Materials & Methods
Materials
Plant material
- *Caralluma adscendens var. attenuata* Wight
- *Caralluma adscendens var. fimbriata* Wallich
- *Caralluma lasiantha* (Wight) N.E.Br.

Chemicals
The chemicals used during the course of the study were of analytical grade. Inorganic salts were obtained from Qualigens, HiMedia, Merck and S.D. fine chemicals, India. All vitamins and plant growth regulators were obtained from Sigma Chemical Co., USA. Agar agar and sucrose were obtained from Qualigens, India. Casein hydrolysate, yeast extract, citric acid, PVP, activated charcoal was purchased from HiMedia Laboratories, India.

Solutions
Hydrogen peroxide, Hydrochloric acid etc.

Glassware
Test tubes (2.5x15 cm), petri plates (55mm and 85mm diameter), Erlenmeyer flask and beakers (100, 250, 500, 1000 ml capacity), pipettes (1, 2, 5, 10 ml capacity) and measuring cylinders of all capacities (10ml-1000ml) were purchased from "Borosil" India. In addition glass screw capped bottles (6x15 cm) were used for some experiments.

Plastic ware
Micro tips (2-200µl, 100-1000µl, 500-5000µl) were obtained from "Sigma Chemical Co" (USA) and plastic beakers of various capacities (50ml, 100ml, 250ml, 500ml and 1000ml) were obtained from "Borosil" India.

Collection of Plant material and maintenance
In the present investigation all the plants belong to Asclepiadaceae. The methodologies followed are same for all plants. *Caralluma lasiantha* and *Caralluma adscendens* var. *fimbriata* shoots were collected along with roots.
form Gooty Hills, Anantapur district, Andhra Pradesh during January, 2004. Shoots along with roots of *Caralluma adscendens* var. *attenuata* were procured in the month of January, 2004 near Sri Krishnadevaraya University campus, Anantapur, Andhra Pradesh.

All the plants along with roots were potted in 30 cm diameter pots and maintained at Sri Krishnadevaraya University garden. The plants were grown at 30°- 40° C with natural daylight and irrigated water as required. After one month of plant establishment in pots, young shoots started growing. Actively growing shoots were used as the source of explant for *in vitro* studies.

**Cleaning of glassware and Storage**

Glassware was soaked in Labolene (detergent) for few hours and washed thoroughly under tap water. Glassware was first rinsed with deionized water followed by distilled water and oven dried (100°C for overnight). To recycle glassware that had contaminated tissue or media prior to washing the culture vials were decontaminated by autoclaving without opening of the closures. The culture vials were soaked in detergent for overnight and then washed with tap water, deionized water, distilled water and oven dried. Even the disposal culture vials were autoclaved prior to discarding them, in order to minimize the spread of bacteria and fungi in the laboratory. Cleaned and dried glassware was finally stored in closed racks until use.

**Media Preparation**

Nutritional requirements for optimal growth on a tissue *in vitro* may vary with the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth (Murashige and Skoog, 1962). There is no single medium that is suitable for all types of plant tissues and organs. So for convenience three media that represent high, medium and low salt concentrations were selected for present investigation.

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) which was developed for culture of tobacco has comparatively high salts
levels, where as Gamborg's B5 medium (Gamborg et al., 1968) was developed for Soybean callus culture and contains a much greater proportion of nitrate compared to ammonium ions and finally Woody Plant medium (WPM) (Lloyd and McCown, 1981) were used in the study to carry out different experiments. The composition of three different media is represented in Table 4.

To maintain the vital functions of a culture, the basic medium consist of inorganic salts (macronutrients and micronutrients), organic compounds (vitamins and amino acids), plant growth regulators, gelling agent and carbon sources were recognized as essential (Gamborg et al., 1968; Schenk and Hildebrandt, 1972; Murashige, 1973). Other organic supplement such CH, CM, YE and antioxidants such as PVP, AC and CA were also added to the medium when required.

**Inorganic salts and organic salts**

Required amounts of macronutrients were weighed specific to each media and dissolved in double distilled water sequentially. Concentrated stock solutions (40X) of inorganic and organic micronutrients were prepared separately. Except iron stock all other stocks are stored in a refrigerator. In order to avoid any problem with iron solubility it was prepared as chelated form. It was prepared by dissolving FeSO$_4$.7H$_2$O and Na$_2$EDTA separately by heating and finally two solutions were mixed and made up to 100ml with sterilized double distilled water and stored at room temperature in amber coloured bottle.

**Plant growth regulators**

The stock solutions of different plant growth regulators such as auxins, cytokinins and gibberellins were prepared at 0.01% concentration using suitable solvent (Table 5) made up to 100ml using sterilized distilled water. Depending on the requirement appropriate amount of PGR stock was added to the basal medium. The stock solution once prepared was not used after three months.
TABLE: 4 Composition of different plant tissue culture media  
(All values expressed as mg/l)

<table>
<thead>
<tr>
<th>Components</th>
<th>Murashige &amp; Skoog MS (mg/l)</th>
<th>Gamborg's B5 (mg/l)</th>
<th>Woody Plant Medium WPM (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄·7H₂O</td>
<td>370</td>
<td>250</td>
<td>370</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>-</td>
<td>170</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>-</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td>3000</td>
<td>-</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>-</td>
<td>400</td>
</tr>
<tr>
<td>CaCl₂ 2H₂O</td>
<td>440</td>
<td>150</td>
<td>96</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>-</td>
<td>134</td>
<td>-</td>
</tr>
<tr>
<td>Ca (NO₃)₂·4H₂O</td>
<td>-</td>
<td>-</td>
<td>556</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>-</td>
<td>-</td>
<td>990</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>3</td>
<td>6.2</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>0.75</td>
<td>-</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>CoCl₂ 2H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>-</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>22.3</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>ZnSO₄ 7H₂O</td>
<td>8.6</td>
<td>2</td>
<td>806</td>
</tr>
<tr>
<td>CuSO₄ 5H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>0.25</td>
</tr>
<tr>
<td>FeSO₄ 7H₂O</td>
<td>27.85</td>
<td>27.8</td>
<td>27.8</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>37.25</td>
<td>33.6</td>
<td>33.6</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.5</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Inositol</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30000</td>
<td>30000</td>
<td>30000</td>
</tr>
</tbody>
</table>
TABLE: 5 Showing equivalent values in Morality for different concentrations (mg/l) of plant growth regulators along with their molecular weights and suitable solvents used in plant tissue culture.

<table>
<thead>
<tr>
<th>Growth regulators Concentration mg/l</th>
<th>Cytokinins (µM)</th>
<th>Auxins (µM)</th>
<th>Gibberellins(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA</td>
<td>KN</td>
<td>2IP</td>
</tr>
<tr>
<td>0.1</td>
<td>0.44</td>
<td>0.46</td>
<td>0.49</td>
</tr>
<tr>
<td>0.2</td>
<td>0.88</td>
<td>0.93</td>
<td>0.98</td>
</tr>
<tr>
<td>0.3</td>
<td>1.33</td>
<td>1.39</td>
<td>1.48</td>
</tr>
<tr>
<td>0.4</td>
<td>1.78</td>
<td>1.86</td>
<td>1.97</td>
</tr>
<tr>
<td>0.5</td>
<td>2.22</td>
<td>2.32</td>
<td>2.46</td>
</tr>
<tr>
<td>0.6</td>
<td>2.66</td>
<td>2.79</td>
<td>2.95</td>
</tr>
<tr>
<td>0.7</td>
<td>3.11</td>
<td>3.25</td>
<td>3.44</td>
</tr>
<tr>
<td>0.8</td>
<td>3.55</td>
<td>3.72</td>
<td>3.94</td>
</tr>
<tr>
<td>0.9</td>
<td>3.99</td>
<td>4.18</td>
<td>4.43</td>
</tr>
<tr>
<td>1.0</td>
<td>4.44</td>
<td>4.65</td>
<td>4.92</td>
</tr>
<tr>
<td>2.0</td>
<td>8.87</td>
<td>9.29</td>
<td>9.84</td>
</tr>
<tr>
<td>5.0</td>
<td>22.19</td>
<td>23.23</td>
<td>24.61</td>
</tr>
<tr>
<td>10.0</td>
<td>44.38</td>
<td>46.47</td>
<td>49.21</td>
</tr>
<tr>
<td>Solvent</td>
<td>1N NaOH</td>
<td>1N NaOH</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>Molecular weight (gm)</td>
<td>25.3</td>
<td>215.2</td>
<td>203.2</td>
</tr>
</tbody>
</table>
Carbon source

All plant tissue culture media required the presence of a carbon source and was added in the form of carbohydrate. In the present study glucose, fructose, sucrose, maltose and galactose are the chief carbon source. Out of these, sucrose was used as a carbon source in different concentration throughout the study.

Additives and antioxidants

Numerous complex nutritive mixtures of undefined composition like casein hydrolysate (CH), coconut milk (CM) and yeast extract (YE) were also added to medium as and when required. Antioxidants such as Citric acid (CA), poly vinylpyrrolidine (PVP), activated charcoal (AC) were added to culture medium in some experiments.

Solidifying agent

For solidification bacteriological agar at 0.8% was used in almost all experiments except rooting (0.6%) experiments.

Preparation of 1 liter Medium

For preparation of 1 liter medium, 500ml of double distilled water was taken in clean Erlenmeyer flask. Macronutrients were added sequentially followed by addition of freshly weighed sucrose and 2.5ml each of micronutrient stock solution to get desired concentration. Required quantity of desired PGRs was added at this stage. After addition of all constituents of media, the pH was adjusted to 5.8 using 1N NaOH or 1N HCl. Medium was made up to one liter. Gelling agent was added as per requirement and media was kept in microwave oven to melt the gelling agent. It was then dispensed into test tubes (15ml) or culture bottles (50ml) and autoclaved at 121°C at 15psi for 15 minutes. All the PGRs used during the course of the present work were added before autoclaving the medium. After sterilization based on the requirement tubes were kept in slant or in vertical position.
Preparation of 1 Liter Murashige and Skoog Medium

Take 500 ml of double distilled water in 1 Liter Erlenmeyer

Weigh, add and dissolve the following macro salts one by one in sequence

<table>
<thead>
<tr>
<th>Micro Stock - I (40X)</th>
<th>mg/100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄·7H₂O</td>
<td>370 mg</td>
</tr>
<tr>
<td>KH₂SO₄</td>
<td>170 mg</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650 mg</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900 mg</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>440 mg</td>
</tr>
<tr>
<td>Myo inositol</td>
<td>100 mg</td>
</tr>
</tbody>
</table>

Weigh, add and dissolve 3% Sucrose

Add 2.5ml of each micro stock I, II, III & IV

Add aliquots of PGR stock of desired concentration

Add DDW upto 900ml in Erlenmeyer flask

Adjust pH to 5.8

Pour medium into graduated cylinder and make up to 1 liter

Add 0.8% agar agar

Melt agar agar with intermittent shaking

Dispense the medium of 15ml each into culture tubes or 50ml each into culture bottles

Autoclave at 15 psi at 121°C for 15 min
Sterilization techniques

The first condition for the success of a culture is asepsis. A few simple precautions to avoid contamination will save valuable time in not repeating experiments.

Preparation of sterile media, containers and small instruments

The nutrient media used in tissue culture were sterilized by autoclaving. In present investigation culture media in glass containers sealed with aluminum foil or plastic closures were autoclaved at 15psi and 121°C for 15 minutes.

Glass culture vials were mostly sterilized along with medium whereas the glassware used for presterilized nutrient medium preparation was sterilized by dry heating in hot air oven at 160-180°C for 3hours.

For aseptic manipulation, the instruments such as forceps, scalpels, and scissors were sterilized by dipping in ethanol, followed by flaming and cooling, which is referred as flame sterilization.

Maintenance of aseptic conditions

Prior to commencing work the hands were washed with antiseptic soap followed by swabbing with ethanol. The laminar airflow chamber was sprayed with ethanol and all paraphernalia used in inoculation such as medium, forceps, scalpel, petri plates etc. were transferred to it and UV light was switched on for 30 minutes. UV light was switched off and air flow was switched on which flow at the velocity of 27±3m/sec. Work in laminar was started after 15 minutes of airflow, so as to remove the O₃ which was built up in the chamber during UV radiation for 30 minutes. In order to minimize the contamination during inoculation hands and inoculating area were swabbed with alcohol frequently, followed by flame sterilization of forceps and scalpel.

Surface sterilization of explant

The explant material, which is used for inoculation generally, loaded with many microorganisms. The growth of explant will be hampered because
microorganisms with brief life cycle grow very fastly on the medium. So, for the development of explant without any problem, the plant material was cleaned and surface sterilized.

Since the plants under investigation belong to same genus the methods followed are same. Actively growing shoots with five to six nodes were the source of explants. The shoots were washed under running tap water to remove soil and dust for 5-10 min. Young shoots were first given a liquid detergent treatment with 1%(v/v) Tween 20 (Merck) for five minutes with constant agitation. After washing off the detergent solution remaining steps of sterilization were carried under aseptic conditions in laminar airflow chamber. After rinsing with sterile double distilled water the explant material was immersed in 70% ethanol for 60sec followed by mercuric chloride (1-0.1%) and hydrogen peroxide (15-30%) at different time intervals (1,3,5 and 10 minutes). After treatment with sterilant the explants were thoroughly rinsed with several changes of sterile double distilled water because retention of such noxious chemicals will seriously affect the establishment of culture.

Transfer of explant

Inoculation was carried out near spirit lamp. Sterilized plant material was transferred on to sterilized petri plate containing filter paper. With the help of forceps and scalpel the explants were dissected and damaged ends were removed blotted on a sterile filter paper. Then explants were inoculated on to culture medium vertically to maintain polarity. The mouth of culture tubes were covered with sterilized aluminum foil and tied with rubber bands. Culture tubes were labeled giving the details of the experiment, name of the explant, date of inoculation etc. For each experiment a minimum of 15 tubes were maintained and all experiments were repeated at least thrice.

Maintenance of culture conditions

Plant cultures are greatly influenced by physical factors such as temperature, relative humidity and light. A constant temperature of 25±2°C was maintained in culture room by air conditioner. A photoperiod of 16 hours
light and 8 hours dark is maintained with an automatic timer. The cultures are provided with a light intensity of 10W/cm².

**Shoot multiplication experiments**

**Explant evaluations**

Surface sterilized juvenile explants such as node, internode and shoot tip explants were cultured on MS medium with BA 2 mg/l and 3% sucrose. Based on percentage of response, shoot number and shoot length best explant for shoot regeneration was identified.

**Media evaluation**

The best explant was selected and cultured on three different media i.e. MS, B₅, WPM supplemented with BA 2mg/l and 3% sucrose. The morphogenic response of the explant on different media was recorded.

**Hormonal evaluation**

After selecting the explant and medium based on percentage of response, shoot number and shoot length, the explants were subjected to various experimental manipulation to assess the morphogenic influence of hormonal combination, different sugars, different concentration of sucrose, organic supplements and antioxidants.

**Callus culture**

**Medium evaluation**

Stem segments were cultured on three different media such as MS, B₅ and WPM supplemented with 2,4-D 2 mg/l for callus initiation. Based on the frequency of response, callus mass and quality the suitable medium was selected for further experiments.

**Hormonal evaluation**

Stem segments were cultured on selected medium supplemented with different hormonal concentration and combinations for screening best combination for callus production.
Organogenesis

Organogenic callus was subcultured on basal medium supplemented with different concentrations of cytokinins and combinations for shoot development. The developed shoots were further cultured for *in vitro* rooting to develop into a complete plantlet.

Somatic embryogenesis

Culturing stem segments on different hormonal concentration induced embryogenic callus, which was further transferred into other hormonal concentrations for the germination of somatic embryos.

*In vitro* rooting

Microshoots with 4-5 nodes were cultured on different media such as full strength and half strength MS, B5 and WPM with and without NAA 0.1 mg/l, 1% sucrose and 0.6% agar. After selecting the best basal medium further the medium is supplemented with different concentration of auxins.

Acclimatization

Well rooted shoots were removed from culture tubes and washed with tap water to remove the traces of agar. The plantlets were transplanted into plastic pots containing sand, farmyard manure and peat moss in 1:1:1 ratio and pots were irrigated with half strength basal liquid medium without sucrose. The plastic pots were covered with a polythene bag to maintain humidity and small holes were made. The width of the holes was slowly increased until the humidity inside and outside the polythene bags comes equal. Later on the polythene bag was removed and well developed plant was further transferred into soil.

Statistical analysis

Each culture tube with one shoot explant was considered as one replicate. Each treatment in each set of experiments consists of 15 replicates and each experiment was repeated three times. Standard error of means was calculated in each experiment. The data was statistically analyzed using one-way analysis of variance (ANOVA), and means were compared using the Tukey test at the 0.05% level of significance.