CHAPTER 2
MATERIALS AND METHODS
Chapter 2. **Materials and Methods**

2.1 **Materials:**

The materials and methods employed to complete the present work in this thesis are as given below:

2.1.1 **Survey and sampling:**

The present research work started with a view to isolate a *Mycorrhizal* species, which is able to mask the drastic effect of salinity to the growing crop; so that the farmer could utilize the potential of the *Mycorrhizal* biofertilizer to enhance the productivity under abiotic stress (salinity) conditions. On the basis of this an experimental work is carried out at School of Life Sciences, North Maharashtra University, Jalgaon. To achieve the given target a hypothesis is followed which state that the species isolated from the given (stress) habitat tends to acclimatize the crop readily in that particular condition with respect to the species isolated otherwise. In accordance with the present situation of stagnating productivity under sugarcane productivity, which is the main commercial crop of western part of Maharashtra; a field survey was done to collect soil sample from the rhizosphere of the healthy sugarcane species growing under salt stress condition for this the materials required for different experiments are as follows: Tube Auger 70 cm in length (stainless steel), shovel, Conductivity meter suitcase, zip-lock plastic bags of 500gm capacity, Hand gloves, marker etc.

2.1.2 **Instrumentation:**

After survey the sample is processed in the lab for that the following instruments were required viz. Compound Microscope (Labomed), Stereozoom microscope (Olympus), Digital camera (Cannon), Sieves of different size (fritsch), glassdish, petriplates, slides and cover slips (Blue star), micropipette, Glass wares: (Borosil), Centifuge machine, Refrigerator, UV-Visible spectrophotometer (Shimadzu Japan) etc.
2.1.3 Chemicals:

All the Chemicals utilized during the research work were of AR grade, spore staining solution PVLG and Melzer reagent and root staining solutions etc.

2.1.4 Greenhouse experiment:

For Greenhouse experiment plastic pots made of polyethylene possessing soil holding capacity of 1 kg were purchased. Greenhouse chamber situated at the premises of the ‘School of Life Sciences’ was utilized for all the pot level experiments. Autoclave (steel made, Pune) was required to sterilize glassware’s and soil sample. Demineralized water was used during the entire experiment from the Millipore India Ltd. Bangalore. For culture maintenance Wheat seeds (Triticum aestivum) variety WH-147, required to test the efficacy of the isolated mycorrhizal species under different salt concentrations were procured from Laxmi seeds company, Aurangabad. Mycorrhizal culture (Glomus Intraradices) was obtained from TERI New Delhi to test its potential.

2.1.5 Field trial:

To test the efficacy of the culture at field level, test plant sugarcane (Saccharum Officinalies) variety Co C 671 was obtained. It is the hybrid obtained by crossing Q 63 and Co 775 and also matures in 10 months period.

2.2 Methods employed:

2.2.1 Field Survey and sample collection:

To identify and isolate the mycorrhizal fungi, which is not only tolerant to the salt condition, but also helps the crop to grow has been done with respect to one of the important commercial crop of the state (sugarcane). For this, a study of VAM spores population and their respective association with cane under saline condition was
carried out. A field survey of total 11 villages was carried out, where the sugarcane has been cultivated in saline soils in Jalgaon district (Fig. 3) is surveyed, with consideration to Southern part of Maharashtra i.e. Sangli, Kolhapur region.

Soil samples for AM were taken in the month of April-May as it is the best season to collect the spores, from the field were the crop is at least 4-7 months old; with the help of shovel soil sample is collected from the rhizosphere region (~30-60 cm) of the sugarcane crop. Care was taken so that to acquire the sample from the crop which was healthy in condition. Soil sampling was done in a random sampling scheme (P. K. Gupta, 2000.) as given below.

![Map of Jalgaon District](image.png)

**Fig. 3** Jalgaon District (Sampling site).
2.2.2 Sampling for soil reclamation:

Saline and alkaline soils samples were collected by using a soil tube-auger and sampling was carried out by the procedure given below.

For soil physio-chemical analysis, tube-auger was taken to the field and sampling was done randomly in which five samples were taken between the rows of growing crops, collected in a plastic zip lock bags, with detail of sample and site. Soil pH and electrical conductivity was checked on the field itself, so that decision has to be made to collect the sample from the cane root. Sample from the rhizosphere region of the cane was taken carefully so that the crop remains viable after taking the sample. It was done with the help of shovel and the sample was collected in a zip lock plastic bags capacity of 100gm. The processing of soil sample was done on the day of collection itself otherwise it was preserved under refrigeration at 5°C for 10 hours, in case the field is far away. A part of soil sample collected from the tube-auger was air dried and processed for further analysis.

2.2.3 Conductivity measurements for salt content: (Richards, 1954)

It is important to measure the salinity content of a soil thereby we can know about the strategy to grow the suitable crop in that condition. Air dried soil sample was passed through 2mm sieve and taken to form a saturated paste with distilled water in 1:1 w/v ratio. The suspension was filtered with the help of Whatman No. 42 filter paper after thoroughly moistening with distilled water in the Buchner funnel. The filtrate was collected if the solution was turbid; the procedure was repeated until a clear filtrate was not obtained. This clean filtrate was then used for determining its electrical conductivity by conductivity meter.
2.2.4 Extraction and estimation of arbuscular mycorrhizal spores from soil.

Propagules of AM fungi consists of chlamydospores or azygospores, vesicles and mycelium or infected root pieces.

Various techniques are used to recover AM propagules from soil. The most basic of these is wet sieving and decanting (Gerdemann and Nicolson, 1963) to remove the clay and sand fractions of the soil while retaining spores and other similar sized soil and organic matter particles on sieves of various sizes.

Equipments and reagents:

Stalking sieves with, nylon or stainless steel mesh and a large range of pore sizes, for isolating spores from the soil sample were used like 40-50 micron (0.04 mm) for small sized spores, 100 micron (0.10mm) for medium sized spores, 250 micron (0.25mm) for very large spores and sporocarps and 400 micron (0.40mm) to separate pebbles and large impurities.

Table 3. Sieves with pore size utilized for extraction of Mycorrhiza.

<table>
<thead>
<tr>
<th>BSS (British Standard Size)</th>
<th>Pore Size(mm)</th>
<th>ISS (International Standard size)</th>
<th>Pore size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.70</td>
<td>10</td>
<td>0.10</td>
</tr>
<tr>
<td>20</td>
<td>0.78</td>
<td>20</td>
<td>0.212</td>
</tr>
<tr>
<td>30</td>
<td>0.5</td>
<td>30</td>
<td>0.30</td>
</tr>
<tr>
<td>60</td>
<td>0.25</td>
<td>60</td>
<td>0.60</td>
</tr>
<tr>
<td>100</td>
<td>0.15</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>200</td>
<td>0.075</td>
<td>200</td>
<td>2.0</td>
</tr>
<tr>
<td>300</td>
<td>0.053</td>
<td>300</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Materials and Methods

Studies on the application of Mycorrhizal fungi for the improvement in plant productivity under abiotic stress conditions

2.2.5 Isolation of spores from soil:

Wet sieving and decanting, which helps in the sorting of the clay and sand fractions of the soil which retain with it the spores of the mycorrhizal fungi of various diameter, generally in μm. This technique is relatively fast as compared to the other ones but it requires further processing to concentrate the sample and this was achieved by sucrose gradient centrifugation technique. The two techniques were described below.

2.2.5.1 Wet sieving and decanting technique:

Soil (250 gm) was mixed in a liter of water and the heavier particles were allowed to settle for a few seconds. Liquid (500-800 ml) was poured through a coarse soil sieve to remove large pieces of organic matter which was then collected. Sieve was than washed in a stream of water to ensure that all small particles have passed through. Pellet recovered was resuspended in the liquid which passed through the coarse sieve, the heavier particles get settled for a few seconds. Suspension was than passed through a sieve fine enough to retain the desired spores generally 38-250μm. Wash the material retained on the sieve to ensure that all colloidal material passes through the sieve. Pellet collected was examined in a watchglass under the dissecting binocular microscope.

2.2.5.2 Sucrose Centrifugation: (Jenkins, 1964)

Soil sample was washed with water to remove large debris. Collect sieved soil and water in a container. Which was than decanted through a 270 or 325 mesh sieve and the residue was collected from the sieve in a beaker. The pellet was suspended in centrifuge tubes and centrifugation was done for 4-5 min in a horizontal rotor. The supernatant liquid was than carefully decanted and re-suspended in a sucrose solution. Again centrifuge for 0.5 – 1 minute. Collected pellet was suspended in distill water for some time and then observed under under stereomicroscope. Count the number of spores in plate/dish and express it as spores/g of the soil sample.
2.2.6 Preservation of spores:

After extracting, the spores were stored for few days in Ringer’s solution.

Table 4. Composition of preservative medium[ Ringer’s solution (100ml)].

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.6 g</td>
</tr>
<tr>
<td>CaCl</td>
<td>0.01 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.01 g</td>
</tr>
<tr>
<td>MgCl</td>
<td>0.01 g</td>
</tr>
</tbody>
</table>

2.2.7 Characterization of spores:

The spores extracted were stained through PVLG and Melzer’s reagent and all the morphological characterization was reported, which helps in the identification of the spore. Image analysis was done with the help of the manual of Schenck and Perez (1990) and also from the description given at the web site of INVAM. Before that care was be taken to obtain the slide of either spore or root specimen to be clear and lucid. The entire major and minor details regarding shape, size, color, hyphal attachment and colonization pattern helps in the identification of that particular species. Here the image analysis is done with the help of ocular and stage micrometry.


The spores were stained in MTT and kept for incubation in dark at 27°C after incubation period the spore was watched for the color reaction under stereo microscope. Spores, which was stained red or pink were treated as viable, otherwise non viable.

\[
\text{% Spore Viability} = \frac{\text{No. of spores which stained red or pink}}{\text{Total no. of spores}} \times 100
\]
Materials and Methods

Studies on the application of Mycorrhizal fungi for the improvement in plant productivity under abiotic stress conditions

2.2.9 Quantitative estimation of intraradical colonization by A. M. fungi.

2.2.9.1 Clearing and staining root specimens (Modified, Phillips and Hayman, 1970).

Clearing and staining procedure was carried out the root samples should be carefully washed, to make it free of soil. It was important that KOH and staining solution volumes were sufficient for the amount of roots being processed and those roots are not tightly clumped together for uniform contact with solutions. To ensure uniform staining, the roots were chopped in to smaller (1-2 cm) segments.

Root specimen was washed under running tap water thoroughly and it was oxidized with the help of KOH. After which it was decanted and washed than it was supplied with alkaline H₂O₂ at room temperature for 10 minutes or until roots were bleached. Again the roots were washed with distilled water. Roots was than treated with 1% HCL and soak for 3-4 min.. After above procedure roots were stained with acid fushsine in lactoglycerol or trypan blue in lactophenol and kept for overnight for staining. The root specimen was than placed in glass petriplate/multiwell plate for destaining. The destaining solution (50% glycerol) was the standard used in step 6, but of course, without the stain.

2.2.10 Sample storage and slide preparation:

Root samples collected from the field was also assessed on the subsequent days, as sometimes it was not possible to clear, stain and estimate on the day itself of collection. For that either the roots was refrigerated at 5ºC or it was preserved in 50% ethanol in tightly sealed vials.

2.2.11 Assessment of root length colonization by AM fungi:

Mycorrhizal colonization was assessed using Biermann and Linderman (1981) method which was known as frequency distribution method, in which the colonization was assessed as proportion of root length colonized by mycorrhizal fungi using a compound microscope.
Materials and Methods

Studies on the application of Mycorrhizal fungi for the improvement in plant productivity under abiotic stress conditions

Roots segment which was to be assessed were cut into 1 cm length and then it was randomly suspended in lactoglycerol in petridish having 1cm grid. It was then observed with ocular micrometer and the stage micrometer at the desired zoom of the compound microscope and observe the root pieces. The proportion of the length of each root segment consisting vesicles, arbuscules or hyphae was estimated to the nearest 10%. Data was recorded frequency distributions from the samples containing 25, 50, 100 root segments. The percentage of the root length colonized in the sample was calculated with the help of frequency distribution.

2.2.12 Estimation of root/hyphal length: (Tennant D., 1975)

Lines intersect (Tennant 1975) method was used to estimate the length of hyphae and roots. For this first the roots were washed and cleaned thoroughly, then dispersed the cuttings in a Petri-dish with grid lines. Enough water was taken to disperse the roots completely. Both the horizontal intersection as well as vertical intersection of the root along the grid line was calculated separately.

Finally the root length was calculated as the sum total of the vertical as well as horizontal intersect was than multiplied with the constant i.e. 11/14* grid size (Grid size is the length of one side of one square of the grid).

2.2.14 Quantification of propagules from soil:

There are several methods for measuring propagules in soil. Extracted spores in water can be pipetted out into a nematode counting slide, which was similar to haemocytometer with 1 ml capacity. The slide was etched into rectangles with 30 parallel lines. Spores per ml were calculated by counting the spores contained in a portion of the slide according to Daniel et al 1982.
2.2.15 Assessment of infectivity potential of inoculum:

In this method total number of Infective propagules was calculated on the entire root length. This was also known as infectivity potential test.

For this 100gm soil was based per 1gm root based inoculum in the pot. Pre-germinated seeds was planted and grown for 1 month period. Which was than harvested and the roots were recovered. Roots were chopped equally in 1 cm length and the total root length was recorded as per Tenant D (1975), which was than cleared in KOH solution and stained. At last the number of entry points was counted which was formed on randomly picked 100 segments of the root.

2.2.16 Pot trial with Glomus sp.:

Arbuscular Mycorrhizal isolate was tested at pot level but before that necessary care was taken, so that pure undamaged and parasite free spores were selected. The dominant salt tolerant species was then cultured through monospecific culture technique, sited as per culture method of ‘INVAM’ web site. Soil analysis was done before performing the pot level experiment. Saturation extract of soil was used for measuring various cations concentrations. Soil organic matter was analysed as per Walkley Method (1947).

Protocol:

1 gm of air-dry soil was added into 500 ml beaker with 1 N potassium dichromate than it was mixed with concentrated sulfuric acid. Which was then allowed to stand for 30 min, after which 200 ml of distill water was added followed by concentrated orthophosphoric acid and it was allowed to cool. 10-15 drops of diphenylamine indicator were added which was then placed on magnetic stirrer. It was then titrated with 0.5M ferrous ammonium sulphate solution, until the color changes from violet blue to green.
Calculations:

Percentage organic matter in soil was calculated by using the following formula.

\[ M = \frac{10}{V_{\text{blank}}} \]

\[ \% \text{ Oxidizable organic carbon} = \left( \frac{V_{\text{blank}} - V_{\text{sample}}}{W_{t}} \right) \times 0.3 \times M \]

\[ \% \text{ Total organic carbon (w/w)} = 1.334 \times \% \text{ Oxidizable organic carbon} \]

\[ \% \text{ Organic matter (w/w)} = 1.724 \times \% \text{ Total organic carbon} \]

where

M = Molarity of ferrous ammonium sulphate sol.

Soil total Nitrogen was estimated as per modified Kjedahl method. Calcium and Magnesium was determined according to Versenate titration method; while available Phosphate was determined by Bray P-1 method. Sodium and Potassium was determined by Flame Photometer method. Micronutrients were determined in the DTPA soil extraction by atomic absorption spectrophotometer.

2.2.16.1 Inoculum Preparation (Raising Monospecific culture):

For pot level trial, soil was steam-pasteurized for 2 hr at 15 lbs pressure, for the three successive days to eliminate the viable micro-organisms. Soil was then mixed with autoclaved sand in 2:1 ratio and used for experimental purpose.

To raise the culture of the isolate, this technique was utilized. For this *sorghum vulgare* seeds were taken which was surface sterilized with HgCl₂ and 70 percent ethanol sol and it was allowed to grow in a sterilized sand and soil mix 1:4.
Materials and Methods

Studies on the application of Mycorrhizal fungi for the improvement in plant productivity under abiotic stress conditions

Hoagland’s nutrient solution was prepared in order to supply the nutrient necessary for its growth. After 10 days of germination, the pot was placed in a glass container and plant was uprooted and made it free of soil particles. It was done carefully so that the tertiary roots remain intact. The dominant healthy spores were isolated from the saline site was carefully taken in the micro syringe which was then applied carefully on the roots of the plant. After this process the plant was immediately planted on another plastic pot of 250 ml capacity in which terragreen and sand was filled in 1:1 ratio. Regular watering was done so that the plant completes its life cycle and after 5 weeks it was transferred into a large size pot. After 3 months the soil was extracted for the given fungal species.

2.2.16.2 Trap culture:

Trap culture is generally used for establishing large quantity of single species i.e. to increase inoculum for its further use. Trap culture was raised from the inoculum which was developed by monospecific culture. Soil, sand, peat or vermiculite mixture was prepared in which soil and sand was mixed in a ratio of 1:1. Before placing, it was subjected to autoclave i.e. at 120°C for 1 hour at 15 psi for three consecutive days. Roots were chopped the into small fragments and mixed with the seeds to be planted, it was generally placed 1 cm below the seeds so that it get infected as soon as the radical emerge from the seed. The culture was grown in green house at 25°C±1 with 60% relative humidity. Half the strength of Hoagland’s nutrient solution was provided to the plant. At the end it was harvested for the large inoculum need for pot experiments and field experiments.

The efficient isolate was then subjected to pot level trials having different concentration of the mixed salts medium i.e. Magnesium chloride, Calcium Chloride, and Sodium Chloride over a range of saline conditions, adjusted to 4ds/m, 6ds/m, 8ds/m, and 10ds/m. Control plants received an equal amount of autoclaved inoculum.
Materials and Methods

All the sets were raised in triplicates, so that we get a total of 30 sets, including inoculum with untreated soil and plant without inoculum with untreated soil. Pots required for experiments were of 1kg capacity and are made up of polyethylene, in which 900gm of autoclaved soil and sand (1:1) was taken. Salt stress was induced with the regular supply of salt solution, which includes MgCl$_2$, CaCl$_2$, and NaCl$_2$ in the ratio 1:2:3. The experiment was performed under controlled environmental conditions (25±1/22±2 °C day/night, 14 h photoperiod, 55-60 % relative humidity). 15 seeds of wheat variety WH 147 per pot were surface sterilized and soaked in water before planting.

Two days old sprouted seedling was sowed just 3 cm above inoculum (5 gm), which was then covered with 1 cm layer of autoclaved soil. Each pot in the greenhouse was provided with the ‘growth substrate’ which was prepared accordingly:

Three, five liter conical flask of Borosil were taken to prepare the growth substrate, Cattle manure was collected separately on a clean polythene sheet which was then air dried and crushed. Farm straw was collected and processed in a blender. Each flask was then supplied with five hundred gram of animal manure and was mixed with 200 gm of husk; final volume made was 2.5 liter. It was then kept for 20-25 days in dark to allow natural process of fermentation. After every third day dried plant leaves was added to the flask. At the end, whole growth substrate was made to dry out and then it was subjected to autoclave. After sterilization it was again suspended with 4 liter of demineralized water and it was supplied on every 4$^{th}$ day.

Plants were harvested after six weeks and shoot and root length, fresh weight and dry weight were recorded for each individual plant. Assessment of Root length was done as per Tennant D (1975) and viability testing of the spores with a vital stain was done by the method of Z, -Q & Hendrix JW (1988). The Mycorrhizal infection unit (MIU) was determined at the end of trial according to Franson and Bethlenfalvay (1989). Estimation of chlorophyll was determined as per Arnon D. I. (1949).
2.2.16.3 Chlorophyll Estimation : (Arnon, D. I., 1949)

The mature green leaf were plucked just below the growing young pair of the apical meristem of the plant; the sample was washed and dried with tissue paper. Sample of leaf was finely cut and well mixed into the clean porcelain mortar. The tissues were grinded to a fine pulp with addition of 20 ml of 80% of acetone. The homogenate was centrifuged at 3000 rpm for 15 min. The supernatant was then transferred to 100 ml volumetric flask. The procedure was repeated for the remaining residue until we get a clear solution. Supernatant was collected and the final volume was made to 50 ml and the ‘Absorbance’ was measured at 645 and 663 nm against the clear solvent (acetone) as blank.

The chlorophyll content was determined using the formula stated below

\[
\text{Chlorophyll 'a' (mg/g fr. wt.)} = (0.0127) \times (\text{OD663}) - (0.00269) \times (\text{OD645}) \times 100
\]

\[
\text{Chlorophyll 'b' (mg/g fr. wt.)} = (0.229) \times (\text{OD645}) - (0.00488) \times (\text{OD663}) \times 100
\]

\[
\text{Total chlorophyll (mg/g fr. wt.)} = (0.0202) \times (\text{OD645}) + (0.00802) \times (\text{OD663}) \times 100
\]

Estimation of root colonization was done. Root samples were cleared with 10% (w/v) KOH, stained with 0.05% (v/v) trypan blue in lactophenol as described by Phillips and Hayman (1970) and examined microscopically. Extent of colonization will be determined, using a gridline intercept method (Giovenetti and Mosse 1980).

The concentration of nutrients in plant tissues was measured in a plant extract both in fresh as well as in dried form. Total plant analysis was quantitative in nature and was done by wet digestion with HNO₃-HClO₄ in 2:1 ratio (Rashid, 1986). After digestion decant the supernatant liquid and then Ca, Mg, Zn, Fe, Cu etc, was determined by atomic absorption spectrophotometer. Sodium was measured with flame photometer as per Haddad and Higginson (1990); and the other metals Shoot Phosphorus concentration was determined colorimetrically (Watanabe and Olsen 1965).
Materials and Methods

Studies on the application of Mycorrhizal fungi for the improvement in plant productivity under abiotic stress conditions

Calculations:

Micronutrient cations in plant:

\[ \text{Zn, Fe, Cu, or Mn (ppm)} = \frac{\text{ppm in extract-blank} \times A}{Wt} \]

Where

\( A = \) Total volume of the extract (ml)
\( W = \) weight of dry plant (gm)

2.2.17 Pot trial with *Glomus intraradices*:

Commercial sample of *G. intraradices* procured from TERI, New Delhi was propagated by pot cultures with soil-sand-vermiculite (1:1:2) and *Sorghum vulgare* as host for 3 months. *Sorghum* seeds were rinsed under running tap water and surface-sterilized by treating it for 1 min in 10% (v/v) NaOCl then in D/W for 3 min, after that 45 s in 50% (v/v) ethanol and again finally with distilled water. The sterilized *Sorghum* seeds were germinated in 10 cm diameter glass vessel containing sterilized sand soaked in water. Two days old plants were transferred to 9 plastic pots and inoculated with 5 gm of Glomus *intraradices* inoculum consisting of root fragments with 60% of their root length infected. Experimental pot cultures were supplied with nutrient solution containing 1.5 mM CaCl\(_2\), 0.25 mM MgSO\(_4\), 0.02 mM KH\(_2\)PO\(_4\) and micronutrients equivalent to \(1/4\) of Hoagland’s solution. All the pots cultures were allowed to grow and propagate at green house for the duration of 4 month after which it is harvested for experimental purpose.

Experimental setup comprised of two factors, fungal treatment and salt concentration. Both AM and non-AM were subjected to total six concentration starting with 2.5ds/m, then 4.5, 6.5, 8.5, 10.5 and 12.5ds/m. Control plants received an equal amount of autoclaved inoculum.
Materials and Methods

*Studies on the application of Mycorrhizal fungi for the improvement in plant productivity under abiotic stress conditions*

All the sets were supplied with 10 seeds of WH 147 and raised in duplicates, so that we get total 28 sets, including inoculum with untreated soil and plant without inoculum with untreated soil. Pots required for experiments were of 1kg capacity, made up of polyethylene, in which 900gm of autoclaved soil and sand (1:1) was taken. Salt stress is induced with the regular supply of salt solution which included Mgcl₂, CaCl₂, and NaCl₂ in the ratio 1:2:3. The experiment was performed under controlled environmental conditions (25±1/22±2 ºC day/night, 14 h photoperiod, 55-60 % relative humidity). Plants were harvested at the end of six weeks and were subjected to physico-chemical analysis as stated under sugarcane isolate above.

2.2.18 Isolation of indigenous isolate from Lonar Lake sample:

Lonar crater is situated about 1 km to south-west of village lonar, comes under district Buldhana, Maharashtra. It is one of the third biggest craters in the world, having peripheral circumference of 6 km. It’s reported that the water of this lake is saline throughout the basin, as it is a natural salt ecosystem there is a great possibility to find a Mycorrhizal isolate here. Sampling of soil was performed from the rhizosphere of a grass species (*Cymbopogon citrates*) and we found the soil to be quite salty (10.5ds/m) with a pH of 9, which is quite alkaline. The mycorrhizal fungus isolated from Lonar Lake was cultured by monospecific culture method stated above. The inoculum was used for pot level trial in which again 2 factors were taken and in all 30 pots were planted with 10 seeds per pot, both the AM inoculated and control were grown in triplicate. Both AM and non-AM were subjected to total five concentration starting with 0, 4.5, 6.5, 8.5, to 10.5ds/m. Again the pot size was same as before i.e. of 1 kg capacity in which 900 gm of soil and sand mixture is taken in 1:1 ratio. Here the culture inoculum used was of 4 gm size. Further experimental part was same as that used in case of sugarcane rhizosphere isolate.
2.2.19 **Pot trial: synergistic effect of Lonar isolate and sugarcane isolate**

It’s evident from the many research work that the synergistic effect was much better than the individual effect so, it was thought to use isolate from rhizospheric soil under sugarcane cultivation and isolate from Lonar lake sample simultaneously. Six gm of each inoculum was mixed in 1:1 ratio and placed below the seedling of wheat variety WH 147. All the experimental condition was same as in case of lonar trial, the only difference was that the salt concentration is changed slightly i.e. seven salt concentration levels has been selected starting from 0ds/m, 4.5 ds/m, 6.5 ds/m, 8.5ds/m, 10.5ds/m, 12.5ds/m and 14.5 ds/m, with 8 seeds planted per pot and the biological growth substrate was modified a little in that we have added 150 gm of jaggery to the manure and 250 ml of cow urine was added every 5th day to the solution. Again rest of the procedure was same for plant harvesting and analysis.

2.2.20 **Pot trial: Testing the synergistic effect at field level:**

The most important part for any agricultural bioinoculant package is its performance at field level. For this the following steps were carried out to test efficacy of the synergistic effect of AMF isolate at field level.

After successful attempt made at pot level, field level trial was organized at village Shivani, Tehsil Bhadgaon, district Jalgaon. This agriculture field is situated about half a kilometer away from the village. Test crop was sugarcane (*Saccharum Officinalies*), of variety Co C 671. It is a hybrid variety which results from the crossing of Q 63 and Co 775 and also matures in 10 months period. Experimental plot was an area of 22.25 m², total 4 blocks were assigned with four treatments, in the pattern that 1 treatment per block, which were assigned at random within blocks of adjacent subject. In all 16 set were used in this experiment. As the field was already saline there was no need to give any salt treatment so four treatments included control, Glomus sp., fertilizer, Lonar sp. + Glomus Sp. Sugarcane seeds were cut between the internodes so that the seed would have single bud per seed.
Materials and Methods

Studies on the application of Mycorrhizal fungi for the improvement in plant productivity under abiotic stress conditions

In each set four seeds were sown while making a distance of 2 feet intra-set and 2.5 feet inter set. In all 64 seeds were sown, growth analysis was done after 3 months period. During and after field trial the effect of inoculants on the growth of sugarcane in this field soil was analyzed by measuring the height, % survival, girth, & no. of leaves, counted as per Deo et.al. (1998). Improvement in the growth parameters of the test plant (sugarcane) with respect to control would suggest the growth-promoting role of the isolate alone and in combination with other salt tolerant species to observe any synergistic effect.

2.2.21 Statistical Analysis of Data:

All the data were subjected to two way ANOVA software (M/S Indostat services Ltd. Hyderabad) with replication and Duncan’s multiple range tests. Significance of the data is tested at various P levels.