MATERIAL AND METHODS
Objective 1: Study of diversity of AM fungi in different Agro-climatic conditions of Mysore region

Study Site:

In the present field survey, four southern districts viz. Hassan, Mandya, Mysore and Chamarajanagar of Karnataka state, India, were selected and samples were collected from different sites randomly (Table 3). Totally 29 study sites were selected which includes 29 samples for this investigation (Fig. 2). Most of the sites in Hassan district and few from Mysore and Chamarajanagar were non-irrigated agricultural field samples, whereas Mandya district samples were all irrigated agricultural field samples.

Geographically, Hassan district lying between 12°13’ & 13°33’ North latitudes and 75°33’ & 76°30’ East longitude and elevated at 934 (3189 ft) MSL, 195kms far away from Bangalore. Total area 6826.15 sq. km. Average annual rainfall is about 1031mm. Hassan district is subdivided into 7 taluks, among them 2 taluks are completely lies in Western Ghats, remaining five districts are plain lands and major food crops grown here are potato, maize, finger millet, common beans and some vegetable crops (Anon, 3).

Mandya district lies at 12.52°N and 76.9°E, elevated at 678 (2224 ft) MSL and 96 km far away from Bangalore. Average annual rainfall is about 700mm. Mandya district also subdivided into 8 taluks, among them we have selected five taluks for collection of samples from agricultural fields. Mandya is one of the Major producers of Sugarcane and Rice in Karnataka state. Other food crops are Maize and other vegetable and pulse crops. Mandya district is well facilitated with channel irrigation, as the Cauvery river pass through this district and K.R.S reservoir situated in Mandya district (Anon, 4).

Mysore is at 770m above sea level and 140kms from Bangalore. It has an area of 6,268 sq kms, geographically lies 11°30’ N to 12°50’ N latitudes and 75°45’ E to 77°45’ E longitudes. Average rainfall annually is around 86 centimeters. Most of the Mysore area is semi-arid region. Majority of the agricultural systems depend on monsoon, except few regions are well facilitated with river water and tube well irrigation. As the region identified as semi-arid, the major food crops of this region are Paddy, Maize, Sorghum, Ragi, Tobacco and few other vegetable crops (Anon, 5).
Fig 2: South Karnataka map showing different sampling sites of the present research work.
Chamarajanagar district has an area of 1235.9 Sq. Kms and lies 11°55′34″N 76°56′25″E and it is almost completely arid zone, average annual rainfall received is about 798.2 mm, district partially irrigated and partially arid zone. The major food crops growing in this region are Maize, Sorghum, pulse crops, Ragi, etc. (Anon, 6).

All the sampled agricultural fields were practicing conventional type farming i.e. use of chemical fertilizers mainly and organic manure rarely, which is generated from their own practice and irrigation is either through normal river water (Channel water) or pumping through underground water table (Tubewell) (Table 3). Semi-arid areas which are designated as non-irrigated were completely dependent on rain only.

Collection of Soil samples

The rhizospheric soil and root samples were collected from all the four districts for a period of 2008 to 2010. Totally 29 sampling sites were noted (Table 3). Approximately 500 g of rhizosphere soil from each site were collected. The rhizosphere samples were taken from a particular plant block covering an area of 100m² randomly from a depth of 10-30cm from different standing crop in the fields. Soil particles adhered to fine roots were removed by generous shaking and roots connected to each sampled plant were also collected to quantify their arbuscular mycorrhizal status and stored in standard FAA solution to assess percent colonization (Fig. 3). The soil samples were combined to make the composite samples, which were air dried and stored at 4°C.

Analysis of Soil chemical properties

Collected soil samples were divided into two subsamples for the purpose of physico-chemical analysis and isolation of spore. Subsamples were air-dried for 2 weeks and stored at 4°C. Examination of soil physico-chemical properties were done at Central Sericultural Research and Training Institute (CSR & TI), Mysore to record different soil parameters like pH, Electrical conductivity (EC), Organic carbon (OC), Phosphorous (P) and Potassium (K).

Isolation and Identification of AM fungi

Isolation

Spores of arbuscular mycorrhizal fungi were isolated by following wet sieving and decanting method described by Gerdemann & Nicolson (1963). The step wise procedure is as follows:
Fig 3: a-h, Collection of soil and root samples from different agricultural fields of Mysore region.
<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Site of collection</th>
<th>Sample name</th>
<th>Crop rotation</th>
<th>Irrigation &amp; type of irrigation</th>
<th>Standing crop at the time of sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Arasiekere</td>
<td>ARSK</td>
<td>Sunflower, Maize.</td>
<td>No.</td>
<td>Sunflower (<em>Helianthus annuus</em> L.)</td>
</tr>
<tr>
<td>2.</td>
<td>Hassan</td>
<td>HSN-1</td>
<td>Ragi, Maize, potato.</td>
<td>No.</td>
<td>Finger millet (<em>Eleusine coracana</em> (L.) Gaertn.)</td>
</tr>
</tbody>
</table>

Table 3: Name of the sampling sites, Standing crop and type of irrigation of sampling sites of Mysore region
<table>
<thead>
<tr>
<th></th>
<th>District</th>
<th>Code</th>
<th>Crops</th>
<th>Irrigation Method</th>
<th>Crops Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Pandavapura</td>
<td>PDP</td>
<td>Vegetables and pulses.</td>
<td>Yes. Tube well irrigation</td>
<td>Black gram (<em>Vigna mungo</em> (L.) Hepper)</td>
</tr>
<tr>
<td>15</td>
<td>S.R. Patna</td>
<td>SRP</td>
<td>Pulses, Rice.</td>
<td>Yes. Channel water irrigation</td>
<td>Horse gram (<em>Macrotyloma uniflorum</em> (Lam.) Verdc.)</td>
</tr>
<tr>
<td>16</td>
<td>Mysore</td>
<td>MYS-1</td>
<td>Maize, tobacco.</td>
<td>No</td>
<td>Maize (<em>Zea mays</em> L.)</td>
</tr>
<tr>
<td>17</td>
<td>Mysore</td>
<td>MYS-2</td>
<td>Sorghum, Maize.</td>
<td>No</td>
<td>Indian bean (<em>Dolichos lablab</em> L.)</td>
</tr>
<tr>
<td>18</td>
<td>Nanjangudu</td>
<td>NJD</td>
<td>Sorghum, Maize.</td>
<td>No</td>
<td>Sorghum (<em>Sorghum bicolor</em> (L.) Moench)</td>
</tr>
<tr>
<td>19</td>
<td>Piriapattana</td>
<td>PRPT</td>
<td>Vegetables.</td>
<td>No</td>
<td>Capsicum (<em>Capsicum annuum</em> L.)</td>
</tr>
<tr>
<td>20</td>
<td>T. Narasipura</td>
<td>TNP</td>
<td>Ragi, Maize, Pulses.</td>
<td>No</td>
<td>Maize (<em>Zea mays</em> L.)</td>
</tr>
<tr>
<td>21</td>
<td>Hunsur</td>
<td>HNSR</td>
<td>Vegetables and pulses.</td>
<td>Yes. Tube well irrigation</td>
<td>Brinjal (<em>Solanum melongena</em> L.)</td>
</tr>
<tr>
<td>22</td>
<td>K.R. Nagara</td>
<td>KRN</td>
<td>Pulses, vegetables.</td>
<td>Yes. Tube well irrigation</td>
<td>Tomato (<em>Solanum lycopersicum</em> L.)</td>
</tr>
<tr>
<td>23</td>
<td>H.D. Kote</td>
<td>HDK</td>
<td>Vegetables, Pulses.</td>
<td>Yes. Tube well irrigation</td>
<td>Brinjal (<em>Solanum melongena</em> L.)</td>
</tr>
<tr>
<td>24</td>
<td>Gundlupet</td>
<td>GPT</td>
<td>Ragi, Sorghum.</td>
<td>No</td>
<td>Sorghum (<em>Sorghum bicolor</em> (L.) Moench)</td>
</tr>
<tr>
<td>25</td>
<td>Begur</td>
<td>BGR</td>
<td>Pulses, Ragi.</td>
<td>No</td>
<td>Pigeon pea (<em>Cajanus cajan</em> (L.) Millsp.)</td>
</tr>
<tr>
<td>26</td>
<td>Chamarajanagar-1</td>
<td>CHN-1</td>
<td>Maize, Sugarcane.</td>
<td>Yes. Tube well irrigation</td>
<td>Maize (<em>Zea mays</em> L.)</td>
</tr>
<tr>
<td>28</td>
<td>Yelandur</td>
<td>YLDR</td>
<td>Maize, Ragi, Pulses.</td>
<td>Yes. Tube well irrigation</td>
<td>Maize (<em>Zea mays</em> L.)</td>
</tr>
<tr>
<td>29</td>
<td>Kollegala</td>
<td>KLGL</td>
<td>Rice.</td>
<td>Yes. Tube well irrigation</td>
<td>Rice (<em>Oryza sativa</em> L.)</td>
</tr>
</tbody>
</table>
**Step 1.** 100 g of air-dried root-rhizosphere soil mixture is added into a glass or plastic container with 1000 ml of tap water.

**Step 2.** The root-soil mixture is vigorously mixed with a glass rod for 30 sec. Soil macro-aggregates should be crushed with hand.

**Step 3.** After 10-second pause enabling to settle heavier particles and organic material, the remaining soil-root-hyphae-spore suspension is slowly poured through a set of two sieves. The procedure should be repeated until the upper layer of soil suspension is transparent. The sieves used are those with pores of diameters of 0.5 (the top one) and 0.045 mm. Most spores retain on the 0.045 mm sieve. The top sieve isolates large sporocarps and spores associated with roots. (Sieve: Sieves with various mesh size. At least the following mesh size are required; 1 mm, 100 μm and 50 μm. Other sizes such as 500μm and 250μm are preferable.)

**Step 4.** The extracts are washed away from the sieves to beaker. The collected spore suspension was added on glass petridish (with the help of ink filler) which contain whatman no.1 filter paper.

**Step 5.** Using a stereo microscope (Labomed-CZM6), spores, aggregates, and sporocarps are picked by means of pipette or needle. The gravity of spores is a little lighter than that of soil particles. Successive decantation of soil suspension followed by sieving with fine mesh can concentrate the spores from soil. Since the spores are globular or sub–globular in 50–500 μm in diameter, in sieving they can be recognized under a dissecting microscope.

**Sucrose Centrifugation Technique**

Spores and minimal amount of organic particles were further purified by resuspending sievings (residue collected from sieves) in the 40% sucrose solution and centrifuge. Sievings are transferred to 10 ml centrifuge tubes with a fine stream of water from a wash bottle and balance opposing tubes, centrifuged at 1,200 to 1,300 x g for 3 min, allowing the centrifuge to stop without braking. The supernatant was removed carefully to avoid disturbing the pellet and then with a finger the organic debris that adheres to the side of the tube were removed. Soil particles were suspended in chilled 1.17 M sucrose, mixed the contents with a spatula and centrifuged the samples immediately at 1,200 to 1,300 x g for 1.5 min. The supernatant was poured through the small mesh sieve, the spores held on the sieve were carefully rinsed with tap water and the spores washed into a plastic petri dish.
When the root-rhizosphere soil mixture contains a large amount organic material, the isolation of spores is preceded with density gradient centrifugation (Furlan et al., 1980).

**Observation of spores**

Spores were mounted with polyvinyl lactoglycerol (PVLG) and Meltzer’s reagent on a slide glass to record the morphological characteristics such as shape, size, colour and the hyphal attachment to spores.

**Composition of PVLG (poly-vinyl lacto-glycerol)**

Polyvinyl alcohol (polymerization 1000-1500), 1.66 g, is dissolved in 10ml of deionized water. Complete dissolution may need 6 hours at 80 ºC. The dissolved polyvinyl alcohol is mixed with 10 ml of lactic acid and 1ml of glycerol. It can be used more than a day after preparation.

**Composition of Melzer’s reagent**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlortal hydrate</td>
<td>100 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Iodine</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>5.0 g</td>
</tr>
</tbody>
</table>

More permanent mounts are made by mixing Melzer's reagent with PVLG in a volume ratio of 1:1 (and storing the mixture in a dark bottle).

**Identification of AMF spores**

The spores and sporocarps thus isolated were observed under compound microscope, and later photographed with Research Microscope (OLYMPUS BX-63). Identification of AM species was carried out by consulting suitable keys of species descriptions as provided by Schenck & Perez (1984) and INVAM. The following diagnostic characteristics of AM fungal spores were used for the identification of the genera and species.

**Sporocarps:** The dimensions, the pattern of spore arrangement inside the sporocarps, the presence or absence of peridium were noted.
**Size and shape of the spores:** Undamaged spores were selected and observed under microscope by mounting them in a drop of water on a clean slide. The shape of the spores and size (dimension) were measured with the help of software provided with photographic microscope.

**Subtending hypha:** The presence or absence of attached hypha(e) (stalk) to spore, thickness, whether simple or bulbous, constricted or funnel shaped, straight or recurved, pore open or occluded were recorded.

**Wall layers:** Ornamentation, the number, the thickness, and the colour of the wall layers were recorded under high magnification.

**Spore contents:** The appearance of cytoplasmic contents, globular, reticulate or vacuolated was also recorded.

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**Objective 2: The status of AM fungi associated with some commonly growing crop plants**

**Collection of Root samples:**

The rhizospheric soil and root samples were collected from naturally growing plants in four districts namely Hassan, Mandya, Mysore and Chamarajanagar of South Karnataka for a period of 2008 to 2010. Totally 29 sampling sites were noted (Table 1). Roots of standing crop were collected by digging the soil and in some cases uprooting the whole plant (Fig 3). Plants were randomly selected in an area of 50 m². Soil particles adhered to fine roots were removed by generous shaking and roots of each sampled plant were collected to quantify their arbuscular mycorrhizal status and stored in standard FAA solution to assess percent colonization.

**Clearing and staining of roots**

The cleaned roots were cut into 1cm long pieces and stained with Trypan blue, according to the procedure described by Philips & Hayman (1970). The roots were autoclaved in 10% KOH for 15 min. at 15 lb pressure. The KOH solution was poured off retaining the bits inside the test tube and the root bits were washed thrice with tap water. The dark coloured roots (pigmented) wherever necessary were treated with alkaline H₂O₂ solution (6% v/v) till the root segments were bleached properly followed by washing thrice again with tap water as before to remove H₂O₂. Later the roots
were acidified with 5N HCL for 3-5 min. and washed with water and stained by immersing the roots in 0.5% Trypan blue prepared in lactophenol keeping in boiling water for 30-60 min, until the roots stained satisfactorily. After removing the excess of stain, if any, with lactophenol, the root bits were mounted serially on a clean glass slide in lactophenol covered with cover slip and observed under microscope for AMF association. The structures like arbuscules, vesicles and mycelium were considered for presence of AMF association.

**Composition of stains and Fixatives**

<table>
<thead>
<tr>
<th><strong>Standard FAA solution:</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formalin</strong></td>
<td>15ml</td>
</tr>
<tr>
<td><strong>Glacial Acetic acid</strong></td>
<td>05ml</td>
</tr>
<tr>
<td><strong>Alcohol (70%)</strong></td>
<td>200ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Lactophenol:</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenol</strong></td>
<td>20ml</td>
</tr>
<tr>
<td><strong>Lactic acid</strong></td>
<td>20ml</td>
</tr>
<tr>
<td><strong>Distilled water</strong></td>
<td>20ml</td>
</tr>
<tr>
<td><strong>Glycerine</strong></td>
<td>40ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Trypan blue stain:</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phenol</strong></td>
<td>10ml</td>
</tr>
<tr>
<td><strong>Glycerine</strong></td>
<td>20ml</td>
</tr>
<tr>
<td><strong>Lactic acid</strong></td>
<td>10ml</td>
</tr>
<tr>
<td><strong>Distilled water</strong></td>
<td>10ml</td>
</tr>
<tr>
<td><strong>Trypan blue</strong></td>
<td>0.25gm</td>
</tr>
</tbody>
</table>

**Assessment of Percent (%) root colonization**

The assessment of mycorrhizal infection was done by the simple slide method (Giovannetti & Mossae, 1980). Root segments were selected randomly from the stained samples and observed for the presence or absence of functional structures (Mycelium, Arbuscules and Vesicles) of AM fungi. A minimum of 100 root segments were used for the enumeration and percent colonization of AM fungi was calculated using the following formula;

\[
\text{Percentage colonization} = \frac{\text{Total Number of root samples colonized}}{\text{Total number of root segments observed}} \times 100
\]
Statistical analysis

Spore density, species richness, isolation frequency (IF), relative abundance (RA) and Shannon-Wiener index of diversity were conducted as follows: spore density was defined as the number of AMF spores and sporocarps in 100gm soil; species richness was measured as the number of AMF species occurred per soil sample; IF= (the number of samples in which a given species was isolated/ the total number of samples) x100%; RA=(the number of a given species spore/the total number of spores) x 100%; Shannon-Wiener index of diversity  

\[ H = - \sum p_i \ln ( p_i ) \]

where \( p_i \) is the proportion of total number of species made up of the ith species (Li et al., 2007). Pearson’s correlation coefficient (r) was calculated between percent colonization, spore population, soil pH, soil ‘P’ and soil ‘K’, using SPSS software (version 17.0).

Objective 3: Strain selection of AM fungi by following trap culture method

Mass multiplication of AM fungi

AM fungi are obligate in nature and cannot be grown in vitro. For the AM fungal inoculum, spores collected form soil can be used. However, spores in soil are not always active in colonizing plants. Therefore, mass multiplication was done to get enough inoculum of monospecific strain by trap culture using suitable host. The methodology followed in the present investigation to get monospecific inoculum is as follows.

Trap culture method

Trapping is necessary to obtain many healthy spores of colonizing fungi for identification and as inoculum to establish monospecific cultures. Spores collected directly from a field soil were used for trap culture.

Potting medium

Various potting materials for horticulture can be also used. However, the materials for potting medium should be low in available phosphate and preferably not rich in organic matter. In some cases the fungi isolated from some specific soils may need the specific soil properties for their growth. In the present work, sterile soil or soil-sand mixture was used. The physico-chemical parameter of the soil used in present study is follows;
<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Sample Name</th>
<th>pH</th>
<th>EC mmhos/cm</th>
<th>OC (%)</th>
<th>Available P (kg/ha)</th>
<th>Available K (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sterilized</td>
<td>7.67</td>
<td>0.44</td>
<td>0.51</td>
<td>22.89</td>
<td>538</td>
</tr>
<tr>
<td>2.</td>
<td>Unsterilized</td>
<td>7.42</td>
<td>0.56</td>
<td>0.97</td>
<td>19.41</td>
<td>493</td>
</tr>
</tbody>
</table>

**Host plant**

Selection of effective host for multiplication of AMF is very important. Many experiments were conducted to find out which short lived plant host such as maize, ragi or sorghum promotes maximum AM infection and spores production for selecting inoculum preparations. Riju & Hosagoudar (2012) observed that maize plants had produced more spore inoculum compared to sorghum plants. Therefore on the basis of these finding Ragi (*Eleusine coracana*) and Maize (*Zea mays*) plant were selected for first generation of trap cultures, later for the next two generations Maize plants was used for further multiplication of inoculum successively.

**Preparation of Pure Culture**

- To purify an isolated fungus, single species spores isolation is needed. Morphologically similar spores or the spores belonging to same genus or species are grouped. (Sometimes even if the spores are morphologically identical, it often contains contaminants whose morphology is very similar).
- In our current research, initially six species of AM fungi were selected for the mass multiplication of inoculum through trap culture method. They are *Glomus fasciculatum*, *Glomus mosseae*, *Glomus microcarpum*, *Acaulospora scrobiculata*, *Acaulospora foevata* and *Acaulospora myriocarpa*. Later only three species were selected on the basis of sporulation and colonization. They are *Glomus fasciculatum*, *Acaulospora scrobiculata*, and *Acaulospora foevata*.
- Seven days old seedling (grown in sterilized soil) placed under stereomicroscope microscope. Single spore was picked up and transferred on fine roots or root tip of the seedling. Similarly 10-15 spores were placed all along the root (Fig. 6 &7).
- The seedling which possessed spores on its root was carefully placed inside the potting medium (initially in paper cups) by making a cavity with the help of glass rod. Then seedling was properly covered with soil and watered regularly. All trap plants were fertilized with Hoagland’s solution once in 25 days.

- After the host plant attained 90 days of growth, watering was stopped and allowed the plants to wilt. After complete wilting of the plants, the root bits of trap plant and dried soil containing spores were mixed well and stored at 4-5°C for further use.

- After recovering soil inocula from all the pots 100gm of soil inoculum from each pot was taken separately to review the spore number and successiveness. The first generation soil inoculum which consisted spores of single species and root bits were again re-cultured for two more generations to increase the number of spores in the soil inoculum(Fig. 8). The stored soil inoculum was directly used for further pot experiments.
Fig 6: a, Preparation of Ragi seedlings (15 days); b, Seedlings were carefully removed from the pot by immersing it in a water container; c, Seedlings were washed and kept in a plastic tray containing water; d-e, Single species spores were collected in eppendorf tubes; f, Potting medium was prepared in small paper cups and labeled.
Fig 7: g, Preparation of potting medium; h-i, Spores were placed along the surface of root of seedling under stereo microscope; j-k, Seedlings containing spores were carefully inserted inside potting medium (a deep cavity earlier made) using glass rod; l, Potting medium containing seedlings containing single species spores.
Fig 8: **m**, Trap cultured plants of second generation; **n-p**, after harvesting third generation trap cultured plants, soil containing roots were mixed thoroughly and stored.
Objective 7: Efficacy of AM fungi on growth and yield enhancement of Common Bean

In the present investigation common bean (*Phaseolus vulgaris*, var. Arka Komal) was selected to study the effectiveness of selected species of AM fungi. Systematic classification of common bean is as follows;

Division : Magnoliophyta  
Class : Magnoliopsida  
Order : Fabales  
Family : Fabaceae  
Subfamily : Faboideae  
Genus : *Phaseolus*  
Species : *Phaseolus vulgaris* L.

Healthy beans seeds were taken and surface sterilized (in 95% ethanol for 30 sec. and then for 90 sec. with 0.5 mg ml$^{-1}$ of HgCl$_2$) before sowing in the pot containing soil medium. Potting medium consists of soil + sand mixture in the ratio of 5:1. Two type of potting medium was used along with the inoculation, viz. sterile soil + sand mixture and unsterile soil + sand mixture. AMF inoculum was added @ 200 gram/pot in different layers starting from middle through top and 3cm soil was added on top layer of inoculum. Surface sterilized bean seeds were sown at the rate of 6 seeds per pot. The following experimental setups were maintained in five replicates for each treatment.

- Sterile soil + Sand (positive control) – (St + C)
- Sterile soil + Sand + AMF (*Glomus fasciculatum*) – (St + I1)
- Sterile soil + Sand + AMF (*Acaulospora foevata*) – (St + I2)
- Sterile soil + Sand + AMF (*Acaulospora scrobiculata*) – (St + I3)
- Unsterilized soil + Sand (negative control) – (USt + C)
- Unsterilized soil + Sand + AMF (*Glomus fasciculatum*) – (USt + I1)
- Unsterilized soil + Sand + AMF (*Acaulospora foevata*) – (USt + I2)
- Unsterilized soil + Sand + AMF (*Acaulospora scrobiculata*) – (USt + I3)
All the pots were placed in poly house condition and fertilized with Hoagland’s solution. The composition of Hoagland’s solution is as follows:

**Hoagland's Solution (Plant Nutrient Solution)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Solution</th>
<th>mL Stock Solution/1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>2M KNO₃</td>
<td>202g/L</td>
<td>2.5</td>
</tr>
<tr>
<td>2M Ca(NO₃)₂ . 4H₂O</td>
<td>236g/0.5L</td>
<td>2.5</td>
</tr>
<tr>
<td>Iron (Sprint 138 iron chelate)</td>
<td>15g/L</td>
<td>1.5</td>
</tr>
<tr>
<td>2M MgSO₄ . 7H₂O</td>
<td>493g/L</td>
<td>1</td>
</tr>
<tr>
<td>1M NH₄NO₃</td>
<td>80g/L</td>
<td>1</td>
</tr>
<tr>
<td><strong>Minors:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>2.86g/L</td>
<td></td>
</tr>
<tr>
<td>MnCl₂ . 4H₂O</td>
<td>1.81g/L</td>
<td></td>
</tr>
<tr>
<td>ZnSO₄ . 7H₂O</td>
<td>0.22g/L</td>
<td></td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.051g/L</td>
<td></td>
</tr>
<tr>
<td>H₃MoO₄ . H₂O</td>
<td>0.09g/L</td>
<td></td>
</tr>
<tr>
<td>1M KH₂PO₄ (pH 6.0)</td>
<td>136g/L</td>
<td>0.5</td>
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1) Make up stock solutions and store in separate bottles with appropriate label.
2) Add each component to 800mL deionized water then fill to 1L.
3) After the solution is mixed, it is ready to water plants.

Plants were irrigated regularly to maintain proper moisture. After 85 days of plant growth, plants from all the pots were uprooted taking are to not to damage root cortical system. Plastic pots were made a cut longitudinally at both the sides and soil attached with the roots was removed carefully. Roots and shoots were separated to get the fresh weight and representative root samples from each treatment were collected and preserved in standard FAA solution for assessment of percent root colonization.

**Assessment of Fresh weight and Dry weight**

The root and shoot portions of the plants were separated. The root portion washed in running tap water to remove all the adhering soil particles and gently pressed in folds of filter paper to remove excess of moisture. The fresh weights of all the treatments were recorded. Later
the samples were wrapped in paper and placed in hot air oven at 72\(^{0}\)C for 48 hrs, then removed and cooled in desiccators and reweighed to get the dry weight.

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**Estimation of Nitrogen, Phosphorus and Chlorophyll**

**Estimation of Nitrogen**

In the present investigation, Kjeldahl method was followed for determination of total N. The methodology followed is as follows (Sawhney & Singh, 2006):

- 1gm of sample in the form dried shoot powder is added to Kjeldahl digestion tube along with 2 g of salt/catalyst mixture of K\(_2\)SO\(_4\) and CuSO\(_4\).
- Similarly, blanks containing only reagents with each set of samples were digested.
- 15 mL of concentrated H\(_2\)SO\(_4\) was added and slowly heat to 200\(^{0}\)C. Once the frothing has subsided, the temperature was brought up to 350\(^{0}\)C and heated until the digest was clears. Further, the digest was heated at 350 to 375\(^{0}\)C for an additional 35 minutes to 1 hour for clearing.
- The digest was cooled and added deionized water to makeup 100 ml in a 100ml volumetric flask.
- 10mL of 2\% H\(_3\)BO\(_3\)was added and few drops of mixed indicator solution (0.01 g of bromocresol green and 0.07 g of methyl red in 100 mL of ethyl alcohol (90\%)) was added to 100-mL flask and placed under the condenser with the condenser tube immersed inside the 2\% boric acid solution.
- The distillation unit programmed for 20 mL of 40\%NaOH per cycle. The Kjeldahl tube containing 10 mL of diluted digested sample was transferred to the distillation apparatus and began distillation. The distillation process was programmed for 3 min. After 3 min the flask containing boric acid solution was taken out.
- The NH\(_3\) which is distilled into the H\(_3\)BO\(_3\) solution was titrated using standard 0.1 M HCl. The end point was reached when the green solution goes to a pink color.

**Calculations:**

\[
\text{Percentage N} = \frac{(T - B) \times N \times 1.401}{\text{Sample taken}}
\]
Where, \( T = \text{mL of sample titrated} \)
\[ B = \text{mL of blank titrated} \]
\[ N = \text{acid normality} \]

**Estimation of Phosphorus**

**Digestion of sample**

Three gram of dried sample was taken, finely ground and transfer to a 100ml kjeldahl flask. 25 ml of mixture of conc. HNO_3, conc. HClO_4 and conc. H_2SO_4 (3:2:1) was added and shaken well ensuring that no dry lumps were left behind. A clean acid–washed glass beads was dropped into the flask to prevent bumping during digestion. The experiment was allowed to stand overnight in a fume-hood and then heated it on a digestion heater and watched out for foaming during the first hour especially in samples with high fat content. After completing the digestion, it was allowed to cool, filtered through Whatman No.40 filter paper into a 100ml volumetric flask. The contents of digested flask were quantitatively transferred by rinsing the flask 3-4 times with deionised water. The residue of silica was washed on the filter paper with very dilute HCL (1:19) diluted with water in order to wash down the salts completely. The volume of the collected filtrate was made up to 100ml.

**Estimation Procedure**

Estimation of phosphorus in the plant material was carried out by Colorimetric Method (Sawhney & Singh, 2006). 2ml of digested sample extract taken in 25ml volumetric flask. Few drops of 2,4-dinitrophenol indicator were added and neutralized the contents with 4N ammonia by adding few drops. Excess of ammonia was neutralized with 2N H_2SO_4 and the volume was adjusted to about two third of the flask with water. 1 ml of sulphomolybdate solution was added into it and the neck of the volumetric flask was washed with distilled water and 0.5 ml of freshly prepared stannous chloride solution was added.

The contents were mixed thoroughly and the volume made up to 25ml. Then within 4 to 20 min. the absorbance at 660 nm was recorded using a spectrophotometer. Same procedure was followed to prepare a standard curve containing 0.2 ppm to 1.0 ppm phosphorus. The amount of phosphorus in the sample was measured comparing to the standard curve and results were expressed as ppm phosphorous/gram dry wt. of the sample.
**Estimation of Chlorophyll**

1 gram fresh leaves were cut into small pieces and homogenize in a mortar with excess acetone, with a pestle. The supernatant was decanted and filtered on Bucher funnel through Whatman No.42 filter paper. Sufficient quantity of 80% acetone was added and repeated the extraction. The filtrates were pooled and the volume was made to 100ml in a volumetric flask. The absorbance of the extract at 645nm and 663nm was read for determination of total chlorophyll. The chlorophyll content was calculated on a fresh weight basis employing the following formula (Arnon, 1949).

\[
\text{Total chlorophyll (in mg.)} = 20.31 \text{A}_{645} + 8.05 \text{A}_{663}
\]

**Estimation of total protein**

**Extraction of protein**

The dry seeds without coat were ground with pestle and mortar and the fine powder produced (100 mg) was added to 1ml of 50mM Phosphate buffer (pH 7.8) for 20 minutes with constant stirring at room temperature on a rotator shaker. The samples were centrifuged at 10,000 g for 20 minutes to clarify the supernatant and finally stored at \(-20^\circ\) C. The supernatant was used for quantification purpose (Pereira et al., 2009).

**Estimation of total protein by Bradford method**

**Bradford reagent:** Coomassie Brilliant Blue G-250 (10mg.) was dissolved in 5 ml of 95% ethanol, to this solution 10 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 100ml. Solution was filtered through Whatman No.1 filter paper and stored in brown bottles.

**Phosphate buffer:** Sodium dihydrogen orthophosphate (0.0718gm.) and disodium Hydrogen orthophosphate (1.2 gm.) was dissolved in 100ml of distilled water and then pH was adjusted to 7.8.

**Preparation of standard curve**

- 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mg BSA (Bovine Serum Albumin) was weighed and made up to 0.1 ml with the buffer solution in respected test tubes.
Then 5 ml of brilliant blue reagent solution was added in all tubes and shaken well, and then absorbance was determined by the spectrophotometer at 595 nm.

Standard curve was prepared from the resulted absorbance (Bradford, 1967).

**Protein assay**

- 100 µl (0.1 ml) protein solutions were pipetted into 12 x 100 mm test tubes.
- 5 ml of Bradford reagent was added to the test tube and the contents were mixed either by inversion or vortexing.
- The absorbance at 595 nm was measured after 2 min and before 1 hr in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent.
- The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

**Statistical analysis**

All the experiments were conducted with five replicate, data were pooled and subjected to analysis by SPSS16.0 statistical software. Analysis of variance (ANOVA) was carried out, the means were compared by Duncan's Multiple Range Test ($P<0.05$).