \mu \text{l of toluene added}] \times 5}{115.5 \times \mu \text{g of sample taken}} \]

(molecular weight of proline 115.5).

2.2.3.11. Electrolyte leakage (EL):

The individual fresh leaf samples from each treatment were cut into small pieces of almost equal length and used for EL measurement. The leaf sample (~0.5g) was placed in test tube, containing distilled deionized water (10ml) and tubes were kept in water bath at 32^0C for 2h. After 2h incubation period, tubes were cooled to 25^0C and initial electrical conductivity (EC) of the medium was measured using conductivity meter (Hanna, HI 8733). Then, the samples in the tubes were autoclaved for 20min and final EC was measured after cooling the tubes at 25^0C. EL of fresh leaf samples was calculated by using the formula (Dionisio-Sese and Tobita, 1998):

\[ \text{EL} = \left( \frac{\text{Initial EC/Final EC}}{100} \right) \times 100 \]
2.2.3.12. AM root colonization:

The root samples were collected separately from each treatment and washed with distilled water. The roots were cut into almost 1 cm pieces and then thoroughly mixed. A sub-samples (0.5 g) was then used to estimate AM root colonization according to the method described earlier (2.2.1.5).

2.2.3.13. Antioxidant enzyme analysis of the test plants:

The fresh shoot (including leaf) and root samples from each treatment were used for antioxidant enzyme analysis. The fresh tissue (500 mg) was frozen in liquid nitrogen and then grounded in pre-chilled mortar and pestle. Then the enzymes were extracted by homogenizing in extracting buffer [50 mM phosphate buffer (5.0 ml, pH 7.0); polyvinylpolypyrrolidone (1%, v/v), ascorbic acid (0.2 mM)]. The homogenate was then centrifuged at 15,000 x g for 20 min at 4°C and the supernatant was used for various antioxidant assays (Abdel Latef AAH and Chaoxing H, 2011).

2.2.3.13.1. Superoxide dismutase (SOD, EC 1.15.1.1) assay:

SOD activity was monitored using the method of Stewart and Bewley (1980). The reaction mixture (3.0 ml) contained phosphate buffer (50 mM, pH 7.8), EDTA (100 mM), L-methionine (13 mM), NBT (75 mM). After addition of tissue extract (50 µl) and riboflavin (2 mM, 20 µl); the reaction mixture was placed under fluorescent tube (15 W) for 10 min. Duplicate tubes with the same reaction mixture were kept in dark and used as blank. Absorbance was measured at 560 nm and one unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction.

2.2.3.13.2. Catalase (CAT, EC 1.11.1.6) assay:

CAT activity was determined by decomposition of H₂O₂ and decreased in absorbance at 240 nm (Aebi and Lester, 1984). This assay mixture (3.0 ml) contained H₂O₂ (10 mM) in phosphate buffer (50 mM, pH 7.8). The reaction was
initiated by adding sample extract (150μl) and change in absorbance was monitored at 240nm for 3min. Unit activity of CAT was the amount of enzyme required to decompose 1μM of H₂O₂ per minute (extinction coefficient of H₂O₂: 40mM⁻¹cm⁻¹ at 240nm).

2.2.3.13.3. Ascorbate peroxidase (APX, EC 1.11.1.11) assay:

APX activity was measured by monitoring decrease in absorbance at 290nm due to oxidation of ascorbate (Nakano and Asada, 1981). The reaction mixture (1.0ml) contained phosphate buffer (pH 7.0), ascorbate (0.5mM), H₂O₂ (0.5mM) and sample extract (10 μl). To initiate the reaction, H₂O₂ was added at last and decrease in absorbance was recorded for 3min. One unit of APX oxidizes ascorbic acid at a rate of 1μM per min at 25°C (Absorbance coefficient for ascorbic acid: 2.8mM⁻¹cm⁻¹).

2.2.3.13.4. Peroxidase (POD, EC 1.11.1.7) assay:

POD activity was measured by monitoring change in absorbance at 470nm due to guaiacol oxidation (Polle et al., 1994). The reaction mixture (3.0ml) contained phosphate buffer (100mM, pH 7.0), guaiacol (20mM) and H₂O₂ (10mM). The reaction was started by adding sample extract (150μl). The unit activity was the amount of enzyme required to form 1μM tetraguaiacol per min.

2.2.3.14. Mineral analysis of the test plants:

Dried shoot and root tissues of plant from each treatment were used for the determination of total nitrogen, phosphorus, potassium, sodium, calcium, magnesium, copper, zinc and chloride contents.

2.2.3.14.1. Determination of total nitrogen content:

Total nitrogen content in the plant tissues was determined by micro-kjeldahl method. Dried powder of root and shoot tissue (including leaf) (0.1g) was mixed with potassium sulphate (1.9g), mercuric oxide (80mg), concentrated sulfuric acid (2ml) and the mixture was digested at 370°C for 1h. Then the reaction mixture was cooled and ammonia free water was added to dissolve the solid.
During this reaction, nitrogen is converted to ammonium sulphate. To this digest, sodium hydroxide-sodium thiosulphate solution was added and distilled to liberate ammonia from the digest. Liberated ammonia was collected in boric acid solution (4% w/v, 5ml) with few drops of mixed indicator and ammonium borate was formed. During absorption of ammonia, the color changed from pink into green. Distilled samples were titrated against standard sulfuric acid (0.02N) until pink color reappeared. Nitrogen content in the samples was calculated by the following formula (Thimmaiah, 2006):

\[
N \text{ (g/kg)} = \frac{\text{[(ml } H_2SO_4 \text{ in sample } - \text{ ml } H_2SO_4 \text{ in blank)} \times (N) \text{ of acid } \times 14.01 \times \text{ final volume } \times 1000]}{\text{weight} \times \text{ aliquot volume (ml)}}.
\]

2.2.3.14.2. Determination of phosphorus content:

Powdered plant root and shoot tissue (including leaf) from each treatment was acid digested as follows:

Ground plant tissue (1g) was mixed with di-acid mixture (10ml) (HNO₃ : HClO₄; 9:4) and vortexed. Sample mixture was placed on hot plate followed by heating at high temperature until liquid became colorless. Then the liquid was cooled and deionized water (20ml) was added. The mixture was filtered and the aliquot of solution was used for phosphorus estimation. The digested sample was placed in 50ml volumetric flask and 10ml of vanadomolybdate reagent (Ammonium molybdate-ammonium vanadate in HNO₃) was added to it. Then the volume of the solution was made up to the mark with deionized water and vortexed. Absorbance of the solution was measured at 420nm after 30min of incubation at room temperature. For the preparation of standard curve, KH₂PO₄ (0 to 5ppm P) solution was used (Bhargava and Raghupathi, 1993).

2.2.3.14.3. Determination of sodium and potassium content:

Di-acid digestion of plant shoot (including leaf) and root tissue (dried) was carried out as per the method for phosphorus estimation. The sample was read in flame photometer (Flame Photometer 128, Systronics) at 598nm. For
preparation of standard curve, NaCl (10ppm) solution was used (Bhargava and Raghupathi, 1993).

Dried shoot (including leaf) and root tissue of plant was digested by di-acid digestion method. The digested sample was then read in flame photometer (Flame Photometer 128, Systronics) at 548nm. Standard KCl solution (0 to 5ppm K), was used to prepare calibration curve (Bhargava and Raghupathi, 1993).

2.2.3.14.4. Determination of calcium and magnesium content:

Di-acid digests of dried plant tissue (shoot including leaf and root) was used for estimation of calcium and magnesium content. After di-acid digestion, the sample was diluted up to 100ml with deionized water. Then, the concentration of calcium and magnesium present in the diluted digest was measured in AAS (Perkin Elmer 603). For calibration, standard CaCO$_3$ (100ppm) and MgSO$_4$.7H$_2$O (1000ppm) were used (Bhargava and Raghupathi, 1993).

2.2.3.14.5. Determination of zinc and copper content:

Dried plant tissue (1g) was di-acid digested according to the method for phosphorus estimation and the digest was diluted to 100ml with deionized water. The diluted digest was fed into AAS (Perkin Elmer 603) to measure the concentrations of zinc and copper. Standard ZnSO$_4$.7H$_2$O (100ppm) and CuSO$_4$.5H$_2$O (100ppm) were used for preparation of standard solutions (Bhargava and Raghupathi, 1993).

2.2.3.14.6. Determination of chloride content:

Chloride content in each powdered plant tissue (shoot including leaf and root) was determined using Spectro Xepos XRF analyzer.

Mycorrhizal responses on enhancement in plant biochemical and mineral contents and antioxidant enzyme activity as well as plant growth improvement was calculated over respective non-mycorrhizal plants as described by Shetty et al., (1995).
2.2.3.15. Statistical analysis:

Data were subjected to analyze the variance for main effects (mycorrhiza and salinity stress) and their interaction (mycorrhiza x salinity stress). Duncan’s Multiple Range Test was used to determine significant ($P<0.05$) difference between means. Statistical software SPSS V. 9.0 was used for the statistical analysis of the data.