MATERIALS AND METHODS

The details of the materials used and experimental techniques (methods) adopted during the investigation are described as following. Proposal for the investigation of quality, quantity and effectiveness of Arbuscular mycorrhizal fungi is displayed in plate 3.

SURVEY OF SOLANACEOUS VEGETABLE CROPS

A survey was conducted from October 2010 to March 2011 in Osmanabad, Latur, Parbhani, Beed, Jalna and Aurangabad districts in the farmers’ field of Marathwada region (17° 35’ N to 20° 40’ N Latitude and 70° 40’ E to 78° 15’ E Longitude) of Maharashtra state. Frequent visits were made to study mycorrhizal association at different growth stages of plants.

Sample Collection

Tomato (*Lycopersicum esculentum* Mill.), Chilli (*Capsicum annuum* L.) and Brinjal (*Solanum melongena* L.) plants were randomly collected from Osmanabad, Latur, Parbhani, Beed, Jalna and Aurangabad area during 2010-2011 for AM fungal association. On the field five mature plants were selected and then uprooted by loosening surrounding soil. About 500 g of rhizospheric soil of these plants of each species from every site was taken in separate polythene ziplock bags, care was taken to get soil sample at a depth up to 15-20 cm. Primary and secondary fine roots of uprooted plants were washed in water to remove adherent soil debris and then preserved in Formalin-Acetic-Alcohol (FAA) (Ethyl alcohol 50 mL, Glacial acetic acid 5 mL, Formaldehyde (37-40%) 10 mL and Distilled water 35 mL) in specimen bottles.
Plate III - Proposal for the investigation of quantity, quality and effectiveness of Arbuscular Mycorrhizal Fungi (AMF)
PLATE - I

Samples collection sites

Map of Maharashtra Showing Geographical Location of Study Sites
MATERIALS AND METHODS

PLATE - II

Field Visit

Rhizosphere Soil Collection

Field Visits for Collection of Rhizosphere Soil
ASSESSMENT OF ARBUSCULAR MYCORRHIZAL FUNGAL COLONIZATION IN ROOTS

AMF colonization in Tomato, Chilli and Brinjal roots collected from different study sites were studied according to Phillips and Hayman (1970) and Dalpé and Séguin (2013).

Root clearing

At the time of root colonization assessment, preserved roots of Tomato, Brinjal and Chilli were washed in water to remove traces of FAA. Nearly 20-30 root segments of 2-3 cm length were added in 50 mL beaker half filled with 10 % KOH to facilitate stain penetration in cortex tissue. Beaker was placed in oven for two hours at 70°C. Roots were heated till depigmentation. In some cases microwave oven (30 seconds) was used for KOH treatment.

Rinsing and acidification of root tissues

The root segments were rinsed 2-3 times in water to dilute KOH residue and then immersed in 15 mL of Hydrochloric acid (5 %) for 2 minutes at room temperature to improve the root staining efficiency.

Root staining

Acidified roots were washed 2 to 3 times in water and immersed in trypan blue (0.05 %) for overnight period. Destaining was done to remove excess stain from root tissues using water.

Slide mounting

Stained root segments were mounted on microscopic slides in polyvinyl lactic acid glycerol (PVLG) [(Polyvinyl alcohol 8.33g, distilled water 50mL, lactic acid 5mL, glycerine 5mL) (A dry powder polyvinyl alcohol was added to
the water in beaker and kept in oven at 60°C to dissolve it. Lactic acid and glycerine was then added. Solution was used after 24 hours) medium (Omar et al. 1979; Koske and Tessier, 1983). Roots were observed under the compound microscope (LOBAMED Vision 2000) and photographed with a Sony digital camera (DSC-W310/BC E37).

A root was considered mycorrhizal when hyphae, vesicles and arbuscules alone or in any combination of these were present. Root colonization was measured according to the Giovannetti and Mosse (1980) using the following formula

$$\text{Root colonization (\%)} = \frac{\text{Number of colonized segments}}{\text{Total number of segments examined}} \times 100$$

Mycorrhizal root length colonization was quantitatively calculated according to McGonigles et al. (1990) as well as Bierman and Linderman (1981).

**ISOLATION AND QUANTIFICATION OF AMF SPORES**

**Sample collection**

Polythene bags of field collected rhizospheric soil of Tomato, Brinjal and Chilli were brought to laboratory. Soil was dried at room temperature for 48 hours. These soil samples were mixed to form a composite soil sample of every plant species from every site. These soil samples were stored at 4°C until processing.

A part of composite soil samples was used for isolation and quantification of AM fungal spores. Spore density and spore diversity was studied from every sample collected of Tomato, Chilli and Brinjal. A portion of the soil was reserved as a source of native AMF for mass multiplication and for study of physicochemical characters of soil.

Field collected soil was rich in organic debris, sucrose density centrifugation technique (Walker et al., 1982) was also some times used to
separate spores from the organic debris. Glomalean fungal spores were isolated from soil by using wet sieving and decanting method of Gerdemann and Nicolson (1963). Few samples were repeated according to Ohms (1975), Gaur and Adholeya (1994) and Muthukumar et al. (1996).

**Spore isolation**

Hundred grams of field collected and dried composite rhizosphere soil was suspended 1000 mL of tap water in glass beakers. Soil macroaggregates were crushed with glass rod. Suspension was left undisturbed for 10 minutes to allow the heavier soil particles to settle down. After 10 min soil suspension was passed through the stack of sieves in a descending order having mesh size 355 µm, 210 µm, 150 µm, 125 µm, 63 µm and 25 µm. The procedure was repeated to recover maximum spores from soil. The remains on each of the sieves were washed. The sievings on each mesh was collected into separate small beakers. Sieveing was then filtered using graduated Whatman filter paper. Filter paper having spores was placed in a petri dish and observed under stereozoom microscope. Spores or sporocarps were collected on a small triangular piece of filter paper by using blunt-tipped needle.

**Spore quantification**

Five replicates from composite soil of each species collected from field were subjected for spore density quantification. Isolated spores from 100 g of soil were recorded. These collected spores were separated according to distinct morphological types. 2-5 spores of same morphotypes were mounted on a clean glass slide with a drop of PVLG. PVLG was allowed to set for 2-5 minutes to become more viscous. A clean cover slip was placed on drop of PVLG and entire spores were observed for morphological study. Later on spores were crushed by applying light to moderate pressure on the cover slip with the tip of a needle. Different wall layers were observed in these crushed spores.
Materials and Methods

Spores were identified according to Schenck and Perez (1990), original descriptions in literature those provided by International collection of vesicular arbuscular mycorrhizal fungi (INVAM.). Characters such as presence or absence of sporocarp, structure of peridium, spore size, shape, colour, wall ornamentation, subtending hyphae and mode of attachment were considered for identification of spore. After identification spore population was estimated.

The corelation between root length colonization percentage and spore density in rhizosphere soil was calculated according to Bewick et al. (2003) and denoted by Pearson correlation coefficient ‘r’.

\[ r = \frac{\sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2 \sum_{i=1}^{n} (y_i - \bar{y})^2}} \]

Herbarium

AMF spores are preserved in the herbarium in pot soil, in vials with suitable medium and permanent slides as voucher specimens. The slides were sealed with DPX on the edges and are kept for drying. After drying voucher specimens of AM fungi were deposited in Department of Botany, Arts, Science and Commerce College NalDurg, Maharashtra, India.

Taxonomy of AM Fungi

Intact AMF spores were mounted on a glass slide in water and observed under binocular compound microscope (100x and 400 x; LOBAMED Vision 2000) and photographed with a Sony digital camera (DSC-W310/BC E37). Morphological characters like colour, shape, surface texture, ornamentation, and dimentions were measured. Spores were slightly cracked by applying pressure and spore wall configuration (outer wall layers and germinal wall layers) was noted. The same process was repeated by mounting the spores in
PVLG and Meltzer’s reagent, the observations were again made after 24 hours after clearing of spores.

Glomalean spores were identified according to Schenck and Pérez (1990), Walker (1983), original descriptions in literature (Vaingankar and Rodrigues 2011; Schüßler 2000; Mosse and Bowen 1968; Koske et al. 1986; Muthukumar et al. 2005; Bukhari and Rodrigues 2006) and culture database established by International Collection of Vesicular Arbuscular Mycorrhizal (http://invam.wvu.edu/the-fungi) fungi.

PHYSICO-CHEMICAL CHARACTERS OF SOIL

A part of compoite soil collected from field was used for physicochemical characterization. Soil was spread out on a tray for air drying. It was sieved over a 2 mm sieve and used for characterization. Soil analysis was done for colour, pH, Electrical Conductivity, Organic carbon %, and major and minor nutrients [Niirogen (kg / hector), Phosphrous (kg / hector), Potassium (kg / hector), Calcium (meq.), Magnesium (meq.), Sodium (meq.), Zinc (ppm), Ferrous (ppm), Mangenese (ppm), Copper (ppm) and Boron (ppm)].

pH of the soil was measured potentiometrically in a 1:5 soil – water suspension by pH meter. Electrical Conductivity (dS/m) which provides concentration of soluble salts in the soil was measured in 1:5 soil-water suspensions by conductivity meter. Organic Carbon was evaluated by Walkely and Black (1934) method by oxidizing organic carbon with potassium dichromate and sulphuric acid. Available Nitrogen was assessed by alkaline permanganate method by using Kjeldhal tube (Subbiah and Asija, 1956). Available Phosphorus in soil was determined by Olsens method by using spectrophotometer (Olsen et al, 1954) and Bray & Kurtz (1945). Water soluble and exchangeable Potassium was calculated by Ammonium acetate method of Hanway and Heidel (1952) using Flame photometer. Calcium and Magnesium
cations were estimated by EDTA titration (GOI, 2011b). Analysis of Ferrous, Mangenese, Copper and Zinc were done by acid digestion of soil (Jackson, 1967).

Pearson correlation was studied between AMF colonization and nutrient availability in the rhizosphere soil. Another effort was made to correlate spore population studied with quantity of nutrients present in soil in natural field conditions.

MASS MULTIPLICATION OF INDIGENOUS AM FUNGI

Spores collected from soil deteriorate with time therefore they are used for only identification at genus level. Morphology of spores is a basis for identification of AM fungi. For detailed observation and identification upto species level, multiplication of AM fungus in pot culture was done (Rodrigues, 2006; Habte and Osorio, 2001). Trap pot cultures were set for increasing number of indigenous inoculum.

Inoculum

Rhizosphere soil of Tomato, Brinjal and Chilli collected from different study sites was mixed to form a composite soil, this composite soil containing spores, mycelia and root fragments were used as inoculum for mass multiplication of AM fungi.

Trap culture

Local crop field soil was collected; it was mixed with locally collected river sand to prepare 1:1 mixture. This mixture was used as substrate for pot culture. Substrate soil was autoclaved for 1 hour at 120°C, it was air dried. Soil sand mixture was autoclaved for three times and used for trap cultures. 15 cm diameter pots were three fourth filled with autoclaved substrate, a layer of 100g of previously prepared soil inoculum was placed over it and later on a thin
layer of substrate soil was placed on it. Five sets of trap cultures were raised to get sufficient inoculum for next experiment.

Surface sterilized (2% sodium hypochloride) and distilled water washed seeds of Maize (Zea mays), Jowar (Sorghum vulgare), Wheat (Triticum aestivum) and Sunflower (Helianthus annuus) was sown in pots. These trap culture pots were placed in open air, the plants were irrigated regularly every week upto field level capacity. Fertilizer in the form of Hogland’s solution minus phosphorus (Ross, 1974) was added two times within the growth period. After 20 days, a portion of the root was checked for AM colonization and after 90 days soil samples were collected from each pot for the identification and estimation of different types of indigenous AMF spores.

Shoot portion was discarded and soil alongwith roots and spores were used as inoculum for further experiment.

GROWTH RESPONSE OF PLANTS TO AMF AND OTHER FUNGAL SPECIES

A pot experiment was carried out to study the interaction of AMF and other rhizospheric biocontrol fungi with respect to growth parameters on Tomato cv. TO1389 (Syngenta India Limited, Pune, Maharashtra), Brinjal cv. Panna ARBH928 (Ankur Seeds Private Limited, Nagpur, Maharashtra) and Chilli cv. SITARA (Monsato Holdings Private Limited, Rangareddy, Andhra Pradesh). The experiment was conducted in the garden of Botany department, Arts Science and Commerce College, Naldurg (15° 26' North latitude and 75° 7' East longitude and an altitude of 678 m above mean sea level).

Local crop field soil was collected it was used as substrate for pot culture. Substrate soil was autoclaved for 1 hour at 120°C, it was air dried. Soil was autoclaved for three times and used for the study of growth response of Tomato, Brinjal and Chilli plants to AMF and other fungal species. 15 cm diameter pots were three fourth filled with autoclaved substrate, a layer of 100g
of AMF multiplied soil inoculum from trap cultures was placed over it and later on a thin layer of substrate soil was placed on it.

The experiment consisted of seven treatments with various inoculums combinations. The details of treatments was as given below.

T1: Control

T2: Indigenous Arbuscular Mycorrhizal Fungi

T3: Indigenous Arbuscular Mycorrhizal Fungi + *Trichoderma viride*

T4: Indigenous Arbuscular Mycorrhizal Fungi + *Trichoderma harzianum*

T5: Indigenous Arbuscular Mycorrhizal Fungi + *Aspergillus niger*

T6: Indigenous Arbuscular Mycorrhizal Fungi + *Alternaria solani*

T7: Indigenous Arbuscular Mycorrhizal Fungi + *Rhizopus stolonifer*

Pot soil and root segments of jowar plant produced during mass multiplication experiment were used as indigenous AMF inoculums while rhizospheric fungi *Aspergillus niger, Alternaria solani, Rhizopus stolonifer* and two *Trichoderma* species viz. *T. harzianum* and *T. viridae* used in this study which were was collected from the crop field soil and isolated and identified in the laboratory.

Mycelial disc of 2 cm diam. of *A. niger, A. solani, R. stolonifer, T. harzianum* and *T. viride* from 7days-old pure culture grown in laboratory were transferred to 50 mL PDA in a 250 mL conical flask. It was incubated at room temperature for 7 days. At the end of the incubation period 100 ml of sterile distilled water was added. Flasks were shaken and the content of conical flask was applied as inoculum in soil for pot experiment.

Previously surface sterilized five seeds of Tomato cv. TO1389, Brinjal cv. Panna (ARBH928) and Chilli cv.Sitara were sown 2 cm below the soil
MATERIALS AND METHODS

These experimental pots were placed in open air, the plants were irrigated regularly every week up to field level capacity. Fertilizers were not supplied at any of the growth stage. After 20 days, a portion of the root was checked for AM colonization and after 90 days plants were harvested for the study of growth parameters.

Harvest method is the basic technique for biomass measurement. Before harvest number and area of leaves was measured by disc method. Fifty leaf disc of known size were taken from randomly selected leaves of plant, discs and remaining leaf blades were oven dried and leaf area was calculated by using formula suggested by (Vivekanandan et al., 1972).

Height of individual plants was measured from the ground level. Plants were uprooted loosening the soil care was taken to get maximum amount of roots. Root length of each plant was measured. Plants (Shoot including leaves, flowers and fruits) were cut at the ground level mark. Fresh biomass of shoot and root was measured using digital balance. Plants (shoot and root) were dried in oven for 24-48 hours at 80°C up to constant weight, cooled and weighed for dry weight. After weighing, dried plant material (shoot and root) was stored in polthene ziplock bags for the study of biochemical effects of AM fungi alongwith biocontrol agents.

The mycorrhizal efficiency (MEI) index was calculated according to Bagyaraj (1994) using dry weight by following formula.

$$\text{Mycorrhizal Efficiency Index} = \frac{\text{Wt. of Inoculated plant} - \text{Wt. of Uninoculated Plant}}{\text{Wt. of Inoculated Plant}} \times 100$$

Interactive effect of other fungi (IEF) with AMF was calculated by considering biomass of AMF (dry weight) treated plant as mycorrhizal control. Interactive effect was calculated by using following formula

$$\text{IEF} = \frac{\text{Wt. of AMF and other fungi treated plant} - \text{Wt. of AMF plant}}{\text{Wt. of AMF and other fungi treated plant}} \times 100$$
BIOCHEMICAL EFFECTS OF AM FUNGI WITH BIOCONTROL AGENTS ON PLANTS

Tomato, Brinjal and Chilli plants collected at harvest during the study of growth response of plants to AMF and other fungal species were dried. Whole dried plant material including root and shoot was grinded in Grinder and Mixture. The ground material was collected in polythene bags and was used for biochemical analysis. Plant material was subjected to analyse nutrients such as Nirogen, Phosphrous, Potassium, Calcium, Magnesium, Sulphur, Sodium, Zinc, Ferrous, Copper, Mangenese, Molybdenum and Boron.

For Assessment of various nutrients Subbiah and Asija (1956), Olsen et al. (1954), Hanway and Heidel (1952), GOI (2011b) and Jackson (1967) methods were used.

Mycorrhizal nutrient efficiency (MNE) was studied to describe the mycorrhizal effect on nutrient uptake by using the formula of Wu and Zou, (2009).

\[ \text{Mycorrhizal Nutrient Efficiency (MNE)}(\%) = \frac{E_i - E_{ni}}{E_{ni}} \times 100 \]

Where \( E_i \) and \( E_{ni} \) were the values of nutrient element of plants treated by AMF and non-AMF.

Interactive Nutrient Effect of other fungi (INE) for nutrient efficiency over AMF was calculated by considering nutrient value of AMF treated plant as mycorrhizal control. Interactive effect (Excess nutrient content in the plant tissue than Mycorrhizal (AMF) plant due to interaction of both fungi) was calculated by using following formula

\[ \text{INE} = \frac{\text{Nutrient elements of AMF and other fungi treated plant} - \text{Nutrient elements of AMF plant}}{\text{Nutrient elements of AMF plant}} \times 100 \]
STATISTICAL ANALYSIS

The data collected from the experiment were analysed statistically following the procedure given by Bewick et al. (2003), McDonald (2009) and Mungikar (1997).