3. MATERIALS AND METHODS

3.1 MATERIALS:

On the priority basis of Rajasthan and Gujarat two species *viz. Tecomella undulata* (Sm.) Seem and *Ailanthus excelsa* Roxb. were selected for present research work. Brief description, distribution and importance of both species are as following:

3.1.1 *Tecomella undulata*:

*Tecomella undulata* (Sm.) Seem is an economically important, timber yielding tree species, which is having some medicinal properties also (Parekh and Chanda 2007; Khatri et al. 2009; Dhir and Shekhawat 2012). It is a medium sized tree with height ranging from 8 to 15 m, deciduous or nearly evergreen with drooping branches and stellately grey-tomentose innovations, otherwise glabrous. The leaves are simple 5.0-12.5 cm in length and 1.0-3.2 cm in width, narrowly oblong, obtuse, and entire with undulate margins. Flowers are inodorous in corymbose few flowered racemes, terminating short lateral branches, pedicels are 6.0-13.0 mm long, calyx 9.5-11.0 mm long, campanulate. Wings are very narrow round the apex of seed, absent at its base (Chal et al. 2011).

*T. undulata* is distributed in Arabia, southern Pakistan and northwestern India. It is naturally occurring in five states of India *viz.* Gujarat, Rajasthan, Punjab, Haryana and Maharashtra. In Rajasthan, this species is distributed in Barmer, Jaisalmer, Jodhpur, Pali, Ajmer, Nagaur, Bikaner, Churu and Sikar (Nandkarni 2000).

3.1.2 *Ailanthus excelsa*:

*Ailanthus excelsa* is one of the important fodder tree species for arid and semi arid areas of India. It is a fast growing, attains a height upto 24 m, with straight cylindrical stem of 1.8-2.4 m girth, spreading branches and large (40-100 cm) pinnate leaves with 15-41 long pointed leaflets, the terminal leaflet normally present and the
basal pairs of leaflets often lobed at their bases. Its wood is useful for packing material and match stick industries (Jat et al. 2011).

*Ailanthus excelsa* is indigenous to Indian peninsula and grows almost throughout the tropical and subtropical parts of the country especially in the dry tracts. In Rajasthan *A. excelsa* is mainly present in Sikar, Jaipur, Jodhpur, Ajmer, Tonk, Dosa and Udaipur (Jat et al. 2011).

### 3.1.3 Selection of Trees:

In order to carry out macro and micropropagation experiments on *Tecomella undulata* and *Ailanthus excelsa* few healthy 10-15 years old trees of both species were selected in the year 2007 growing in AFRI campus and experimental area of F.G.T.B. field (Figure 1), along with the G.P.S. locations in tables (3 & 4).

**Micropropagation:**

**Table 3: Details of trees marked as resource mother stock of both the species used for micropropagation.**

<table>
<thead>
<tr>
<th>T. undulata</th>
<th>GPS location</th>
<th>Flower colour</th>
<th>A. excelsa</th>
<th>GPS location</th>
<th>Male/Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree 1</td>
<td>Altitude 218 m Latitude 26°13’40.0” Longitude 073°01’50.7”</td>
<td>Orange</td>
<td>Tree 1</td>
<td>Altitude 217 m Latitude 26°13’44.5” Longitude 73°01’53.7”</td>
<td>Male</td>
</tr>
<tr>
<td>Tree 2</td>
<td>Altitude 218 m Latitude 26°13’48.0” Longitude 073°01’50.1”</td>
<td>Yellow</td>
<td>Tree 2</td>
<td>Altitude 218 m Latitude 26°13’44.5” Longitude 73°01’53.5”</td>
<td>Male</td>
</tr>
<tr>
<td>Tree 3</td>
<td>Altitude 218 m Latitude 26°13’49.5” Longitude 073°01’49.2”</td>
<td>Yellow</td>
<td>Tree 3</td>
<td>Altitude 219 m Latitude 26°13’44.3” Longitude 073°01’53.9”</td>
<td>Female</td>
</tr>
<tr>
<td>Tree 4</td>
<td>Altitude 218 m Latitude 26°13’51.0” Longitude 073°01’48.6”</td>
<td>Orange</td>
<td>Tree 4</td>
<td>Altitude 219 m Latitude 26°13’43.0” Longitude 073°01’54.7”</td>
<td>Female</td>
</tr>
</tbody>
</table>
Macropropagation:

**Table 4: Details of trees marked as resource mother stock for macropropagation of *Tecomella undulata*.

<table>
<thead>
<tr>
<th>T. undulata</th>
<th>GPS location</th>
<th>T. undulata</th>
<th>GPS location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree 1</td>
<td>Altitude 213 m</td>
<td>Tree 6</td>
<td>Altitude 215 m</td>
</tr>
<tr>
<td></td>
<td>Latitude 26°14’01.3”</td>
<td></td>
<td>Latitude 26°14’02.0”</td>
</tr>
<tr>
<td></td>
<td>Longitude 073°02’18.8”</td>
<td></td>
<td>Longitude 073°02’17.7”</td>
</tr>
<tr>
<td>Tree 2</td>
<td>Altitude 214 m</td>
<td>Tree 7</td>
<td>Altitude 215 m</td>
</tr>
<tr>
<td></td>
<td>Latitude 26°14’01.4”</td>
<td></td>
<td>Latitude 26°14’02.1”</td>
</tr>
<tr>
<td></td>
<td>Longitude 073°02’18.0”</td>
<td></td>
<td>Longitude 073°02’18.3”</td>
</tr>
<tr>
<td>Tree 3</td>
<td>Altitude 214 m</td>
<td>Tree 8</td>
<td>Altitude 215 m</td>
</tr>
<tr>
<td></td>
<td>Latitude 26°14’01.5”</td>
<td></td>
<td>Latitude 26°14’02.3”</td>
</tr>
<tr>
<td></td>
<td>Longitude 073°02’17.4”</td>
<td></td>
<td>Longitude 073°02’18.6”</td>
</tr>
<tr>
<td>Tree 4</td>
<td>Altitude 215 m</td>
<td>Tree 9</td>
<td>Altitude 213 m</td>
</tr>
<tr>
<td></td>
<td>Latitude 26°14’01.4”</td>
<td></td>
<td>Latitude 26°14’02.1”</td>
</tr>
<tr>
<td></td>
<td>Longitude 073°02’16.2”</td>
<td></td>
<td>Longitude 073°02’18.9”</td>
</tr>
<tr>
<td>Tree 5</td>
<td>Altitude 215 m</td>
<td>Tree 10</td>
<td>Altitude 215 m</td>
</tr>
<tr>
<td></td>
<td>Latitude 26°14’01.8”</td>
<td></td>
<td>Latitude 26°14’01.8”</td>
</tr>
<tr>
<td></td>
<td>Longitude 073°02’17.5”</td>
<td></td>
<td>Longitude 073°02’19.2”</td>
</tr>
</tbody>
</table>

### 3.2 METHODS:

Both micro and macropropagation approaches were adopted to develop clonal propagation technique in *T. undulata*, whereas in case of *A. excelsa* only micropropagation methods were used. These micro and macropropagation methods used in present research work are described in details in the following paragraphs.

#### 3.2.1 Micropropagation:

Routine tissue culture methods were used in present studies, which involve aseptic media preparation, explant collection, surface sterilization and inoculation to establish aseptic cultures. These shoot cultures were multiplied and maintained by
repeated subculturing on fresh medium (MS + 1 mg/l BA) as mother stock of shoots for conducting shoot multiplication and rooting experiments.

3.2.1.1 Media Preparation:

Different basal media *viz.* Murashige & Skoog medium (Murashige and Skoog 1962), B₅ medium (Gamborg *et al.* 1968), Woody Plant Medium (Lloyd and McCown 1980), White’s medium (White 1963) and Hoagland’s nutrient solution (Hoagland and Arnon 1950) were used according to the experiments. But MS and B₅ medium were used in experiments more frequently. Composition of MS, B₅, WPM and Hoagland medium and concentrated stock solutions were prepared as given in following Tables (5 to 8). According to the experiments different supplements like plant growth regulators, amino acids, antibiotics etc. were added to the basal medium.

Concentrated salts and vitamin stock solutions were prepared using analytical grade salts (Hi-Media). Use of concentrated stocks enhances the accuracy as well as save time of media preparation. Stocks were prepared with distilled water and stored in refrigerator at 5 °C. Fresh media were prepared by diluting the stocks to a level of desired concentration. Sucrose (3 % w/v, Hi-Media) was added in medium as energy source unless otherwise mentioned in the experiments. Plant growth regulators were added according to the experimental requirement. The pH of medium was adjusted to 5.8 with 1N NaOH and 1N HCl. Media were gelled using agar (0.8 % w/v, Hi-Media) after adjusting pH.
Table 5: Composition of MS medium and volume required to prepare one liter medium from concentrated stocks.

<table>
<thead>
<tr>
<th>Stocks</th>
<th>Media ingredients</th>
<th>Ingredie nts concentration in medium</th>
<th>Ingredients Conc. in Stock and degree of Conc.</th>
<th>Volume to be added to prepare 1 liter medium.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mg/l)</td>
<td>(g/l)</td>
<td>(X times)</td>
</tr>
<tr>
<td>A</td>
<td>NH₄NO₃</td>
<td>1650</td>
<td>82.5</td>
<td>(50X)</td>
</tr>
<tr>
<td>B</td>
<td>KNO₃</td>
<td>1900</td>
<td>95.0</td>
<td>(50X)</td>
</tr>
<tr>
<td>C</td>
<td>H₃BO₃</td>
<td>6.2</td>
<td>1.2</td>
<td>(200X)</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
<td>170</td>
<td>3.4</td>
<td>(200X)</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>0.83</td>
<td>0.166</td>
<td>(200X)</td>
</tr>
<tr>
<td>D</td>
<td>Na₂Mo₄.2H₂O</td>
<td>0.25</td>
<td>0.25</td>
<td>(1000X)</td>
</tr>
<tr>
<td></td>
<td>CoCl₃.6H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>(1000X)</td>
</tr>
<tr>
<td>E</td>
<td>CaCl₂</td>
<td>440.00</td>
<td>88.0</td>
<td>(200X)</td>
</tr>
<tr>
<td>F</td>
<td>MgSO₄.4H₂O</td>
<td>370.00</td>
<td>74.0</td>
<td>(200X)</td>
</tr>
<tr>
<td></td>
<td>MnSO₄.4H₂O</td>
<td>223.30</td>
<td>44.7</td>
<td>(200X)</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄.4H₂O</td>
<td>8.60</td>
<td>1.72</td>
<td>(200X)</td>
</tr>
<tr>
<td>G</td>
<td>CuSO₄.5H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>(1000X)</td>
</tr>
<tr>
<td>H</td>
<td>Na₂EDTA</td>
<td>37.30</td>
<td>7.46</td>
<td>(200X)</td>
</tr>
<tr>
<td></td>
<td>FeSO₄.7H₂O</td>
<td>27.30</td>
<td>5.46</td>
<td>(200X)</td>
</tr>
<tr>
<td>I</td>
<td>Thiamine HCl</td>
<td>0.1</td>
<td>0.1</td>
<td>(1000X)</td>
</tr>
<tr>
<td></td>
<td>Nicotinic Acid</td>
<td>0.5</td>
<td>0.5</td>
<td>(1000X)</td>
</tr>
<tr>
<td></td>
<td>Pyridoxine</td>
<td>0.5</td>
<td>0.5</td>
<td>(1000X)</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>2.0</td>
<td>2.0</td>
<td>(1000X)</td>
</tr>
<tr>
<td>J</td>
<td>Myo Inositol</td>
<td>100</td>
<td>10</td>
<td>(100X)</td>
</tr>
</tbody>
</table>

Sucrose (3 %) and Agar (0.8 %) were added separately into medium unless otherwise mentioned in the experiment.
# Materials and Methods

Table 6: Composition of B₅ medium and volume required to prepare one liter medium from concentrated stocks.

<table>
<thead>
<tr>
<th>Stocks</th>
<th>Media ingredients</th>
<th>Ingredients concentration in medium (mg/l)</th>
<th>Ingredients Conc. in Stock and degree of Conc. (X times)</th>
<th>Volume to be added to prepare 1 liter medium (ml)</th>
<th>Inorganic salts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(NH₄)₂SO₄</td>
<td>134.00</td>
<td>6.7</td>
<td>50X</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>KNO₃</td>
<td>2500</td>
<td>125</td>
<td>50X</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>KI, H₃BO₃</td>
<td>0.75, 3.00</td>
<td>0.15, 0.6</td>
<td>200X, 200X</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NaMoO₄, CoCl₂.H₂O</td>
<td>0.25, 0.025</td>
<td>0.25, 0.025</td>
<td>1000X, 1000X</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CaCl₂.2H₂O</td>
<td>150</td>
<td>30</td>
<td>200X, 5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>MgSO₄.7H₂O, MnSO₄.4H₂O, ZnSO₄.7H₂O</td>
<td>250, 10.00, 2.00</td>
<td>50, 2, 0.4</td>
<td>200X, 5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>CuSO₄.5H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>1000X, 1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Na₂EDTA.2H₂O, FeSO₄.7H₂O</td>
<td>37.25, 27.85</td>
<td>7.45, 5.57</td>
<td>200X, 5</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>NaH₂PO₄</td>
<td>130.50</td>
<td>26.1</td>
<td>200X, 5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Nicotinic acid, Thiamine HCl, Pyridoxine</td>
<td>1.00, 10.00, 1.00</td>
<td>1, 10, 1</td>
<td>1000X, 1000X</td>
<td></td>
</tr>
</tbody>
</table>

Sucrose (3 %) and Agar (0.8 %) were added separately into medium unless otherwise mentioned in the experiment.
Table 7: Composition of Woody plant medium and volume required to prepare one liter medium from concentrated stocks.

<table>
<thead>
<tr>
<th>Stocks</th>
<th>Media ingredients</th>
<th>Ingredients concentration in medium</th>
<th>Ingredients Conc. in Stock and degree of Conc. (X times)</th>
<th>Volume to be added to prepare 1 liter medium (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NH₄NO₃</td>
<td>400.00 mg/l</td>
<td>50X</td>
<td>20 ml</td>
</tr>
<tr>
<td>B</td>
<td>K₂SO₄</td>
<td>990.00 mg/l</td>
<td>100X</td>
<td>10 ml</td>
</tr>
<tr>
<td>C</td>
<td>H₃BO₃, KH₂PO₄</td>
<td>6.2 mg/l, 170.00 mg/l</td>
<td>200X</td>
<td>5 ml</td>
</tr>
<tr>
<td>D</td>
<td>CaCl₂.2H₂O</td>
<td>96.00 mg/l, 19.2 mg/l</td>
<td>200X</td>
<td>5 ml</td>
</tr>
<tr>
<td>E</td>
<td>MgSO₄.7H₂O, MnSO₄.4H₂O, ZnSO₄.7H₂O</td>
<td>370.00 mg/l, 74 mg/l, 22.3 mg/l, 8.6 mg/l</td>
<td>200X, 200X, 200X</td>
<td>5 ml, 5 ml, 5 ml</td>
</tr>
<tr>
<td>F</td>
<td>CuSO₄.5H₂O</td>
<td>0.25 mg/l</td>
<td>1000X</td>
<td>1 ml</td>
</tr>
<tr>
<td>G</td>
<td>Na₂EDTA.2H₂O, FeSO₄.7H₂O</td>
<td>37.2 mg/l, 7.44 mg/l, 27.8 mg/l</td>
<td>200X, 200X</td>
<td>5 ml, 5 ml</td>
</tr>
<tr>
<td>H</td>
<td>Ca(NO₃)₂.4H₂O</td>
<td>556 mg/l</td>
<td>200X</td>
<td>5 ml</td>
</tr>
<tr>
<td>I</td>
<td>Na₂MoO₄.2H₂O</td>
<td>0.25 mg/l</td>
<td>1000X</td>
<td>1 ml</td>
</tr>
<tr>
<td>J</td>
<td>Thiamine HCl, Nicotinic acid, Pyridoxine, Glycine</td>
<td>1.00 mg/l, 0.5 mg/l, 0.5 mg/l, 2.0 mg/l</td>
<td>1000X, 1000X, 1000X</td>
<td>1 ml, 1 ml, 1 ml</td>
</tr>
<tr>
<td>K</td>
<td>Myo insitol</td>
<td>100 mg/l</td>
<td>200X</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

Sucrose (3 %) and Agar (0.8 %) were added separately into medium unless otherwise mentioned in the experiment.
Table 8: Composition and concentration of salts of Hoagland solution.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (mg/l)</th>
<th>Constituent</th>
<th>Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>940 mg</td>
<td>MnSO₄·H₂O</td>
<td>34 mg</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>520 mg</td>
<td>CuSO₄·5H₂O</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>KNO₃</td>
<td>660 mg</td>
<td>ZnSO₄·7H₂O</td>
<td>2.2 mg</td>
</tr>
<tr>
<td>NH₄H₂PO₄</td>
<td>120 mg</td>
<td>(NH₄)MoO₂·4H₂O</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>28 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Plant growth regulator’s concentration varies according to the objective of experiments. The media were dispensed in culture tubes (Borosil 2.5 X 15 cm) and conical flasks (Schott Duran 150 ml). These vessels were plugged with non absorbent cotton. Vessels containing media were autoclaved in a horizontal/vertical autoclave at 15 lb/cm² and 121 °C for 20 minutes for complete sterilization. After autoclaving the media were kept for 2-3 days before using for fresh shoot culture establishment, shoot stock maintenance, shoot multiplication or rooting experiments.

3.2.1.2 Explant Collection and Sterilization

Single node explants of 2.0-2.5 cm length and 0.4-0.5 cm width for *Tecomella undulata* and 0.6-0.9 cm width for *Ailanthus excelsa* were excised from 10-15 years old selected mature trees (Figure 2). In *A. excelsa* the nodal explants were collected from coppice shoots. After removing leaves, the explants were thoroughly washed with running tap water followed by distilled water containing few drops of Tween-80 and subsequently treated with Bavistin (100 mg/100 ml) and Streptomycin (50 mg/100 ml). Next step of surface sterilization with Sodium Hypochlorite (2.5 % v/v) solution for 5-7 minutes followed by 3-4 times washings with sterile distilled water were carried out under aseptic conditions in a laminar airflow cabinet.

3.2.1.3 Inoculation and Culture Condition:

Autoclaved culture media, glasswares and other required accessories for inoculation were surface sterilized by exposing them for 20 minutes to UV light in a laminar air flow cabinet. Forceps, spatula and scalpel holders were surface sterilized
by heating them in flame. The explants were slightly trimmed at both ends before planting them on autoclaved culture medium. The nodal shoot segments were inoculated by keeping them vertically on the medium and basal portion of segments were inserted in the medium. In some experiments, filter sterilized (0.2 μ filter) antibiotics viz. Ciprofloxacin (100, 250 and 500 mg/l) and Levofloxacin (100, 250 and 500 mg/l) were also added to the basal medium to overcome the bacterial contamination.

Cultures were incubated at 2000 lux light intensity for 8 hrs upto 27 October 2009 and thereafter 16 hrs photoperiod, using fluorescent cool white light produced by tube (Philips) and regulated by electric timer (Vista Biocell). Temperature of the culture room was controlled with temperature controller units (Saveer) attached with Air conditioners and sensor were installed in the centre of culture room. The monthly pattern of temperature and relative humidity were recorded with data logger (Novus) programmed to record the data (temperature and relative humidity) at 15 minutes interval. Annual pattern of culture room is shown in Graph 1.

Graph 1: Monthly pattern of temperature (°C) and relative humidity (%) of tissue culture laboratory from June 2011 to May 2012.
3.2.1.4 Subculturing and shoot stocks maintenance:

The healthy shoot cultures were multiplied and maintained by repeated subculturing at 4 weeks intervals on fresh shoot multiplication medium (MS + 1 mg/l BA). These shoots were multiplied aseptically by excising apical shoots to remove apical dominance and to enhance axillary buds proliferation and their multiplication. A small clump of 3-4 shoots was preferred for subculturing on medium containing MS + 1.0 mg/l BA for shoot multiplication and shoot culture stock maintenance. Mother shoot stock cultures were maintained in 150 ml conical flasks to provide shoots to experiments on multiplication and rooting.

The in vitro grown shoot stocks were maintained for almost five years and recorded the multiplication rate at each subculture. Average multiplication rate of 12 month is given for each year from 2007 to 2011 in Graph 2. In order to obtain high shoot multiplication and good shoot growth, various experiments were conducted on methods of propagule preparation, effect of plant growth regulators, additives and sucrose etc.

Graph 2: *T. undulata* average shoot multiplication rate of stock cultures on MS + 1 mg/l BA medium in five successive years (2007-2011).
3.2.1.5 In vitro Rooting:

Long shoots of minimum 1.6 cm length were isolated from in vitro grown shoot stocks of *Ailanthus excelsa* and *Tecomella undulata* just before subculturing them on fresh medium and used for rooting experiments. Individual shoots without callus were inoculated in the rooting medium. Mainly two methods were used for rooting experiments. One is by growing these shoots directly on rooting medium containing different levels of auxins or other root promoting additives. Second is by pre-conditioning of these shoots, keeping their basal end in auxin solutions for fifteen minutes (pre-treatment) and then subculturing them on different basal media lacking auxins. Auxin solution were prepared by dissolving auxin in little amount of alkali (NaOH) and diluted with distilled water to make desired strength. Autoclaved auxin solutions were used for pretreatment. The excised shoots were carefully isolated and pretreated in test tubes with filter paper bridges containing auxin solution at concentrations defined in the experiment. After pre-treatment the shoots were inoculated into the rooting medium devoid of auxin with the help of flame sterilized forcep. The forcep was allowed to cool as it can damage the tender in vitro shoot. The shoots were given dark treatment for 5 days after inoculation in all cases as described in previous literature (Rathore *et al.* 1991). In each experiment a minimum of 10 explants were used.

3.2.1.6 Ex vitro Rooting:

*Ex vitro* rooting is always preferred as it combines the rooting and hardening step, which reduces the cost of tissue culture plants also. *Ex vitro* root induction was performed by dipping the basal portion of microshoots in auxin solution for 15 minutes and subsequently transferred such treated shoots to thermocol cups filled with autoclaved vermiculite enriched with half strength of MS salt solution and kept in mist polyhouse with RH 80 ± 10 % and 32 ± 5 °C temperature.

3.2.1.7 Hardening of plants:

Hardening was carried out in culture room conditions. Rooted plantlets were transferred to autoclaved vermiculite along with ½ strength liquid MS medium devoid of sucrose. The plantlets were kept in closed jam bottles and maintained in
culture room for 15 days for their growth. The plantlets were provided ½ strength liquid MS medium in an interval of 5 days for proper initial growth. After 15 days, caps were removed for 5-10 minutes and plantlets were monitored closely. Jam bottles were again closed, in case any wilting was observed. In this way, plantlets were observed for few hours every day by removing the cover of bottles. This period of low humidity exposure was gradually increased to harden plantlets so that they can withstand normal conditions. Acclimatization was carried out in mist chamber and maintained the humidity between 80 ± 10 % (30 sec misting at 15 minutes interval). The hardened plantlets were then transferred to polythene bags containing soil: compost (3:1) and kept in green house. These plantlets were irrigated with normal tap water. Such plantlets have been transferred to the field after proper hardening in mist polyhouse (1 month) and in shade house (1-2 month).

3.2.2 Macropropagation:

Various research experiments were carried out for induction of rooting in stem cuttings collected from mature trees. This methodology involves maintenance of mother trees, selection, preparation and treatment of stem cutting with auxins and fungicides, raising them in root trainer or in polybags inside mist polyhouse, hardening in shade house and finally establishing them in field.

3.2.2.1 Collection and Preparation of Stem Cuttings:

Stem cuttings were collected from 15-16 year-old-trees growing in AFRI’s experimental area. These trees were marked as resource mother stock (table 4) for collecting stem cuttings for macropropagation studies. To induce new rejuvenated sprouts some trees were lopped, some were pollard at 1.5 meter height and some were cut at 30 cm height from the ground to induce new sprouts. All these managed trees were watered at 15 days interval to increase the number of new sprouts and their further growth. Trees were also treated with fungicide and insecticide to prevent the attack of pathogens and insects.

Stem cuttings were collected from such managed trees growing in AFRI’s experimental area. Fresh stem cuttings were collected having 0.5-2.0 cm thickness,
with 3 to 5 nodes unless otherwise mentioned in the experiment. Various experiments were conducted on *T. undulata* stem cutting to see the effect of season, auxins (IAA, IBA, NAA etc.) genotypes, number of nodes and thickness on rooting. The stem cuttings were harvested with the help of sharp secateurs and were kept in wet gunny bags to avoid desiccation during transportation from collection site to mist polyhouse. The branches were cut into approximately 8.0-12.0 cm long shoot segments. The lower end is cut close to the node in a slanting manner, to increase the surface area for absorption of nutrients and also to expose more meristematic tissues. All the leaves were removed from the cuttings to reduce water losses due to evaporation. The apical (distal) portion of stem cuttings were cut straight across the stem just 2.5 cm above the node.

### 3.2.2.2 Pre Treatment:

All the stem cuttings prepared for experiments were first treated with fungicides (Bavistin 0.1 gm/l) to kill surface fungi and their subsequent growth during root induction period. The lower portions of stem cuttings were treated with different concentration of auxins as described in each experiment. Upper portions of cutting were covered with choupatia paste (petroleum jelly, red lead and copper carbonate in the ratio 2:1:1) to prevent fungal attack. This paste also partially checks the desiccation from the upper cut end. In one experiment upper cut ends of stem cuttings were sealed with wax also.

Auxins (IBA) solutions were prepared by dissolving them in very small volume of absolute alcohol or 1 N NaOH and finally obtained the desired concentration by adding distilled water. Auxin treatment is either given for different duration at fix concentration or at different concentration for fix duration. The cuttings were planted in such a way that at least one bud remains inside the soil and upper portion bears 3 to 5 buds. The proximal end of cuttings was firmly embedded in the soil.

### 3.2.2.3 Potting Mixture:

A mixture of sand: compost (3:1) ratio was filled in polythene bags. The soil mixture used was sterilized by formalin (5 %). In most of the experiments polythene bags were used but in one experiment black color 150 cc and 20 celled root trainers
(Hyko trays) were used. These polybags/root trainers were placed in a raised platform inside mist polyhouse.

3.2.2.4 Environmental Conditions:

The temperature and relative humidity (RH) of mist polyhouse were controlled with the help of cooling pods and exial fans system to maintain 60-90 % RH and 25-35 °C temperature.

Mist polyhouse was covered with 25 % shade agronet to reduce excess of sunlight. The actual light intensity in the polyhouse ranged between 5500 ± 100 lux in winter and 8700 ± 100 lux in summers as measured by lux meter. The stem cuttings were maintained in mist polyhouse for 3 months for observing sprouting and rooting response.

3.2.2.5 Hardening and Field transplantation:

After removing polythene bag carefully, fifteen macropropagated and hardened plants were transferred to the field into 45x45x45 cm³ size pits along with the earth ball attached to root system. These clonal plants were transferred to field in the months of July (rainy period). Field established macropropagated plants were watered immediately and thereafter at an interval of seven days for 2 months (except in rainy day) and then at an interval of 15 days for 4 months. Thereafter, they were irrigated on monthly basis for subsequent six months. After one year these plants were left in natural conditions, where they are growing well.

3.2.3 Recording of Observation:

In case of micropropagation studies, observations were recorded on shoot initiation, shoot multiplication, shoot elongation, callus growth, shoot tip necrosis, leaf fall, root induction, root length and root number in regular intervals as described in each experiment. Survival rate was recorded during hardening. Qualitative character such as callusing, leaf fall and shoot tip necrosis were recorded by assigning relative scores viz. - = no callusing/no necrosis; ++ = low; +++ = moderate; ++++ = heavy.
Data of macropropagation studies were recorded on number of stem cutting sprouted and rooted at an interval of 15 days till termination of experiment. Survival percentage was recorded during hardening and field trials and growth data on annual basis were also recorded till 2011.

3.2.4 Statistical Analysis:

The experimental unit is explants/stem cutting, which can be assigned at random to a treatment group. Thus in present studies, completely randomized design (CRD) were used for statistical analysis. This design can accommodate any number of treatment groups, and unequal numbers in each group. Data collected and shown in percentage were transformed by square root if it was ranging either from 0 to 30 % or 70 to 100 % and if data were less than 10 %, 0.5 value added to all percent values before statistical analysis. After analysis data were converted back to original percentage values in tables. Data were evaluated by ANOVA test and means were compared with Duncan’s test to determine the significance of differences using SPSS (version 8.0) software.