MATERIALS AND METHODS

3.1 Study sites

Achanakmar (Chhattisgarh)

Achanakmar is situated in Bilaspur district of the Indian state of Chhattisgarh. Its geographic coordinates are 22°25'0''N 81°51'0''E. This biosphere reserve is recognized by UNESCO as biodiversity hotspot. It comprises 557.55 km² of forest, and is linked by the hilly Kanha-Achanakmar Corridor to the tiger reserve in Kanha, Madhya Pradesh. The Achanakmar wildlife sanctuary was established in 1975, under provisions of the Wildlife Protection Act of 1972. Achanakmar has been declared a Tiger Reserve under the Project Tiger in 2009. It is part of Bilaspur Forest Division in northwest Chhattisgarh, around 55 km north-west of Bilaspur. The sanctuary is close to Amarkantak and the source of the Narmada River. Forest vegetation mainly comprises of *Shorea robusta, Terminalia tomentosa, Pterocarpus marsupium* and *Bambusa* spp. The sanctuary is home to leopards, gaur, chital, Bengal tigers, striped hyena, Jackals the sloth bear, sambar, nilgai, four horned antelope, chinkara, blackbuck, and wild boar, among other species.

Amarkantak (Madhya Pradesh)

Amarkantak is a pilgrim town in Anuppur District in the Indian state of Madhya Pradesh, India and its geographic coordinates are 22.67ºN 81.75ºE with an elevation of 1048 meters. Soil is ferruginous and clayey, with an acidic or neutral reaction. Monsoon starts in July and lasts up to September but there is some rain in winter also. The temperate climate and the equitable distribution of rain make Amarkantak an ideal plateau for dense vegetation cover. The forest is sal-dominated; there are associate species such as *Mallotus philipensis, Buchanania lanzan, Ougeinia oojinesis, Terminalia chebula, Bauhinia spp, Grewia spp, Gardenia latifolia, Anogeissus latifolia*, together with climbers and shrubs such as *Bauhinia vahillii, Dioscorea smilax, Diospyros melanoxylon, Celastrus paniculata* and such ground flora as *Desmodium, Moghania, Sida, Crotalaria, leucas, Pogostemon bengalensis, Strobilantihes, Setaria, Oplismenus* etc.
Jabalpur (Madhya Pradesh)

Jabalpur is a district place in the Indian state of Madhya Pradesh; its geographic coordinates are 23.17ºN 79.95ºE. It has an average elevation of 411 meters. It has a humid subtropical climate, typically of North-Central India. Summer starts in late March and last up to June. May is the hottest month with average temperatures reaching up to and beyond 45ºC. They are followed by monsoon season, which last until early October, with a total precipitation of nearly 55 inch. Winter starts in late November and last until early March. The Narmada river bringing in fresh water from Amarkantak has developed Jabalpur district into an agrarian economy. The land of the Narmada basin with its fertile alluvial soil gives good yields of sorghum, wheat, rice and millet in the villages around Jabalpur. Tectona grandis, Shorea robusta and Eucalyptus spp. etc are also found in Jabalpur. There are many Non wood forest products also found in the forest such as Diospyros melonoxylon, Terminalia chebula, Buchanaia lanzan, flower and seeds of Madhuca indica and flowers, seeds, bark and roots of various plant species.

Jharsuguda (Odisha)

Jharsuguda district is situated at the north western part of Odisha. It is surrounded by Sundargarh district in the north, Sambalpur district in the east, Bargarh in the south and Chhatishgarh state in the west. It has an average annual rainfall of 1652 mm and situated in between 21 51’ 00” latitude and 84 02’ 00” longitudes. The climate of this place is tropical sub-moist humid and soil is acidic sandy-loam, yellowish brown which retain less amount of water. Due to high porosity of soil the rain water goes down fast and upon exposure to sunlight it respires back to the atmosphere. Intermittent heavy rainfall followed by direct sunlight maintains a condition of high humidity. Major forest tree species present at the site include Shorea robusta, Terminalia elliptica, Diospyros melonoxylon, and Schleichera oleosa etc while major associated flora present at the site includes Phoenix acaulis, Flemingia chapper, Saccopetalum tomentosum.
Fig. 1. A view of a sal forest (a) during rainy season; (b) deposition of litter in summer.
Fig. 2 (a-d sal fruit) (a-b) single fruit (c) viviparous germination (d) fallen on forest floor
Motinala (Madhya Pradesh)

Motinala is a forest division in the Mandla and Balaghat district of Madhya Pradesh state; its geographic coordinates are 22°21'0"N, 80°54'0"E. It is around 80 km. from Jabalpur. Climate is warm and temperate with an average annual temperature 23.4°C and rainfall is 1338 mm. The site has a dense forest cover of *Shorea robusta*. Some other tree species *Diospyros melonoxylon*, *Terminalia chebula*, *Buchania lanzan*, and *Madhuca indica* are also found.

3.2 Host Plant

**Sal (Shorea robusta Gaertn.f.)**

Sal forests of different densities were selected for the present study. The forests of Achanakmar-Amarkantak have high density (Figs.1a-b) while forests of Motinala, and Jharsuguda has moderate to low density.

3.3 Collection and preservation of ECM

The samples were collected from Achanakmar, Motinala, Amarkantak Biosphere Reserve (Chhattishgarh and Madhya Pradesh) and Jharsuguda (Odisha). Their natural habitat photographs were taken using a digital camera (Sony cyber shot 12 mega pixels).

These samples were collected in sterilized cellulose paper bags. For proper identification, washed 2-3 times with water, surface disinfected with 0.1% sodium hypochlorite for 10 minutes, and then washed again in repeated changes of sterilized water to remove the disinfectant. Samples were then preserved in FAA (Formaldehyde Acetic acid Alcohol solution) in the transparent plastic bottles. Samples were then preserved in FAA in the transparent plastic bottles and brought to the laboratory of Forest Pathology Division, Tropical Forest Research Institute Jabalpur, Madhya Pradesh, India.

*Preparation of Formaldehyde Acetic acid Alcohol (FAA) Preservative*

For preparation of FAA, ethyl alcohol (50%) 900 cc was used. In order to prepare it 450 ml distilled water was added in 450 ml of absolute alcohol.
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Subsequently 30 ml formaldehyde and 50 ml Acetic acid was added to 50% alcohol solution. It was then kept overnight at room temperature.

3.4 Calculation of frequency, occurrence and abundance (Agarwal, 2008)
Frequency and abundance of ectomycorrhizal fungi growing on forest floor under sal trees were determined by applying the following formulae:

\[
\text{% Frequency} = \frac{\text{Number of quadrates containing particular fungus}}{\text{Total number of quadrates}} \times 100
\]

\[
\text{% Occurrence} = \frac{\text{Number of fruit bodies of individual fungus in all the quadrates studied}}{\text{Total number of colonies of all the species}} \times 100
\]

\[
\text{Abundance} = \frac{\text{Total number of individuals in all quadrates}}{\text{Number of quadrates in which individual species occur}} \times 100
\]

3.5 Sterilization

Sterilization of glasswares

Glassware were dipped overnight in acidic potassium dichromate (K$_2$Cr$_2$O$_7$) solution washed with clean tap water air dried and wrapped in news paper then sterilised in hot air oven at 180°C for 3 hrs.

Sterilization of Media

Culture media were sterilized in an autoclave at 121°C at 15lbs/inch$^2$ for 30 minutes.
3.5.1 Washing and disinfection

Fresh tender fruit bodies of ECM fungi were selected for isolation. The soil was removed at different stages, first by paint brush (Camel 1.0 hair brush), then under water and later washed in running water for about a minute keeping the material in a strainer. After removing the extraneous soil, the material was again washed in running water and process was repeated 3 to 4 times.

Final washing was done in 2 to 3 changes of sterilized water when any adhering extraneous body on fruit body of ECM was removed with the help of a camel – hair brush under a magnifying glass.

The fruit body of ECM was then surface disinfected with 1% sodium hypochlorite for 3 to 4 minutes and washed in repeated changes of sterilized water to remove the disinfectant. Sodium hypochlorite is a mild disinfectant and possesses a much slower action. Mercuric chloride (0.1%) which is a strong disinfectant may be used but prolonged exposure of this disinfectant may kill delicate fruit body of ECM.

3.5.2 Solarization of soil

In the month of May-June 2011, 10 m x 1.0 m raised nursery bed was irrigated then covered with a polythene sheet. In solarization moist heat (approx 90-110°C) generated from the excess moisture and CO₂ produced through respiration of microorganisms and germination of seeds which may cause green house effect. Polythene cover protects it from the outer environment. The above procedure is repeated in the same nursery bed for a month. After 30 days polythene cover was removed and solarised soil was kept in polythene bags for its further use in experiments (Fig.29, b).

Effect of solrization on % germination, Growth Index and Dry Plant Biomass

In order to study the effect of solarized soil following four treatments were made in a four replicate while each replication contained 50 seeds, after germination seedlings were uprooted and dry plant biomass was estimated (Table 6).

1. Solarized soil
2. Solarised soil+ natural sal forest soil
3. Un solarised soil+natural sal forest soil

4. Unsolarized soil

3.6 Isolation of fungi

Equipments

- Microscope (Labovision Biofantasy Microscope Sl. No.: 3177661) and accessories used for taxonomic study and identification.
- Hot Air Oven (Technica India)
- Weighing machine (Aisconet Licens)
- Autoclave (Sonar)
- Laminar Air Flow (Thermodyne)
- BOD incubator (Remi)
- Sterilizing agents: Autoclave, Alcohol (Absolute), Spirit, Sodium hypochlorite and Hot Air Oven.
- Staining agents: Lacto phenol, Cotton blue
- Phenol – 10g; Lactic acid – 10g; Glycerol – 20g; Distilled water – 100ml.
- Lacto phenol cotton blue - To the phenol of above composition 0.1g of cotton blue dye was added. The solution was diluted by adding lacto phenol if required.

Culture Media

- Norkrans (1950) Medium
- Potato Dextrose Agar Medium (PDA)

Media preparation

Norkrans (1950) medium all components were weighed carefully and dispensed in a conical flask (500 ml. Borosil) and final volume was made 250 ml (04 sets) with
double distilled water plugged tightly with non adsorbent cotton and autoclaved at 121$^\circ$C and 15 lbs/inch$^2$ for 15 minutes.

**PDA medium** 39g of powdered PDA medium (commercially available as MicroXpress from Tulip Diagnostics) was weighed and dispensed in 1000 ml double distilled water wrist shaken and distributed equally in 500 ml conical flasks (04 sets) plugged tightly with non adsorbent cotton and autoclaved at 121$^\circ$C and 15 lb/inch$^2$ for 15 minutes.

### 3.6.1 Isolation of ECM fungi

Fresh and active fruit bodies of ECM only were selected for isolation. For the isolation of ECM thin pieces (~ 5.0-2.0 mm) were taken from different parts of fruit body.

After 30 days of incubation mycelial growth were obtained on PDA and Norkran’s, (1950) medium; for sub-culturing these were transferred into slants of the same medium and incubated at 25 ± 2° C for 15 days. After incubation growth appeared in slants and called as Mother culture or Stock culture.

**Mycorrhizal cultures**

Cultures of the following ectomycorrhizal fungi were prepared for use the present study:

**Russula michiganensis** Shaffer *Brittonia* 14:3 (1962) 254-284p. (Figs.4 a-c) forming mycorrhizal association with sal (*Shorea robusta*), collected from Amarkantak sal forest (Acc.No. – TFC 2787).

**Lycoperdon compactum** G.H. Cunn. =*Morganella compacta* (G. Cunningham) Kreisel & Dring (Figs. 4 d-f) forming mycorrhizal association with sal, collected from Amarkantak sal forest (Acc.No. – TFC 2785).
Materials and Methods

Fig. 3. Various steps of mycorrhizal inoculums production.
Fig. 4. Isolation and preparation of pure culturing of ECM fungi, a. Fruit bodies of *Russula michiganensis* (ECM1), b. Microphotograph of basidiospores (40x), c. Pure cultures on MMN medium. d. Fruit body of *Lycoperdon compactum* (ECM2), e. Microphotograph of basidiospores (40x), f. Pure culture on PDA agar medium.
3.6.2 Isolation of AM fungi (Sylvia, 1994)

To obtain viable AM spores, wet-sieving decanting and sucrose floatation technique was applied. Soil sample were collected and processed for isolation of AM spores. 100 g soil was mixed in luke warm water in a large beaker and allowed the heavier particle to settle down. The suspension was gently stirred several times and poured through coarse sieve (710µm) to remove large pieces of organic matter. The particles were re-suspended and suspension was decanted through 500µm, 250µm, 105µm and 53µm sieves to retain the AM spores of different sizes. The sieving collected was transferred into a 50ml centrifuge tube and centrifuged with distilled water for 5 minutes at 1750 rpm. The supernatant was decanted carefully and pellets were re suspended in 50% sucrose solution and it was again centrifuged for 15 second at 3000 rpm. The supernatant with spores were poured into a fine sieve (45 µm) and rinsed with water to remove sucrose (Sylvia, 1994). The spore were picked up with the help of specially designed tweezers and observed under dissecting microscope with strong illumination.

Quantitative estimation of AM spores

Spores and AM fungi were placed in small petridishes marked with gridline in water with 5.0% commercial grade formaldehyde. The dish is placed under stereo zoom microscope and numbers of AM spores were counted.

Density gradient centrifugation

The contents of the sieve obtained as above were transferred to 50 ml centrifuge tubes and centrifuged for 3-4mins at 3000rpm in swing head type centrifuge. The supernatant was decanted carefully and pellets were re-suspended in 45% sucrose solution. It was again centrifuged for 15 seconds at 3000-4000 rpm. The sucrose was decanted in a 38-45µm sieve very thoroughly, rinsed with water to remove sugar. The mixture was then poured into Petri dish for study under the stereo zoom microscope (Sylvia, 1994).

Cone method

Filter paper cone was kept in a funnel after all the water trickles down transfer the filter paper cone after unfolding in similar sized petriplate. The spores and
extrametrical hyphae were collected from filter paper and counted either wet or after air drying under a dissecting binocular microscope (Mukerji et al. 2002).

3.6.3 Isolation of growth promoting and litter decomposing microbes

Rhizospheric soil and litter samples were collected separately from the sal forest of Achanakmar Amarkantak Biosphere Reserve. The samples were subjected to serial dilution and plating technique for the isolation of soil fungi and phosphate solublizing bacteria (Warcup, 1950). 1g of each sample was taken separately and mixed in 9 ml of distilled water and shaken thoroughly to obtain a homogenous solution. Then 1 ml of this suspension was taken into 9 ml of distilled water and again mixed thoroughly. The above was further diluted to make dilution i.e. 1:100, 1:1000, 1:10000 and 1:100000. The whole process was carried out in laminar air flow chamber to avoid contamination. Potato Dextrose Agar (PDA) was used for isolation of litter fungi. Plates were incubated at 25±2°C.

Following growth promoting and litter decomposing microbes were used in experiments.

- *Aspergillus niger* (Acc.No. – TFC 03)
- *Cladosporium oxysporum* (Acc.No. – TFC 12) (Litter decomposer)
- *Trichoderma viride* (Acc.No. – TFC 10)

3.7 Identification of Isolated microbes

3.7.1 Identification of ECM

*Microscopic study*

For the microscopic study of the collected samples, carefully a thin section was cut across the gills, obtained manually with the help of a parallel razor blade and it was placed on the clean-clear glass slide and staining process was done by the lacto phenol-cotton blue stain. It was then examined under advanced research microscope and microphotographs were taken. These were identified with the help of standard monographs available in the library and taxonomic expertise from the Forest Pathology Division was used.
Above method were repeated for each sample.

Size, shape, ornamentation of the basidiospore, basidia, cystidia and other hymenial elements were observed (By the help of ocular-stage scale; size of spores, basidia, basidiospore, cystidia and other hymenial elements was measured).

**Micrometry:**

Calculation of least count;

\[ X = \frac{S}{O} \times 0.01 \]

Where; \( S \) = Stage micrometer division;

\( O \) = Ocular division; \( X \) = least count

1 part of stage division = 10 µm

<table>
<thead>
<tr>
<th>Ocular lens</th>
<th>Least count</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x</td>
<td>11.11</td>
</tr>
<tr>
<td>10x</td>
<td>9.5</td>
</tr>
<tr>
<td>40x</td>
<td>2.5</td>
</tr>
<tr>
<td>100x</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Each sample was identified on the basis of above observation and with the help of standard literature Bakshi (1974); Cunningham (1979); Surcek (1998); Mukerji et al., (2002); Mohanan (2011).

**3.7.2 Identification of AM**

Genera and species of AM fungi were identified on the basis of morphology of their resting spores by consulting taxonomic manual Schenck and Perez (1990).

**3.7.3 Identification of growth promoting microbes**

Isolated soil fungi were identified on the basis of their morphological characters. Identification was confirmed by consulting taxonomic literature (Barnet and Hunter, 1972; Gilman, 1957; Ellis, 1971).
3.8 Maintenance of cultures

3.8.1 Preparation of ECM inoculum

ECM cultures were maintained by subculturing in Norkrans and PDA medium and maintained in a refrigerator at 4 ± 1°C.

For the preparation of ECM inoculum superior quality wheat grains were taken and it were soaked overnight in water after that wheat grains were boiled in water (did not over cooked). Water was drained off by filtering it through a coarse rectangular sieve (1.0 m X 1.5 m). These wheat grains were spread over news paper sheet and allowed to cool and dry under ceiling fan. Gypsum salt, 25 mg/kg of Bavistin® (80%w.p.) and 10 mg/kg of streptomycin sulfate were mixed in air dried wheat grains, then wheat grains were filled in narrow mouth long neck bottles, plugged and autoclaved at 121°C & 15 lbs/inch² for 30 minutes. After autoclaving, it were allowed to cool and then it were stored under refrigeration for one night.

On next day from Mother culture or Stock culture, 6-8 mycelial discs were inoculated in the bottles and incubated at 25±2°C for 45 days.

After 45 days of incubation milky-white colored thick mycelial growth appears on wheat grains which bind them loosely. This culture is known as spawn (seed) and will be used further to inoculate sal rootlets (Fig.5). Bulk density of ECM cultures was expressed as g/l.

3.8.2 Preparation of AM inoculum

Feeder roots of Sal were collected and thoroughly washed in sterile water to remove all soil traces and external mycelium. About 5 g of these feeder roots were planted in sterilized potting mixture containing soil, sand and organic matter in 2:2:1 ratio. Maize seeds were surface sterilized with absolute ethyl alcohol and sown on this potting mixture. After three months, roots of the same trap plant were harvested, washed and sheared to get the AM inoculum (Walker, 1994). This method was used to propagate AM spores present in natural sal forest soil. This AM inoculum will be used with solarised and un-solarised soil at the time of seed sowing.
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Fig. 5. Spawn of mycorrhizal cultures *Russula michiganensis* (ECM$_1$) and *Lycoperdon compactum* (ECM$_2$)
**Materials and Methods**

**Estimation of % colonization of root by AM**

**Collection of root sample**

The finer feeder roots, preferential sites of AM development were collected in sterilized polythene bags for mycorrhizal assessment from experimental plot of Forest Pathology Division of Tropical Forest Research Institute Jabalpur and Achanakmar Amarkantak Biosphere Reserve Madhya Pradesh and Chhattishgarh in the month of August 2011.

**Clearing and staining of roots** (Phillips and Hayman, 1970; Koske and Gemma 1989).

**Reagents used**

(i) 10% KOH (10 g KOH dissolved in 90 ml distilled water).
(ii) 2% HCL (2 ml conc. HCL in 98 ml distilled water).
(iii) H₂O₂
(iv) NH₄OH

First of all root samples were washed under running water to remove adhered soil particles then transferred in 10% KOH solution, heated at 90°C for 20-40 minutes, allowed to cooked and poured off KOH, again washed with tap water (three times) then bleached with alkaline H₂O₂ (30 ml 10% H₂O₂+ 0.5g of NH₄OH+567 mi of water), again washed in tap water (3 times) then acidified with 1% HCl (3-4 times). Roots were then stained with 0.05% trypan blue in lactophenol, heated at 900°C for 10-15 minutes, excess stain was removed by dispersing the stained root in lactophenol then microscopic observation of roots were taken.

**Estimation of root colonized percentage**

Stain used: Melzer’s reagent and polyvinyl alcohol-lactic acid-glycerol (PVLG).

De-staining reagent Lactic acid + glycerol (1:1 ratio)

The stained root was examined under stereomicroscope for the presence of mycelium, arbuscules, intramatrical vesicles or spores and extrametrical
Materials and Methods

sporocarps, if any, of AM fungi. Total root colonization percentage was determined as follows:

\[
\text{% Colonization} = \frac{\text{Number of roots colonized}}{\text{Total number of roots examined}} \times 100
\]

3.8.3 Preparation of inoculums of Growth promoting microbes

Fungal inoculums viz. Aspergillus niger, Cladosporium oxysporum and Trichoderma viride were bulk cultured on Potato Dextrose Broth in a BOD incubator at 25±2\(^\circ\)C. This inoculum was used to decompose sal litter in the present study.

3.9 Decomposition of sal litter (Soni and Jamaluddin, 1990)

Sal litter collected from Achanakmar Amarkantak Biosphere Reserve was packed in a mosquito net-bags (200g litter/bag) and decomposed using different fungal combinations as follows

(i) Control
(ii) Aspergillus niger
(iii) Cladosporium oxysporum
(iv) Trichoderma viride
(v) Aspergillus niger + Trichoderma viride + Cladosporium oxysporum
(vi) Aspergillus niger + Cladosporium oxysporum
(vii) Aspergillus niger + Trichoderma viride
(viii) Cladosporium oxysporum + Trichoderma viride

All the treatments were made in a replicates of four. Suspension of microbial consortium was made in distilled water and litter was dipped into them and kept in net bags for decomposition under field condition. Initial weight of litter was noted after dipping it into different treatments and weight loss was recorded after definite intervals (6 months).
3.10 Effect of different treatments including ECM on germination and growth of sal

The experimental seedlings given the following treatment (in a replicate of three) and are arranged in RCBD in polythene bags on a cemented platform (Figs. 27, 28)

(i) Unsolarised soil
(ii) Unsolarised soil + Natural sal forest soil
(iii) Unsolarised soil + Sal litter
(iv) Unsolarised soil + ECM
(v) Solarised soil
(vi) Solarised soil + Natural sal forest soil
(vii) Solarised soil + Sal litter
(viii) Solarised Soil + ECM
* ECM = *Lycoperdon compactum*

3.11 Inoculation and planting of seedlings

The experimental seedlings given the following treatment (in a replicate of three) and are arranged in RCBD in polythene bags on a cemented platform. In order to study the growth response of these seedlings to different treatments these seedlings were later on planted at the Forest Pathology Division nursery of Tropical Forest Research Institute, Jabalpur (Fig 30).

(ix) Control
(x) SL (Sal Litter)
(xi) Vc (Vermicompost)
(xii) ECM₁ (*Russula michiganensis*)
(xiii) ECM₂ (*Lycoperdon compactum*)
(xiv) SL + ECM₁
(xv) Vc + ECM₁
(xvi) SL + ECM₂
(xvii) Vc + ECM₂

Sal litter collected from sal forests was added to the local solarised soil in a ratio of 1:4 (w/w) and drenched with 20 ml (v/v) dose of microbial consortium (*Trichoderma*...
viride + Cladosporium oxysporum + Aspergillus niger $10^7$-$10^{10}$ cells/ml approx). The vermicompost was used as a substitute of litter+ microbial consortium of inoculums to compare the result and cost benefits. The potting mixture was filled in black polythene bags (17.8 x 22.8 cm). One gram of inoculum was placed just below seeds during seed sowing in pre-monsoonal period. After six months seedlings were planted in the microplots with 1x0.75m spacing at the institute campus in RCBD. Four blocks each with all the above mentioned treatments were made. The seedlings were watered with tube well water as and when required. The whole experiment was carried out at the nursery of Forest Pathology Division of Tropical Forest Research Institute, Jabalpur Madhya Pradesh (Fig.31).

3.12 Measurement of seedlings Height, Diameter at collar height, Leaf Area and Growth Index

**Height (H)**

Sal is a slow growing timber plant species. Height of seedlings was measured at an interval of one-one month using a standard Nataraj 621 centimeter scale.

**Diameter at collar height (DC)**

Diameter of sal seedlings was measured using a Varnier calipers and expressed as 0.2, 0.3, and 0.5……..cm.

**Leaf Area (LA)**

The leaf area was calculated by deriving a mathematical equation as follows.

Maximum length and breadth of 10 different leaves were taken and mathematical value of length x breadth were calculated. The actual leaf area of these leaves were also calculated by taking outlines of leaves on graph paper and counting the number of squares inside the outline of leaves. Thus an equation is obtained by taking average of L x B and actual areas obtained by graph paper (Fig.7a&b).

$$\text{Leaf area by graph/ LxB} = \frac{64.1}{87.6} = \frac{1}{1.36}$$

$$\text{Leaf area of sample x} = \frac{(LxB)}{1.36} \text{ (cm}^2)$$
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Fig. 6 Determination of leaf area by Graph paper method

Growth Index (GI)

Growth Index of each of the sal seedlings was separately calculated by using the following formulae

\[
G.I. = \frac{H \times DC \times LA}{H+DC+LA}
\]

Where,

GI = Growth Indices, H = Height of sal seedling, DC = Diameter at Collar Height,
LA = Leaf Area

3.13 Estimation of Dry Plant Biomass (DPB)

Representative seedlings from each treatment were up-rooted washed under running tap water to remove adhered soil particles and air dried to remove surface water. Entire seedlings were weighed first, and then it was cut into root and shoots using a scissor and weighed separately on an electronic balance. These seedlings were kept in a large paper envelop, labeled and dried in an oven at 70°C until constant weight, then weighed. The biomass was expressed in the unit of gram.
3.14 Estimation of phosphorous in sal leaves

Healthy representative leaves from each treatment were plucked and kept in a paper envelope, labeled and dried as above in an oven, powdered and used as sample for phosphorous estimation Fiske and Subba Row (1925).

Preparation of reagents

30% H₂O₂

30 ml of H₂O₂ was mixed with 70 ml of distilled water to get 30% H₂O₂.

H₂SO₄ (10N)

27.7 ml of (98% H₂SO₄, Density 1.84 g/ml equivalent wt. 49.0, Normality 36.80) was added in 72.3 ml of distilled water to get 10N H₂SO₄.

Amino naphthol sulphonic acid

0.5 g of 1-aminonaphthol sulphonic acid was dissolved in 195 ml of 15% sodium bisulphate solution in which 5 ml of 20% sodium sulphite solution was added. The solution was kept in amber colored bottles.

Molybdic acid

0.25 g of Ammonium molybdate was dissolved in 175 ml of distilled water in which 75 ml of 10N sulfuric acid was added.

Stock K₂HPO₄

100 mg of Potassium Di-hydrogen Phosphate (K₂HPO₄) was dissolved in 100 ml of distilled water (1 mg/ml) to get stock that will be used to make standard curve.

Preparation of standard curve

1.0 ml of stock was added in 10 ml of distilled water (0.1mg/ml) and following concentration gradient were made with distilled water

0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 of K₂HPO₄
0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, 0.0 of D.W.
5 ml from the above dilutions were taken. To the above solutions 1.0 ml of molybdic acid + 0.4 ml of 1-aminonaphthol sulphonic acid were added and the final volume was made 10 ml using distilled water. Optical densities (O.D.) were taken and a standard curve was plotted which follows the Beer Lambert law (Fig.7).

![Figure 7: Standard curve of KH$_2$PO$_4$](image)

**Fig.7. Standard curve of KH$_2$PO$_4$**

**Procedure**

**Digestion of sample**

In a clean volumetric flask 100 mg of oven dried sal leaves sample was taken. To it added a 2 ml of H$_2$SO$_4$ (Conc.). Heated the sample on a hot plate until it turned black, allowed to cool and added H$_2$O$_2$ (30%) drop wise, heated again till it takes yellow color, allowed to cool and added 3-4 drops of H$_2$O$_2$ until a clear solution was obtained.

**Estimation of phosphorus**

Peroxide digested material was transferred into a volumetric flask washed 3-4 times with distilled water, and the final volume was made 100 ml. 5 ml aliquot from the flask was transferred into a large test tube. To it added 1 ml of molybdic acid (2.5% Ammonium molybdate in 10N H$_2$SO$_4$) carefully followed by addition of 0.4 ml of 1-amino 2 Naphthol 4 sulphonic acid. The solution turned blue. To it added distilled
water and final volume was made 10 ml, allowed to stand for 10 minutes. Five ml of the above solution was transferred into colorimetric tube and optical density (O.D.) was measured at 620 nm. The values of O.D. obtained so were multiplied by a standardized value of 1.22 to get % phosphorous in leaf sample.

3.15 Statistical analysis

Data obtained on, seed germination, plant biomass, survival, plant height, diameter at collar height, phosphorus content in leaves, growth indices and leaf litter decomposition were analyzed by one way analysis of variance (ANOVA) using Sx Statistics software.