MATERIALS AND METHODS

The Maharashtra state is second largest state in India. It is mainly divided into three regions, i.e. Western Maharashtra, Marathwada and Vidarbha region. In Maharashtra state there are 35 districts. Pune district is located in Western Maharashtra. Average rainfall of the Pune District is between 350 and 4000 mm. Most of the rain is received during June to September. The maximum temperature in summer is 44°C and minimum temperature in winter is less than 5°C. Relative humidity ranges from 40 % to 62 %. In the present study, the survey of arbuscular mycorrhizal (AM) fungi from rhizosphere and non rhizosphere soil of tomato variety “Vaibhav” was carried out from three localities of Pune district of Maharashtra, India for three consecutive years i.e. 2009-2010, 2010-2011 and 2011-2012. The selected localities include Fergusson College botanical garden Pune (18˚ 31´ 22.19´´ N to 73˚ 50´ 23.90´´ E longitude), Paud (18˚ 31´ 35.78´´ N to 73˚ 36´ 56.55´´ E longitude) and Urali Kanchan (18˚ 28´ 46.49´´ N to 74˚ 08´01.99´´ E longitude).

Arbuscular mycorrhizal fungi (AMF) have received very little attention, despite the significance of this group of Zygomycetes fungi. Therefore it was decided to undertake a survey of this group of fungi associated with tomato and study their effect on biochemical contents of tomato. During present investigation the AM fungi associated with vaibhav variety of tomato were reported from three selected sites. The studies include taxonomy, physicochemical parameters of soil, percentage root colonization, and observation of various structures in roots, number of AMF propagules and number of arbuscular mycorrhizal species per locality. The mixture of indigenous AM fungi was used to study the effect on vegetative and reproductive growth of the host, tomato. The changes in the biochemical contents of leaves, green fruits and the red fruits were studied with respect to mycorrhiza.

Selection of plant variety

Tomato is one of the major crops cultivated on large scale in Pune district of Maharashtra, India. At present there are many varieties are available in the market. We chose “Vaibhav” variety as it was available throughout the year. The seedlings were bought from Scientific Seedling Nursery located at Urali Kanchan, Pune. The seedlings were a month old. They were grown in coco peat which was devoid of mycorrhiza. The plants were transplanted to the fields containing indigenous species of AM fungi immediately and watered at regular interval. These plants were named as
the mycorrhizal plants. Half of the plants were transplanted in the autoclaved soil and were named as non mycorrhizal plants or control plants.

**Collection of soil samples and roots**

The soil samples were collected from the three selected localities from Pune district. The localities were botanical garden of Fergusson College, Paud and Urali Kanchan. The collection of soil samples was done at random from the rhizosphere and non rhizosphere soils of tomato. The area of the plot was approximately of one acre. The area around the roots of the plants is called as the rhizosphere. This area shows fibrous roots and root hairs and hence this area is rich with the spores of AM fungi. The soil away from the roots is called as the non rhizosphere soil and it has less number of spores of AM fungi. For the comparative account, both rhizosphere and non rhizosphere soils were collected. At the same time, the roots of tomato were collected to study the percent root colonization. The samples were not collected in the first month because one month old seedlings of tomato need at least three weeks for the colonization of AM fungi after transplantation. The plants were grown in all three seasons i.e. monsoon, winter and summer during the period of one year. The life cycle of the plant is short and lasts for three or three and a half months. The first soil samples and the roots were collected when the plants were 60 days old. The second collection was done when the plants were 90 days old and the last collection was done at the time of uprooting the plant. (105 days old). The soil samples were stored in the sterile polythene bags in laboratory at 4°C. These soil samples were used for isolation and identification of spores AM fungi. For calculation of number of propagules, 100g soil was used at a time. The roots were used for studying the percent root colonization.

**Isolation of AM spores from the soil**

The isolation of spores of AM fungi was carried out by wet sieving and decanting method (Gerdemann and Nicolson, 1963) from 100g rhizosphere and non rhizosphere soil. One hundred gram soil was suspended in one liter of tap water. The mixture was stirred well and the coarse particles were allowed to settle for 15-20 minutes. The supernatant was decanted through a series of sieves arranged in descending order of mesh size (400, 250, 175, 125 and 75 µm). The spores from each sieve were collected in a tap water separately in a beaker. The supernatant from each
beaker was then separately filtered through Whatman No.1 filter paper. The filter papers were placed in the Petri-plate, care being taken to ensure that they remain moist. The contents of the filter papers were examined for spores and sporocarps under binocular microscope.

**Picking and Mounting of spores AM fungi**

The quantification of the spores was done under the binocular microscope. The filter papers were spread under the microscope, magnification was adjusted and the spores were picked with the help of needle. The spores were counted by visual assessment method. The spores were picked using needles and mounted in a drop of mounting medium, polyvinyl alcohol lacto glycerol (PVLG). The slides of sporocarps, broken and unbroken spores were prepared for examination. Generally, the spores having the hyphal attachment were preferred.

For quantification of spores, to maintain the accuracy, the filter papers were divided into sixteen smaller fields with pencil and the screening of each field was done thoroughly.

**Preparation of polyvinyl alcohol lacto glycerol**

This is a sticky mounting solvent for mounting the spores of the AM fungi. It was prepared by mixing 100 ml lactic acid, 10 ml glycerol and 16.6 g polyvinyl alcohol in 100 ml distilled water. This mixture was heated in a hot water bath till the crystals of polyvinyl alcohol dissolved completely. The process needed 12 hours. The solution was stored in a dark brown bottle.

**Mounting of AM spores in PVLG:**

Intact and crushed spores were mounted on a glass slide in a drop of polyvinyl alcohol lacto-glycerol (PVLG). The spores were transferred to a drop of PVLG on the slide using needle or paint brush leaving enough space to label. The PVLG was allowed to settle for 3-5 minutes to become more viscous. The spores mounted in PVLG were used to study the gross spore morphology. The slides were then placed in an oven at 60 °C for 24 hours to remove the oil droplets and air bubbles. The cover slip was then sealed with nail polish. The slides were labeled and stored in a flat tray.
Identification of mycorrhizal spores

The spores were first mounted in water for morphological measurements. The permanent slides were prepared using PVLG. The important features of the spore like colour, size and shape, number of wall layers, visible contents, size and shape of the subtending hypha etc. were observed under Leica make upright Trinocular Research microscope (Model DM750) with EC3 digital camera. The microphotographs were taken under 4x, 10x and 40x. The morphological characters of the spores were compared with the characters given in the manual for Identification of VAM fungi by Schenck and Perez (1990).

Percent root colonization

The roots of tomato plants collected after every 30 days were washed to remove all the soil and dirt and preserved in FAA, and then they were cut into 1 cm pieces and washed in water. These 1 cm pieces of roots were bleached using alkaline hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) at room temperature for 10-20 minutes. Later these roots were thoroughly washed with tap water to remove the traces of H\textsubscript{2}O\textsubscript{2}. The roots were then cleared in 10 % KOH at 121 °C for 10 minutes in an autoclave. The KOH solution was poured off and then roots were rinsed in tap water to remove traces of KOH, 1 % HCl was added in the test tube to soak the root pieces for 3-4 minutes and then solution was poured off. The root segments were then stained in 0.1 % trypan blue and kept overnight. The root segments were then mounted in PVLG and examined under microscope for percent AM colonization. The slides showing hyphae, arbuscules, vesicles and chlamydospores were sealed with nail polish for permanent mounting (Phillips and Hayman, 1970).

Measurement of Percentage root colonization:

Slide method:

Root segments (1 cm long) stained in 0.1 % trypan blue were mounted on glass slides. The presence or absence of AM colonization (arbuscules / vesicles / hyphae) was scored. The percentage root colonization was calculated using the following formula.

\[
\text{Percentage root colonization} = \frac{\text{Number of root segments colonized}}{\text{Number of root segments observed}} \times 100
\]

Grid-line intersect method: (Giovannetti and Mosse, 1980)
Roots stained in 0.1% trypan blue were dispersed in a Petri-plate marked into 1cm grid-squares. At least 100 intersections were examined for each sub-sample under a microscope at 10X x 40X magnification. Each intersection was counted as colonized, non-colonized or unclear; two values for proportion of root length colonized were calculated. For the lower estimate, the unclear scores were treated as not colonized; for the upper estimate, they were treated as colonized.

**Soil Analysis**

All the soil samples were analyzed for pH, electric conductivity, organic carbon, nitrogen, phosphorous and potassium content (Tandon, 1993).

1. **Hydrogen ion concentration (pH):**
   
   In a beaker mixed 40 g of dried and sieved soil with 40 ml water of distilled water (1:1). The mixture is stirred well using glass rod until it is thoroughly mixed and then allowed the mixture to form a supernatant. The supernatant is used to measure the pH by dipping pH paper in it and then compared with standard colour as final value.

2. **Electrical Conductivity:**
   
   The electrical conductivity (EC) of soil measured via electrodes inserted directly into extracting soil water using conductivity meter. Electrical conductivity is measured in mmhos/cm³. Soil capacity to conduct the current is determined to check the quantity of soil as it affects uptake of nutrients, water and minerals by plants.

3. **Estimation of Organic Carbon:**
   
   Organic Carbon was estimated by the modified Walkley and Black method, (1934)

**Organic Carbon by Wet Digestion:**
   
   Organic matter (OM) in the soil is oxidized with the mixture of potassium dichromate (K₂Cr₂O₇) and concentrated H₂SO₄ utilizing the heat of dilution of H₂SO₄. Unused K₂Cr₂O₇ is back-titrated with ferrous sulphate (FeSO₄.7H₂O) or ferrous ammonium sulphate (FeSO₄·(NH₄)₂SO₄·6H₂O).

**Reagents used:**

1 N Standard potassium dichromate solution (K₂Cr₂O₇):
   
   Dissolve exactly 49.04 g reagent grade K₂Cr₂O₇ (dried at 105°C for 2 Hr.) in distilled water and diluted to 1 liter in volumetric flask.

0.5 N Ferrous sulphate (FeSO₄·7H₂O) or ferrous ammonium sulphate solution:
   
   Dissolve 140 g of FeSO₄·7H₂O or 196.1 g reagent grade
FeSO$_4$(NH$_4$)$_2$SO$_4$,6H$_2$O in about 800 ml water, add 20 ml conc. H$_2$SO$_4$, cool and dilute to 1 liter in volumetric flask.

**Diphenylamine indicator:**
Dissolve 0.5 g diphenylamine in a mixture of 20 ml water and 100 ml of conc. H$_2$SO$_4$.

**Sulphuric acid:** Concentration not less than 96 % (Sp.Gr.1.84). If high amount of chloride (Cl$^-$) is present in the samples add Ag$_2$SO$_4$ at the rate of 15 g / liter to the acid.

**Orthophosphoric acid (85 %)** and/or sodium fluoride chemically pure/pure grade.

**Procedure:**
Accurately weighed 1 g (0.01 g accuracy) of 0.2 mm (80 meshes) soil and placed it in a dry 500 ml Erlenmeyer flask (corning / Pyrex). Include two blanks to standardize FeSO$_4$ or FeSO$_4$, (NH$_4$)$_2$SO$_4$ solution. Add exactly 10 ml potassium dichromate solution- Swirl the flask gently and keep it on asbestos sheet. Rapidly add 20 ml concentrated H$_2$SO$_4$ by directing stream into the suspension. Swirl the flask again 2 to 3 times. Allow the flask to stand on the asbestos sheet for 30 minutes.
Add about 200 ml distilled water. After addition of 10 ml of phosphoric acid or 0.5 g sodium fluoride and 1 ml of diphenylamine indicator, titrate the contents with ferrous (ammonium) sulphate solution till the colour flashes from blue violet to green. If the burette reading is 0 - 4 ml, repeat with less soil. If it is 17 ml or higher repeat with more soil.

**Calculation:**

Organic carbon (%) = \[ ((10 \times (B - T) ÷ B) \times (0.003 \times 100) ÷ (wt. \ of \ soil \ g) ] \]

Where
B - Volume (ml) of FeSO$_4$, 7H$_2$O or FeSO$_4$, (NH$_4$)$_2$SO$_4$, 6H$_2$O solution required for blank titration.
T - Volume of FeSO$_4$, 7H$_2$O or FeSO$_4$, (NH$_4$)$_2$SO$_4$, 6H$_2$O solution needed for titration of soil sample.

Actual organic carbon (%) = Organic carbon estimated × 1.3.

There is incomplete oxidation of organic matter in this procedure. The organic carbon is multiplied by 1.3 on the assumption that there is 77 % recovery.

Organic matter (%) = Actual C (%) × 1.724

The Van Bemmelen factor of 1.724 is used because organic matter contains 58 % C.

**Estimation of Nitrogen**
Estimation of nitrogen in soil, Kjeldahl method, modified by Jackson, (1958) was followed

Reagents:
i. Digestion mixture: 10g of potassium sulphate (K$_2$SO$_4$) and 1 g of copper sulphate (CuSO$_4$, 5H$_2$O) were ground to a fine powder and mixed thoroughly.

ii. Conc. Sulphuric acid (H$_2$SO$_4$).

iii. Boric acid (H$_3$BO$_3$) solution: A 4% solution of boric acid made in warm distilled water.

iv. Sodium hydroxide solution, 10 N.

v. Standard hydrochloric acid solution, 0.1 N and

vi. Methyl red indicator solution (alcoholic).

**Method:-**

To a 100 ml Kjeldahl flask, added 3 g of digestion mixture and 5 ml of treated soil solution. This was kept on a suitable flame until it was clear (greenish-blue) and continued heating for 5 more minutes. It was cooled and diluted to about 15 ml using 10 N, NaOH to under neutralize. Chilled the flask in ice water, wiped the bottom and attached it to a distillation apparatus, fixed a 100 ml conical flask containing 10 ml of boric acid solution and a couple of drops of methyl red indicator, at the receiving end. Added 20 to 30 ml of distilled water to the boric acid solution in the conical flask to ensure that the receiving end of the funnel dips into the boric acid solution. Added 5 to 8 ml of 10 N NaOH from the side tube into the Kjeldahl flask to over neutralize the solution. Heated the flask for distillation and distilled about 20 ml or so of solution. Drew the receiver conical flask down and then stopped heating. When distillation stopped, washed the end of receiving funnel that was dipping into boric acid solution. Titrated the contents of the conical flask with 0.1 N HCl solution and noted the volume of HCl used.

A blank without soil was run and the value was reduced from the titration figure.

**Calculation:**

\[
\text{Nitrogen} = \frac{1.4 \times \text{Vol.of HCl} \times \text{Normality of HCl} \times 100}{\text{Dilution factor} \times (0.5)\%}
\]

4. **Estimation of Available Phosphorus:**

For determining plant available P in soils, two methods are commonly used in India. The Olsen’s method (Olsen et al., 1954) is used for neutral-alkaline soils while the Bray and Kurtz P$_1$ method (Bray and Kurtz, 1945) is used for acidic soils.
Olsen’s (NaHCO₃) Method:
Sodium bi carbonate (NaHCO₃) solution extracts some exchangeable or surface adsorbed Al-P, Fe-P, calcium phosphate and other phosphates.

Reagents:

Sodium bicarbonate (Olsen’s reagent) 0.5M NaHCO₃, pH 8.5: Dissolve 84 g NaHCO₃ in water and make up to 2 liter. Mix thoroughly. Adjust to pH 8.5 with 1 M NaOH (4 g NaOH/100 ml) solution. Usually 20 - 25 ml NaOH solution is required for 2 liter NaHCO₃ solution. Store in glass or polyethylene bottle.

Reagent A: Dissolve 12 g ammonium molybdate ((NH₄)₆Mo₇O₂₄, 4H₂O) in 250 ml of distilled water. Dissolve 0.2908 g antimony potassium tartrate (K(SbO)C₄H₄O₆.H₂O) in 100 ml water. Add these two solutions to 1000 ml of 2.5 M H₂SO₄, mix thoroughly and make to 2000 ml. Store in Pyrex glass Bottle in a dark at cool place.

Reagent B: Dissolve 1.056 g ascorbic acid (C₆H₈O₆) in 200 ml reagent A and mix. Do not keep it for more than 24 h at room temperature. Prepare daily fresh as required

Sulphuric acid 2.5 M: Dilute 140 ml of conc. H₂SO₄ to 1 liter distilled water.

Standard stock P solution: Dissolve exactly 0.439 g potassium dihydrogen orthophosphate (KH₂PO₄) A.R. grade (dried in oven at 60°C for 1 h and cooled in desiccators) in half a liter of distilled water. Add 25 ml of 7NH₂SO₄ (approx.) and make to one liter with distilled water. This gives 100 ppm P standard stock solution. From this, a 2 ppm solution is made by diluting it 50 times.

Standard Curve:

To prepare the standard curve, 1, 2, 3, 4, 5 and 10 ml of 2 ppm P solution are taken in 25 ml volumetric flasks. To these 5 ml of the extracting solution (Olsen’s or Bray and Kurtz P₁) is added. When Olsen’s extractant is used, acidify 5 ml aliquot with 2.5 M H₂SO₄ to pH 5.0. Add distilled water to make the volume to 20 ml and then add 4 ml of reagent B. Make the volume with distilled water mix. Prepare a blank with NaHCO₃ solution, distilled water and 4 ml of reagent B. After waiting for 10 minutes, read the intensity of the blue colour in a photoelectric colorimeter using 730-840 nm filters or on a spectrophotometer at 882 nm (Watanabe and Olsen, 1965). Some workers prefer reading at 730 nm on spectrophotometers as the higher wavelength (882 nm) is not found sensitive.

Procedure:

Weigh 2.5 g 2 mm air dried soil (0.1 g accuracy) into a 150 ml Erlenmeyer flask.
Add a little of Darco G 60 or equivalent grade P-free activated charcoal. Then add 50 ml of Olsen’s reagent (soil to solution ratio of 1:20) and shake on the reciprocating shaker for 30 min (180 oscillations/min). Similarly, run a blank without soil.

Filter through Whatman no. 40 or 42 filter paper into a clean and dry beaker. Shake the flask immediately before pouring suspension into funnel. Place a 5 ml aliquot of the extract in a 25 ml volumetric flask and acidify with 2.5 M H\textsubscript{2}SO\textsubscript{4} to pH 5.0. Add distilled water to 20 ml and then add 4 ml of reagent B. After waiting for 10 min, read the intensity of blue colour on a spectrophotometer or colorimeter as described for standard curve above.

**Calculation:**

$$\text{Available P (kg/acre)} = \frac{R \times \text{volume of extract}}{\text{Volume of aliquot} \times \frac{2.24 \times 1000000}{\text{Wt. of soil} \times 1000000}}$$

Where,

$R$ - $\mu$g P in the aliquot (obtained from standard curve)

5. **Estimation of Exchangeable Potassium:**

The term available potassium (K) conventionally refers to exchangeable + water soluble K. The exchangeable K constitutes the major portion of available K except in saline or saline-sodic soils. Available K or exchangeable K, along with Ca and Mg are usually determined in neutral normal ammonium acetate (N NH\textsubscript{4}OAc) extract of soil. The extraction is carried out by shaking followed by filtration or centrifugation. The K is estimated by using a flame photometer and Ca and Mg either by EDTA - titration or by using atomic absorption spectrophotometer. In soils with appreciable amount of soluble K, Ca and Mg, these cations are estimated in a saturation extract (Jackson, 1958) and deducted from N NH\textsubscript{4}OAc extractable K, Ca and Mg to obtain respective exchangeable cations.

**Reagents and Standard Curve:**

**Ammonium acetate: 1.0 N, pH 7.0:** To 700 ml of distilled water, add 57 ml 99.5 % glacial acetic acid (CH\textsubscript{3}COOH) and then 69 ml of conc. ammonium hydroxide (NH\textsubscript{4}OH). Dilute to a volume of 900 ml and adjust pH to 7.0 by the addition of more of 3N NH\textsubscript{4}OH or 3N CH\textsubscript{3}COOH and make up to 1 liter. Store in Pyrex or polypropylene bottle. Alternatively, dissolve 154 g ammonium acetate (CH\textsubscript{3}COONH\textsubscript{4}) in water and dilute to 1.8 liters. Mix thoroughly. Adjust pH to 7.0 with dilute NH\textsubscript{4}OH or HOAc as required and make to 2 liters.
**Potassium chloride standard solution:**

Make a stock solution of 1000 ppm K by dissolving 1.908 g of AR grade potassium chloride (dried at 60 °C for 1 h) in distilled water and diluting up to 1 liter. Prepare 100 ppm standard by diluting 100 ml of 1000 ppm stock solution to one liter with the extracting solution.

**Standard curve:** Pipette 0, 5, 10, 15 and 20 ml of 100 ppm solution into 100 ml volumetric flasks and bring the volume to mark with extracting solution. The solutions contain 0, 5, 10, 15 and 20 ppm K respectively.

**Procedure:**
Shaking and filtration can be done by the method of Schollenberger and Simon, (1945) or shaking and centrifugation as described by Knudsen et al., (1982)

**Shaking and filtration (Schollenberger and Simon, 1945)**
Place 5 g soil in a 150 ml Erlenmeyer flask and pour in 25 ml of neutral N ammonium acetate (pH 7.0). Shake on a reciprocating shaker (180 + oscillations / min) for 5 minutes and immediately filter through Whatman No. 1 filter paper. First few ml of the filtrate may be rejected.

**Shaking and centrifugation (Knudsen et al., 1982)**
Place 10 g of < 2 mm air dried soil (or use 5 g if the soil contains greater than 500 ppm K) in a 50 ml centrifuge tube. Add 25 ml of NH₄OAc stopper and shake the tube for 10 min. Centrifuge the tube at 2000 rpm for 10 min until the supernatant liquid is clear. Decant the supernatant liquid into a 100 ml volumetric flask. Make three additional extractions in the same manner. Dilute the combined extracts to 100 ml with NH₄OAc.
Mix the solution and determine K in the extract in the flame photometer using K filter after necessary setting and calibration of the instrument (Use 0 and 20 ppm working K concentrations).
Read similarly the different concentration of K in flame photometer and obtain the standard curve by plotting the reading against the different concentrations of K.

**Calculation:**

\[
\text{Available K (kg/ha)} = \frac{R \times \text{Volume of extract} \times 2.24 \times 1000000}{\text{wt. of soil taken} \times 1000000}
\]
Where,

\[ R = \ldots \text{ppm of K in the extract (obtained from standard curve)} \]

**Study of growth parameters**

The tomato plants were grown in the fields at different localities. Growth parameters were measured at the interval of 15 days from second month (60 days, 75 days, 90 days and 105 days). Height of the shoot, number of leaves, number of branches, number of bud bunches length of leaflets, number of fruits and the height of the roots of mycorrhizal and non mycorrhizal plants were measured to study the comparative account of growth.

**Isolation and quantification of Chlorophyll by Arnon’s method**

Chlorophyll is a green photosynthetic pigment in the leaves of all the plants. Chlorophyll was extracted from the leaves of mycorrhizal and non mycorrhizal plants by Arnon’s method (1949). Fresh leaves of mycorrhizal and non mycorrhizal plants were plucked and one gram samples each were weighed. The leaves were crushed in 20 ml 80% chilled acetone using chilled mortar and pestle. The slurry was centrifuged at 5000 rpm for five minutes. The supernatant was collected and the residue was again homogenized with 80% acetone and centrifuged. This was continued till the residue lost all its green pigment and finally turned nongreen. The supernatants were collected and the final volume was made up to 100 ml by using 80% acetone. The 80% acetone was used as a blank and the absorbance of the samples was read at 645 and 663 nm using UV-visible spectrophotometer.

**Isolation and extraction of DNA**

The process of isolation was done by using the method proposed by Murmur et al., (1961). The DNA was isolated from 2 g sample from leaves of mycorrhizal and non mycorrhizal plants. The material was ground in 5 ml C TAB buffer which was a cationic surfactant and also removed the carbohydrates. The paste was transferred to the microfuge tubes and the tubes were incubated at 65°C in water bath for 15 minutes. The tubes were centrifuged at 12000 rpm for 5 minutes at 4°C. The supernatant was transferred to the eppendorff tubes and equal amount of PCI (Phenol-chloroform-Isoamyl alcohol) was added. This was done to remove the proteins and pigments from the material. Phenol precipitated the proteins and chloroform formed a
sharp phase difference. The precipitated proteins accumulated on the phenol and DNA remained in the aqueous interphase.

The samples were centrifuged at 10000 rpm for 2 minutes at 4°C. To the upper aqueous phase 1/10th volume of sodium acetate was added and incubation was done for 5 minutes. The DNA formed a sodium salt and precipitation took place. To the samples, 500 µl of pre-chilled isopropanol was added so that the DNA threads started appearing. The samples were centrifuged at 10000 rpm for 2 minutes at 4°C. The centrifugation led to formation of pellet. To this mixture, 500 µl of ice cold ethanol was added and the mixture was again centrifuged at 10000 rpm for 2 minutes at 4°C. The pellet was dried by inverting the eppendorff tube on a tissue paper. The pellet was re suspended in 100 µl TE buffer and then stored at 4°C. The absorbance was read at 260 nm using TE buffer as blank.

**Estimation of protein**

The protein content of leaves, green fruits and red fruits was estimated by Lowry *et al.*, (1951) method. The plant tissues from mycorrhizal and non mycorrhizal plants were washed and 0.5 g of each sample was crushed in 5 ml 0.1M phosphate buffer (pH-7). The contents were centrifuged at 5000 rpm for 2 minutes. The supernatants were used as the source of protein. The stock solution was prepared by dissolving 50 mg BSA (Bovine Serum Albumin) in 50 ml distilled water. Working standard was prepared by diluting 10 ml stock to 50 ml by distilled water (200 µg/ml). This working standard was taken in a series of test tubes and final volume was adjusted to 1 ml with distilled water. For protein estimation, 0.1 and 0.2 ml of tomato sample was used and the total volume of these test tubes was adjusted to 1 ml with distilled water. To all the test tubes, 5 ml of reagent C was added including the blank and the test tubes were allowed to stand for 10 min after gentle mixing. Then 0.5 ml of diluted Folin-Ciocalteau reagent was added to all the test tubes and all the test tubes were incubated for 30 minutes in dark.

Phosphomolybdic-Phosphotungstic components of Folin-Ciocalteau reagent got reduced by tyrosine and tryptophan in the protein to produce blue colour. Protein in the sample also reacted with the alkaline cupric tartarate to produce blue colour. The blue colour was read at 660 nm against the blank (distilled water, reagent C and Folin Ciocalteau reagent). The standard graph was drawn with the help of the BSA
readings. The protein readings were plotted on the standard graph and the calculations were done for 1 g sample.

**Estimation of total carbohydrates**

Total carbohydrates in the leaves, green tomatoes and red tomatoes were determined by phenol sulphuric acid method proposed by Krishnaveni *et al.*, (1984). For the estimation, 100 mg of mycorrhizal and non mycorrhizal tissue was weighed. The tissue was hydrolyzed by adding 5 ml 2.5N HCl and boiling in hot water bath for three hours. After cooling, it was neutralized using solid sodium carbonate until effervescence ceases. The final volume was made to 100 ml and centrifuged. From this sample, 0.1 and 0.2 ml solution was pipetted out in two separate test tubes. The volume of the test tube was made to 1 ml by using water. To the test tubes, 1 ml phenol and 5 ml 96% H$_2$SO$_4$ was added. The tubes were kept for 10 minutes and shaken well. These test tubes were kept in hot water bath at 20-30°C for 20 minutes. The absorbance was recorded at 490 nm after cooling by using the mixture of 1 ml water, 1 ml phenol and 96% H$_2$SO$_4$.

For the standard readings, glucose solution was used. A standard glucose solution was prepared by dissolving 100 mg in 100 ml water. Working standard was prepared by diluting 10 ml of standard glucose by 90 ml water. In hot acidic medium, glucose gets dehydrated to hydroxymethyl furfural which forms green colour with phenol. The amount to total carbohydrates is calculated by using the standard graph drawn with the help of readings obtained using working standard.

**Estimation of reducing sugars**

The reducing sugars were estimated by dinitrosalicylic acid method proposed by Miller (1972). The reducing sugars were estimated from the leaves, green and red tomatoes of mycorrhizal and non mycorrhizal plants. From the materials, 1 g of the tissues were weighed and crushed in hot 5 ml 80% ethanol twice. The mixtures were centrifuged at 5000 rpm for 2 minutes and the supernatants were collected. The supernatants were evaporated on a hot plant till 2-3 ml amount was left. Little amount of distilled water was added to the supernatants and the reducing sugars were dissolved properly. The final volume was adjusted to 10 ml. These extracts were again centrifuged at 5000 rpm for 5 minutes.
The standard glucose solution was prepared by dissolving 100 mg glucose in 100 ml distilled water (1mg/ml). The working standard was prepared by diluting 10 ml stock with 100 ml distilled water (100µg/ml). From the working standard, 0.5 to 3 ml was pipetted out and the volumes were equalized to 3 ml by adding distilled water. From the leaf supernatants, 0.5 ml and from the fruit supernatants 0.1 ml was used for the sugar estimation. To each test tube, 3 ml of DNSA reagent was added and the contents were heated in a boiling water bath for 15 min. To the warm contents, 1 ml of 40% Rochelle salt solution was added. The reducing sugars (glucose, fructose) reduced the orange DNSA to 3-amino-5-nitrosalicylic acid to produce red colour. The colour intensity indicated the amount of reducing sugars which were read at 510 nm against blank of only distilled water and DNSA using UV-visible spectrophotometer. A standard graph was drawn and the amounts of reducing sugars of samples were calculated using it.

**Estimation of Peroxidase**

The peroxidase amount of the leaves of mycorrhizal and non mycorrhizal plants was estimated using the method proposed by Putter (1974) and Malik (1980). From the samples 1 g of leaves was weighed and grinded in 3 ml 0.1M phosphate buffer (pH 7.0). The buffer was chilled and the mortar and pestle was also pre chilled. The homogenate was centrifuged for 15 minutes at 5°C at 10,000 rpm in cold centrifuge. The supernatant was stored in ice and it was used as the source of enzyme. The absorbance was measured at 436 nm against the blank of 3 ml buffer, 0.05 ml guaiacol and 0.1 ml enzyme. The blank was devoid of substrate H₂O₂. In the second cuvette, 0.1 ml enzyme extract, 3 ml buffer, 0.05 ml and 0.03 ml H₂O₂ was added for reading the absorbance. The absorbance increased by 0.05. The stop watch was started and the time required to increase the absorbance by 0.1 was measured. Guaiacol reacted with hydrogen peroxide and got oxidized in presence of peroxidase. The rate of guaiacol oxidation was measure of peroxidase activity which was measured at 436 nm using UV- spectrophotometer.

**Estimation of Polyphenol oxidase**

The enzyme was extracted from 0.5 g leaves of mycorrhizal and non mycorrhizal plants by a rapid and sensitive method proposed by Esterbaner (1977).
The samples were crushed in 5 ml chilled medium containing 50 mM Tris-HCl, pH 7.2, 0.4 M Sorbitol and 10 mM NaCl using chilled mortar and pestle. The slurries were centrifuged at 10,000 rpm for 10 minutes at 5\(^\circ\)C in cold centrifuge. The supernatants were used as the source of enzyme. In a clean cuvette, 2.5 ml phosphate buffer and 1 ml of substrate catechol was added. In the second cuvette, in addition to the above mixture, 0.5 ml enzyme was added to initiate the reaction. The absorbance was immediately recorded at 495 nm. The decrease in the absorbance was noted after every 30 seconds for 5 minutes.

**Estimation of acid phosphatase**

The acid phosphatase was measured using the leaves, green fruits and red fruits of mycorrhizal and non mycorrhizal plant by using method proposed by Lowry et al., (1954). From the fresh tissues 1 g sample was weighed and it was homogenized in 10 ml of ice cold 50 mM citrate buffers (pH 6.5) in a cold mortar and pestle. The slurry was filtered through four layered muslin cloth and then centrifuged in a cold centrifuge at 10,000 rpm for 10 minutes. The supernatant was used as the source of enzyme.

The substrate was prepared by dissolving 1.49g EDTA, 0.84 g citric acid and 0.03 g p-nitrophenyl phosphate in 100 ml distilled water and the pH was adjusted to 5.3. For the standard readings, p-nitrophenol was used.

From the substrate, 3 ml was incubated at 37\(^\circ\)C for 5 minutes in the incubator. Then, 0.5 ml of extract was added to it. Immediately, 0.05 ml was removed from it and it was mixed to 9.5 ml of 0.085N sodium hydroxide. This was treated as the blank. The remaining substrate and enzyme mixture was further incubated for 15 minutes at 37\(^\circ\)C. After 15 minutes, 0.5 ml was drawn and mixed with 9.5 ml sodium hydroxide. Phosphatase enzyme hydrolyzed the p-nitrophenol phosphate and produced yellow colour in the alkaline conditions. A series of standards was taken in the test tubes, diluted with 10 ml water and the absorbance was measured at 405 nm to draw the standard curve. The sample readings were plotted on the standard curve. The specific activity of the enzyme was expressed as m moles of p-nitrophenol released per minute per mg protein.
Estimation of alkaline phosphatase

The alkaline phosphatase was measured from the leaves, green and red fruits of the mycorrhizal and non mycorrhizal plants by using the method proposed by Lowry et al., (1954). For this, 1 g plant tissues were weighed and crushed in ice cold 50 mM glycine NaOH buffer with pH 10.4 in cold mortar and pestle. The slurry was filtered through four layered muslin cloth and then centrifuged in a cold centrifuge at 10,000 rpm for 10 minutes. The supernatant was used as the source of enzyme.

The substrate was prepared by dissolving 375 mg glycine, 10 mg magnesium chloride, 165 mg p-nitrophenyl phosphate in 42 ml of 0.1N sodium hydroxide. This solution was diluted to 100 ml by using distilled water. The pH was adjusted to 10.5.

From the substrate, 3 ml was incubated at 37°C for 5 minutes in the incubator. Then, 0.5 ml of extract was added to it. Immediately, 0.05 ml was removed from it and it was mixed to 9.5 ml of 0.085N sodium hydroxide. This was treated as the blank. The remaining substrate and enzyme mixture was further incubated for 15 minutes at 37°C. After 15 minutes, 0.5 ml was drawn and mixed with 9.5 ml sodium hydroxide. Phosphatase enzyme hydrolyzed the p-nitrophenol phosphate and produced yellow colour in the alkaline conditions. A series of standards was taken in the test tubes, diluted with 10 ml water and the absorbance was measured at 405 nm to draw the standard curve. The sample readings were plotted on the standard curve. The specific activity of the enzyme was expressed as m moles of p-nitrophenol released per minute per mg protein.

Estimation of Ascorbic acid

Ascorbic acid is commonly called as vitamin C. It is water soluble and heat labile. Estimation of vitamin C was done by volumetric method proposed by Sadasivam, Balsubramanian (1987) and Harris and Ray (1935).

Stock standard was prepared by dissolving 100 mg ascorbic acid in 100 ml oxalic acid (1mg/ml). Working standard was prepared by diluting 10 ml of stock standard to 100 ml with distilled water (100µg/ml). In a conical flask 5 ml of working standard and 10 ml of 4% oxalic acid was taken and titrated against DCPIP (2,6
dichloro phenol indophenol) dye from the burette. The end point was appearance of pink colour which persisted for a few minutes.

The sample was prepared by crushing 1 g of mycorrhizal and non mycorrhizal fruits (green and red) in 4% oxalic acid and centrifuging at 500 rpm for 2 minutes. The final volume was made to 100 ml. For titration 5 ml of this sample extract was added to 10 ml of 4% oxalic acid and was titrated against the DCPIP dye. Ascorbic acid reduced the blue dye to colourless leucobase. The ascorbic acid was oxidized to dehydro ascorbic acid. The end point was appearance of pink colour as the dye took the pink colour in the acidic medium. For the same reason, oxalic acid was taken as a titrating medium. The amount of DCPIP was equal to the amount of vitamin C.

**Estimation of phenols**

The phenols were estimated from the leaves, green and red fruits of mycorrhizal and non mycorrhizal plants by method suggested by Malik and Singh (1980). For phenol estimation 1 g of each sample was crushed in 10 ml 80% ethanol. The homogenates were centrifuged at 10,000 rpm for 20 minutes. The supernatant was taken. The residue was re extracted by using 80% ethanol. The sample was again centrifuged and the supernatants were pooled. The supernatants were evaporated to dryness. The residue was dissolved in 5 ml distilled water. For preparing standard phenol solution, 100 mg catechol was dissolved in 100 ml water and for the working standard the stock was diluted ten times. A series of the working standard was pipetted out in the test tubes and the volume in all the test tubes was made to 3 ml. From the supernatants, 0.1 ml was pipetted out and the volume was made to 3 ml. To all the test tubes, 0.5 ml of Folin-Ciocalteau reagent was added ad after three minutes, 2 ml of 20% Na2CO3 was added to all the test tubes. After mixing the contents thoroughly, the test tubes were placed in boiling water bath for one minute. The absorbance was measured at 650 nm against the reagent blank when the test tubes cooled. The standard curve was obtained from the series of standard readings and the absorbance of the samples was plotted on this curve.

**Isolation of lycopene**

Lycopene is a red coloured pigment distinctly found in the red tomato fruit. It is a carotenoid and its formula is C_{40}H_{56}. Lycopene was estimated using the method
proposed by Ranganna (1976). From the mycorrhizal and non mycorrhizal red fruits, 5 g tissues along with the pulp and the skin were weighed. The tissues were crushed using mortar and pestle. Acetone was repeatedly added to the pulp to extract the red pigment till the pulp turned colourless. A separating funnel was arranged containing 20 ml petroleum ether. All the acetone extracts were pooled and then added to the separating funnel. Acetone and petroleum ether were gently shaken. Some petroleum ether may get evaporated during this procedure. Hence more 20 ml ether was added to the separating funnel. The 20 ml 5% sodium sulphate solution was added to this mixture, the funnel was gently shaken and was allowed to stand for half an hour. The two layers got separated and the colour was noticed in the upper ether layer. The two phases were separated. The pigment was re-extracted from the lower phase by adding 20 ml ether. This was continued till the aqueous phase was colourless. Petroleum ether extracts were pooled and washed once with distilled water. This petroleum ether was poured in a brown bottle containing 10 g anhydrous sodium sulphate. The bottle was kept aside for 45 minutes. Then the petroleum ether was decanted in a flask containing a funnel having cotton wool. Sodium sulphate slurry was washed with petroleum ether till it was colourless. The volume of petroleum ether was made to 100 ml and the absorbance was measured against pet ether as blank at 503 nm using UV-spectrophotometer.

**Mass multiplication**

The rhizosphere soil was used for the trap culture of AM fungi. The spores were isolated and identified in the laboratory. The spores of five genera were identified these genera were *Acaulospora, Entrophosphora, Glomus, Gigaspora* and *Scutellospora*. The *Acaulospora, Scutellospora* and *Glomus* species were used for the pure culture by using two monocots i.e. maize (*Zea mays*) and jowar (*Sorghum vulgare*).

The rhizosphere soil and the roots of tomato were obtained from all the three sites. The soil and the sand mixture were filled in the pots in the proportion of 3:1. The roots were chopped into pieces and mixed well in this mixture. Sterilized maize seeds were sown in the soil and were allowed to grow for three months. The spores sporulated and increased in number during this period. These spores were later used for the pure culture. To confirm the enhanced growth of mycorrhizal plants, control
maize plants were also grown simultaneously. There was a marked difference in the growth of mycorrhizal and control plants.

The pure cultures were done using the monocotyledonous seed as their life cycle is short and secondly they produce fibrous roots. Maize seeds and jowar seeds were used for the pure culture. The seeds were washed with running tap water and then were washed with 0.1% HgCl$_2$. The seeds were washed by distilled water three times. The Petri plates with blotting papers were autoclaved. Sterile distilled water was used to wet the blotting papers. The seeds were transferred to the Petri plates under the aseptic conditions and allowed to germinate for two days. The seeds sprouted within two days and the small radicles were seen on the third day.

The spores were freshly isolated from the soil, identified and then surface sterilized with 0.02% streptomycin and then were washed with distilled water (Norris et al., 1992). The sterilized spores were inoculated of studded on the radicles under the aseptic conditions. The radicles were allowed to grow in the Petri plates for three days. The radicles grew further and this was accompanied with sprouting of the plumule and the leaves were developed on the plumule. The points where the spores were studded on the radicles turned brown in colour. This indicated the infection and the start of colonization of fungi. These seedlings were now ready to shift to the soil.

The mixture of soil and sand were prepared in the proportion of $3:1$. The sand maintains the aeration in the soil. This mixture was autoclaved and filled in the pots. The seedlings were transferred in the pots. The seedlings were grown in the shade for the first week and they were watered regularly. The care was taken during watering and the mixing of the soil samples was avoided. The plants were grown for the two months. The soil samples and the roots were used to confirm the pure culture. The soil was preserved in polythene bags. This soil was used for identification of AM fungi up to species level.

These soil samples were used for production of AMF inoculum on large scale and as a biofertilizer for other crop plants like chili, fenugreek etc.

**Statistical Analysis**

The vegetative and biochemical data of both the control and mycorrhizal plants at all the three surveyed sites was collected at an interval of 10 to 15 days. The
average of the data was calculated which was followed by calculation of its standard deviation. The level of significance was calculated with the help of students T test at probability level 0.05.