4. Results

4.1. DIRECT ORGANOGENESIS

4.1.1. Effect of plant growth regulators

The nodal explants cultured on MS basal medium (without cytokinins) did not show any morphogenetic response and failed to produce shoots even after 7 weeks of culture. On the other hand MS basal medium containing TDZ and BAP (0.5-3.0 mg L\(^{-1}\)), resulted in the induction of multiple shoots from explants after 2 weeks of incubation (Fig. 2 A). Among the various concentration of TDZ and BAP, effective shoot regeneration (15.0 ± 0.39) was obtained in TDZ at 2.0 mg L\(^{-1}\) level (Table 1). However, the cultures continuously growing in the same medium for long duration especially above two or more subcultures, showed abnormalities.

<table>
<thead>
<tr>
<th>Plant growth regulators mg L(^{-1})</th>
<th>Regeneration (%)</th>
<th>Number of shoots (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDZ 0.5 - -</td>
<td>75</td>
<td>4.7 ± 0.40</td>
</tr>
<tr>
<td>1.0 - -</td>
<td>80</td>
<td>10.6 ± 0.90</td>
</tr>
<tr>
<td>2.0 - -</td>
<td>93</td>
<td>15.0 ± 0.39</td>
</tr>
<tr>
<td>3.0 - -</td>
<td>78</td>
<td>7.4 ± 0.40</td>
</tr>
<tr>
<td>- 0.5 -</td>
<td>70</td>
<td>3.6 ± 0.40</td>
</tr>
<tr>
<td>- 1.0 -</td>
<td>80</td>
<td>9.5 ± 0.35</td>
</tr>
<tr>
<td>- 2.0 -</td>
<td>88</td>
<td>12.0 ± 0.42</td>
</tr>
<tr>
<td>- 3.0 -</td>
<td>74</td>
<td>5.7 ± 0.22</td>
</tr>
<tr>
<td>2.0 - 0.25</td>
<td>85</td>
<td>14.3 ± 0.17</td>
</tr>
<tr>
<td>2.0 - 0.5</td>
<td>95</td>
<td>26.4 ± 0.22</td>
</tr>
<tr>
<td>2.0 - 1.0</td>
<td>90</td>
<td>18.3 ± 0.14</td>
</tr>
<tr>
<td>- 2.0 0.25</td>
<td>82</td>
<td>12.5 ± 0.15</td>
</tr>
<tr>
<td>- 2.0 0.5</td>
<td>87</td>
<td>17.2 ± 0.19</td>
</tr>
<tr>
<td>- 2.0 1.0</td>
<td>78</td>
<td>10.5 ± 0.26</td>
</tr>
</tbody>
</table>

Data represented mean, mean ± SE (standard error) of three replicates, each with 20 explants.

In vitro culture studies on medicinal plant of Aerva lanata (L.) Juss. Ex Schult. 52
Figure 2: Direct organogenesis from nodal segments. A) Multiple shoot induction from nodal explant on MS medium containing 2.0 mg L⁻¹ TDZ after 3 weeks of culture. B&C) Multiple shoot formation on MS medium containing 2.0 mg L⁻¹ TDZ combination with 0.5 mg L⁻¹ NAA after 5 weeks of culture. D) In vitro rooting in MS medium containing 1.0 mg L⁻¹ IBA after 4 weeks of culture. E) Induction of in vitro flowering on MS medium containing 1.0 mg L⁻¹ TDZ in combination with 0.5 mg L⁻¹ NAA after 5 weeks of culture. Scale bars: 1.5 cm (A-C), 1.0 cm (D, E).
4. Results

In the present study, synergistic effect of auxin in combination with cytokinin was also evaluated (Table 1). TDZ in combination with NAA markedly enhanced shoot multiplication. However, BAP in combination with NAA did not improve the parameters evaluated. Among all the combinations tried, the maximum percentage of multiple shoot formation was observed in 2.0 mg L⁻¹ TDZ in combination with 0.5 mg L⁻¹ NAA (26.4 ± 0.22; Fig. 2 B). The regenerated shoots were subjected to different treatment of IAA or IBA for rooting (Fig. 3A, B). The best rooting response (7.01 ± 0.56 of ~ 95.2 % of roots per shootlet) was obtained in 1.0 mg L⁻¹ IBA. Flower bud initiation started on the axis of some branches of in vitro regenerated shoots after 4 weeks on a treatment of 1.0 mg L⁻¹ TDZ in combination with 0.5 mg L⁻¹ NAA. The frequency of flowering was found to be 80 % and the maximum number of flower buds per plantlet was 5-9 (Fig. 2E).
4. Results

A) 

![Graph showing percentage of rooting response against different concentrations of PGRs (IAA and IBA).]

B) 

![Graph showing number of roots per shoot against different concentrations of PGRs (IAA and IBA).]

**Figure 3:** Effect of auxins on *in vitro* root induction. A) Percentage of root induction. B) Mean number of roots per shoot. Data represented mean ± SE (standard error) of three replicates, each with 20 explants.
4.1.2. Effect of explant on direct organogenesis

4.1.2.1. Effect of explant on shoot multiplication

In the present study, attempts have been made to optimize the suitable explant and growth regulator treatment for shoot multiplication. Multiple shoot formation was observed from all tested *ex vitro* derived explant types (vegetative nodal, flower bud nodal and mature flower nodal segments) after 15-35 days of culture (Table 2). Among the various *ex vitro* explants, vegetative nodal segments showed maximum number of shoots (3.55 ± 0.22) on MS medium containing with 1.0 mg L⁻¹ BAP (Fig. 4 A, B) and flower bud nodal explant produced only a single shoot (Table 2, Fig. 4 D). No shoot initiation was obtained from mature flower node explant.

Multiple shoot formation ability was tested in all *in vitro* raised explant types (vegetative nodal, flower bud nodal and mature flower nodal segments) after 15-35 days of culture. The highest shoot multiplication frequency (87 %) and maximum number of shoots (18.65 ± 0.39) were obtained from vegetative nodal segments on MS medium containing 1.0 mg L⁻¹ BAP followed by young flower bud node segments (Table 2; Fig. 5 A, B). However, young flower bud nodal segments showed the lowest shoot multiplication frequency (72 %) on the same medium (Fig. 5 E, F). These explants did not proliferate any further shoots beyond 6 weeks. No shoot initiation was obtained from mature flower node explant (Fig. 5 G)
### Table 2: Effect of explants on shoot multiplication

<table>
<thead>
<tr>
<th>PGRs mg L(^{-1})</th>
<th>Vegetative nodal segments</th>
<th>Young flower bud nodal segments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ex vitro</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAP</td>
<td>KIN</td>
</tr>
<tr>
<td>0.2</td>
<td>-</td>
<td>56</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>66</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>75</td>
</tr>
<tr>
<td>0.2</td>
<td>-</td>
<td>48</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>57</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>-</td>
<td>65</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>76</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>87</td>
</tr>
<tr>
<td>0.2</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>64</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>73</td>
</tr>
</tbody>
</table>

Data represents mean ± SE of three replicates; each experiment was repeated thrice. Means separation within column by Duncan’s multiple range test at P < 0.05.

**Figure 4:** Effect of *ex vitro* explant on direct organogenesis.  
A) Vegetative nodal explant of *ex vitro* explant on MS medium containing 1.0 mg L\(^{-1}\) BAP. B) Multiple shoot formation on MS medium containing 1.0 mg L\(^{-1}\) BAP. C) Root formation in MS medium containing 1.0 mg L\(^{-1}\) IBA. D) *Ex vitro* raised flower bud nodal explant cultured on MS medium containing 1.0 mg L\(^{-1}\) BAP. Scale bars: 0.5cm (A-D).
Figure 5: Effect of *in vitro* explant on direct organogenesis. A) Vegetative nodal explant of *ex vitro* explant on MS medium containing 1.0 mg L\(^{-1}\) BAP. B) Multiple shoot formation on MS medium containing 1.0 mg L\(^{-1}\) BAP. C) Root formation in MS medium containing 1.0 mg L\(^{-1}\) IBA. D) Basal callus formation along with roots in MS medium containing 1.0 mg L\(^{-1}\) NAA. E\&F) Flower bud nodal explant of *in vitro* raised plant on MS medium containing 1.0 mg L\(^{-1}\) BAP. G) Mature flower bud nodal explant of *in vitro* raised plant on MS medium containing 1.0 mg L\(^{-1}\) BAP. *Scale bar:* 0.5cm (A), 1cm (B-D), 0.5cm (E-G).
4.1.2.2. Root induction

All the regenerated microshoots (4–5 cm) were excised and transferred to full and half strength MS medium containing either IBA or NAA for rooting. Optimum root induction was obtained when microshoots (obtained both from in vitro and ex vitro plantlets) were cultured in 1/2 strength MS medium containing 1.0 mg L\(^{-1}\) IBA (Fig. 5C). Microshoots within 1.0 mg L\(^{-1}\) NAA containing medium also produces roots, however callus formation also observed along with roots (Fig. 5 D)

4.1.2.3. Histological analysis of organogenesis

4.1.2.3.1. Ex vitro nodal explants

Histological studies of *A. lanata* ex vitro nodal explants during organogenesis revealed difference in regeneration potential depending on the vegetative and flowering stages of the mother plant. Generally, regeneration capacity is poor when explants are obtained during flowering stage rather than vegetative stage. Longitudinal section of *ex vitro* nodal explants obtained during vegetative stage revealed the presence of axillary meristem with leaf primordia in their nodal axis region and well developed cortical and vascular bundles were present below this region (Fig. 6 D). C.S of this explant shows, the presence of the distinct inner and outer zones. Epidermis is uniseriate and fully developed. Epidermis hairs are abundant, thick and straight. Underneath the epidermis, fully developed cortex with uniform, thick walled compact cortical cells were observed. Groups of well developed meristematic cells are present in the pericycle region. Vascular bundles are endarch with well developed xylem and phloem (Fig. 6 G, J).
4. Results

Nodal explants obtained during reproductive stage were characterized by poor regeneration capacity and showed distinct anatomical characters. Presence of a doom shaped floral meristem was observed in this nodal region (E, F). Abundant growth of long uniseriate eglandular trichomes were observed around the flower meristem. C.S of cortex consists of continuous cylinder of collenchyma cells (Fig. 6 H, I). Thick layer of sclerenchyma tissue was observed in the later stage of flower developments. Pith region is fully developed with thick cell wall and several resin canals. Fully developed vascular bundles with layers of sclerenchyma sheath were observed in the stelar region (Fig. 6 K, L). The cross section of vegetative nodal segments along with multiple shoots revealed the presence of distinct meristematic zone. Initiation and development of shoots were observed from this meristematic zone (Fig. 6 M, N).
4. Results

Figure 6: Histological analysis of ex vitro explant during direct organogenesis
A-C) Vegetative node, flower bud node before flowering and flower bud node after flowering
4.1.2.3.2. *In vitro* nodal explants

Nodal segment explants obtained from *in vitro* raised plants showed interesting morphological and anatomical features. They produced numerous shoot buds from the apical region instead of the single axis bud development in the *in vitro* explant. Similar to the *ex vitro* explants, flowering nodal segments obtained during reproductive stage of *in vitro* plants also showed poor regeneration ability.

Longitudinal section of *in vitro* raised nodal segments obtained during vegetative stage revealed the presence of one axial bud with leaf primordia in the nodal region (Fig. 7 D). C.S of the explants showed the presence of distinct inner and outer zones. Epidermis is uniseriate with thin walled cells and poor cuticle development. Epidermal hairs are uniseriate thin and wiry. Underneath the epidermis, broad, irregular thin walled cortical parenchyma cells were observed. They contain plastids with chlorophyll and other pigments. Vascular bundles are limited with poorly developed xylem and phloem tissues. Pith is parenchymatous with thin walled cells (Fig. 7 G, J).

Nodal explants obtained during reproductive stage showed similar anatomical pattern with *in vivo* nodal explant of the same stage. Presence of floral meristem, eglandular trichomes, collenchyma and sclerenchyma sheaths were observed both *ex vitro* and *in vitro* nodal segments during this reproductive stage. However, the collenchyma and sclerenchyma tissues of *in vitro* nodal segments obtained during reproductive stage showed thinner walls than that of *in vivo* nodal explants. Further, they possess less number of eglandular trichomes around the flower meristem (Fig. 7 E, F). Presence of
parenchyma cells in the cortex and pith region of *in vitro* nodal segments of flowering stage were apparently less than those of *in vitro* nodal segments of vegetative stage. The average area of xylem, vascular bundle, collenchyma tissue and sclerenchyma tissues were increased in reproductive *in vitro* nodal segments than those of vegetative nodal segments (Fig. 7 K, L)

After subculture, nodal segments which were obtained from vegetative stage of *in vitro* raised plants showed more meristematic activity at the base and produced numerous shoot buds. Some of the parenchyma cells of in the cortex and pith were enlarged and showed callus like appearance. The diameter of the explants was several times increased more than those of the normal nodal explants. Increases in rate of cell growth and cell division were indicated not only by their increased volumes of nodal segments but also by the formation of adventitious shoots and the presence of callus like region in the explants. The cross section of vegetative nodal segments along with multiple shoots revealed the presence of distinct meristematic zone. Initiation and development of shoots were observed from the meristematic zone (Fig. 7 M-O)
Figure 7: Histological analysis of in vitro explant during direct organogenesis
A-C) Vegetative node, flower bud node before flowering and flower bud node after flowering
D) L.S of vegetative nodal region
E) L.S of floral nodal region just before flowering and F) after flowering.
G-I) C.S of vegetative nodal region, floral node just before flowering and after flowering stage.
M, N) Cross section showed multiple shoot formation from vegetative node of shoot meristem origin on vascular bundle connectivity. Scale bars: 0.5 cm (A-C), 200 μm (D-L), 50 μm (M-O).
4.1.2.3.3. Ex vitro and in vitro leaf explants

In cross sections taken from leaf (ex vitro and in vitro) of A. lanata, the following significant properties were observed. Leaves are small, 2 cm long and 1 cm wide, smooth-edged, mucronate in shape oval. The leaf plate is thin and the base is tapered. The side veins reach the edge of the leaf plate. The leafstalk is low-ribbed. The upper epidermis cells are larger than lower epidermis cells. The cell walls are rectilineal and slightly anfractuose. The outer walls are thickened. The indumentums consist of simple conical shaped multicellular hairs. By veins, the epidermal cells are tetrahedral and stretched along the axis. The mesophyll is dorsoventral. Below the upper epidermis, there are 2-3 layered narrow palisade cells ending above the bundles. The spongy parenchyma consists of 3-5 layers and they contain chlorophyll and ends above the bundles. The conducting bundles consist of small vessels and phloem cells. The midrib makes a small projection on the upper side of the leaf and a big one on the lower side. These projections consist of several rows of angular collenchyma (Table 3; Fig. 8 A-F).

![Figure 8: Ex vitro (A-C) and in vitro leaf segments D-F)](image)

**Figure 8: Ex vitro (A-C) and in vitro leaf segments D-F)** Showing mesophyll and bulged midrib. Cu: cuticle, eh: eglandular hair, ue: upper epidermis, pp: palisade parenchyma, vb: vascular bundle, x: xylem, ph: phloem, sc: sclerenchyma, le: lower epidermis. (**Scale bar**: 100 μm (A-F)).
### Table 3: Histological analysis of leaf segment

<table>
<thead>
<tr>
<th>Explant</th>
<th>Mesophyll type</th>
<th>Palisade layer</th>
<th>Spongy layer</th>
<th>Middle vascular bundle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex vitro leaf</td>
<td>Dorsoventral</td>
<td>2-3</td>
<td>4-5</td>
<td>Collenchyma: 3-4, Parenchyma: 3-5, Present, Present, Present</td>
</tr>
<tr>
<td>In vitro leaf</td>
<td>Dorsoventral</td>
<td>1-2</td>
<td>2-3</td>
<td>Present, Present, Present</td>
</tr>
</tbody>
</table>

#### 4.1.2.3.4. Stomata of *ex vitro* and *in vitro* leaf

The blade, in face view, has adaxial epidermal cells with polygonal contour and sinuous abaxial cells showing primary pit fields. In transaction, the epidermis is uniseriate and coated by a smooth and thin cuticle. Anomocytic stomata were seen on both surfaces, although the latter type is predominant on the abaxial face. The inner and outer periclinal cell walls of the guard cells are thick and the cuticle forms ledges on the upper side. Stomata are even with the other epidermal cells or slightly raised above the surface (Fig. 9 A-D).

![Figure 9: Stomata of *ex vitro* and *in vitro* leaf: A & B – Stomata *ex vitro* leaf (anamostytic type), C & D – Stomata *in vitro* leaf (anamostytic type), Scale bars: 50 µm (A-D).](image-url)
4.1.2.3.5. Trichomes of ex vitro and in vitro stem

Both non-glandular and glandular trichomes which could occur singly often were located in small epidermal depression. There were two types of the glandular trichomes (Fig. 10 A-H). One of them was capitate stalked with uniseriate stalk which consisted of 3-5 cells. The other one was capitate-sessile. They were covered by a cuticle with dense cytoplasm.


4.1.3. Effect of liquid and solid medium on shoot proliferation

4.1.3.1. Effect of plant growth regulators on multiple shoot induction

One month old in vitro raised shoot buds and nodal segments were used for multiple shoot induction. They were cultured on MS solid medium containing different concentration (0.3-0.9 mg L⁻¹) of TDZ and BAP. Among the various treatments, 0.6 mg L⁻¹ TDZ and 0.6 mg L⁻¹ BAP significantly showed higher ratio of shoot induction (5.69 and 2.60 shoots for explants) compared to control and other treatments (Table 4). Optimum percentage (83%) of multiple shoot induction was obtained on MS medium supplemented with 0.6mg L⁻¹ TDZ alone (Fig.11 A, B). Higher concentration of TDZ (above 0.6 mg L⁻¹) or BAP showed reduction in the ratio of multiple shoot induction (Table 4).
Table 4: Effect of PGRs on multiple shoot induction from different explants

<table>
<thead>
<tr>
<th>PGRs (mg L⁻¹)</th>
<th>Shoot bud explants</th>
<th>Nodal explants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of shoot response</td>
<td>No. of shoots/explants</td>
</tr>
<tr>
<td>TDZ 0.3</td>
<td>- 66</td>
<td>1.56 ± 0.18</td>
</tr>
<tr>
<td>TDZ 0.6</td>
<td>- 76</td>
<td>2.60 ± 0.24</td>
</tr>
<tr>
<td>TDZ 0.9</td>
<td>- 68</td>
<td>1.50 ± 0.24</td>
</tr>
<tr>
<td>- 0.3</td>
<td>63</td>
<td>1.26 ± 0.20</td>
</tr>
<tr>
<td>- 0.6</td>
<td>70</td>
<td>1.57 ± 0.66</td>
</tr>
<tr>
<td>- 0.9</td>
<td>58</td>
<td>1.20 ± 0.57</td>
</tr>
</tbody>
</table>

Data represented mean ± SE was carried out using Duncan’s multiple range (DMRT) test at $P < 0.05$ level. All the experiments were carried out three times with at least 20 explants.

4.1.3.2. Effect of solid and liquid medium on proliferation

A comparative analysis of multiple shoot formation in solid (Agar 0.8%) and liquid medium was also studied. After 5 weeks, liquid medium containing 0.6 mg L⁻¹ TDZ in combination with 0.3 mg L⁻¹ NAA and 0.2 mg L⁻¹ IBA showed higher ratio (29.37 ± 0.64) of shoot multiplication (Fig. 11 F) compared to agar gelled medium (12.56 ± 1.24; Fig. 11 C). Further, subculture of these shootlets in liquid medium showed higher ratio of response in terms of shoot length, root length, number of shoots, fresh weight and dry weight (Table 5). Gradual decrease in the number of shoots per explant (13.37 ± 2.67) was observed when MS liquid medium containing lower concentrations of 0.3 mg L⁻¹ TDZ in combination with 0.3 mg L⁻¹ NAA and 0.2 mg L⁻¹ IBA, after 5 weeks of culture (Fig. 11 F). Further, cultures on MS liquid containing 0.6 mg L⁻¹ TDZ in combination with 0.3 mg L⁻¹ NAA, also showed poor respondents (7.34 ± 1.12) of multiple shoot formation data not shown (Fig. 11D).
Table 5: Effect of liquid and solid medium

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Solid</th>
<th>Liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean number of shoots per explants</td>
<td>12.56 ± 1.24</td>
<td>29.37 ± 0.64</td>
</tr>
<tr>
<td>Shoot length (cm)</td>
<td>4.36 ± 0.14</td>
<td>5.76 ± 1.44</td>
</tr>
<tr>
<td>Root length (cm)</td>
<td>3.56 ± 1.04</td>
<td>4.66 ± 0.32</td>
</tr>
<tr>
<td>Fresh weight (% w/w)</td>
<td>0.54 ± 0.03</td>
<td>0.86 ± 0.63</td>
</tr>
<tr>
<td>Dry weight (% w/w)</td>
<td>0.13 ± 0.02</td>
<td>0.28 ± 1.03</td>
</tr>
</tbody>
</table>

medium – MS medium containing 0.6 mg L\(^{-1}\) TDZ, 0.3 mg L\(^{-1}\) NAA and 0.2 mg L\(^{-1}\) IBA.

Data represented mean ± SE was carried out using Duncan’s multiple range (DMRT) test at \(p < 0.05\) level. All the experiments were carried out three times with at least 20 explants.

Figure 11: Shoot multiplication in solid and liquid medium. A, B) Multiple shoot induction on MS solid medium containing 0.6 mg L\(^{-1}\) TDZ after 3 weeks. C) Multiple shoot formation on MS solid medium containing 0.6 mg L\(^{-1}\) TDZ, 0.3 mg L\(^{-1}\) NAA and 0.2 mg L\(^{-1}\) IBA. D) Multiple shoot formation on MS liquid medium containing 0.6 mg L\(^{-1}\) TDZ, 0.3 mg L\(^{-1}\) NAA E) Multiple shoots with spontaneous root proliferation in MS liquid medium containing 0.3 mg L\(^{-1}\) TDZ in combination with 0.3 mg L\(^{-1}\) NAA and 0.2 mg L\(^{-1}\) IBA. F) MS liquid medium containing 0.6 mg L\(^{-1}\) TDZ, 0.3 mg L\(^{-1}\) NAA and 0.2 mg L\(^{-1}\) IBA. Scale bars: 0.5 cm (A, B), 1.5 cm (C-F).
4.1.3.3. Effect of media volumes on multiple shoot proliferation

Of different volumes of liquid medium used in 150 ml conical flask, 30 ml medium showed maximum number of shoot proliferation (Table 6). Further increase or decrease in the volume has led to reduction of the number of shoots. The present study also optimized the suitable culture time resulting in the number of shoots and short period of time leading to lower number of shoots. When shoots attained maximum level, no significant number of shoot multiplications had extended duration. 35 days of culture time was suitable for maximum shoot proliferation (29.37 ± 0.64 shoots for explant) in 30 ml liquid medium. It was also observed that increasing liquid medium to 40 ml promoted hyperhydricity while reducing it to 10 ml led to desiccation of shoots within 2 weeks of subculture.

Table 6: Effect of media volume on multiple shoot formation

<table>
<thead>
<tr>
<th>Volume of medium (ml)</th>
<th>Mean number of shoots per explants during different days of subculture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 days</td>
</tr>
<tr>
<td>10</td>
<td>2.34±0.50</td>
</tr>
<tr>
<td>20</td>
<td>3.25±0.60</td>
</tr>
<tr>
<td>30</td>
<td>4.54±0.24</td>
</tr>
<tr>
<td>40</td>
<td>2.14±0.14</td>
</tr>
<tr>
<td>50</td>
<td>1.38±0.12</td>
</tr>
</tbody>
</table>

Medium-MS liquid medium containing 0.6 mg L⁻¹ TDZ, 0.3 mg L⁻¹ NAA and 0.2mgL⁻¹ IBA

Data represented mean ± SE was carried out using Duncan’s multiple range (DMRT) test at $P < 0.05$ level. All the experiments were carried out three times with at least 20 explants.
4.1.4. Direct organogenesis from leaf explant

4.1.4.1. Effect of growth regulators on direct organogenesis

Leaf explants were cultured on MS basal medium alone or containing various concentrations of TDZ for the induction of shoot regeneration. Leaf explants cultured in concentrations of growth regulators except those in basal medium enlarged considerably and turned green within two weeks of culture (Fig. 12 A, B). All the explants in basal medium turned brown and died within two weeks of culture. Sporadic shoot formation was observed when basal medium was enriched with TDZ (Fig. 12 C). After 4 weeks, more adventitious shoots were observed on leaf explants cultured on media containing 1.0 mg L\(^{-1}\) TDZ compared to the other TDZ concentrations, with an average of 23.6 ± 0.16 shoots per leaf explants and frequency of shoot regeneration of 90 %. Increasing the concentration of TDZ above 1.0 mg L\(^{-1}\) resulted in a marked reduction in shoot formation in leaf explants. In this current study, low concentrations (0.25 - 1.0 mg L\(^{-1}\)) of TDZ had a significant effect on the percentage of shoot bud regeneration from leaf segments, and higher concentration exhibited inhibitory effect (Table 7).
Figure 12: Direct organogenesis from leaf explants. A, B) Initiation of adventitious shoot buds (arrowhead) from leaf explants on MS medium containing 1.0 mg L⁻¹ TDZ. C) Development of the shoot bud after 2 weeks of culture period. D, E) Leaf section shows meristematic region (arrowhead). F) Development of shoot bud after 3 weeks of culture. *Scale bars:* 2 mm (A, B), 5 mm (C), 100 μm (D, E) and 2.5 cm (F).
Table 7: Effect of TDZ on shoot regeneration from leaf explant

<table>
<thead>
<tr>
<th>PGRs (mg L(^{-1}))</th>
<th>Percentage of response</th>
<th>Mean number of shoots/explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0</td>
<td>0.0(^f)</td>
</tr>
<tr>
<td>0.25</td>
<td>50</td>
<td>8.7 ± 0.15(^d)</td>
</tr>
<tr>
<td>0.5</td>
<td>70</td>
<td>15.6 ± 0.16(^b)</td>
</tr>
<tr>
<td>1.0</td>
<td>90</td>
<td>23.6 ± 0.16(^a)</td>
</tr>
<tr>
<td>1.5</td>
<td>60</td>
<td>11.7 ± 0.15(^c)</td>
</tr>
<tr>
<td>2.0</td>
<td>40</td>
<td>6.7 ± 0.15(^c)</td>
</tr>
</tbody>
</table>

Data represented mean, mean ± SE (standard error) of three replicates, each with 15 cultures. Means having the same letter in a column were not significantly different by Duncan’s multiple range test \((P < 0.05)\).

Consistent subculturing of the \textit{in vitro} raised plants culture after every 4 weeks on fresh MS medium containing 1.0 mg L\(^{-1}\) TDZ led to improved shoot proliferation response (Fig. 12 F). Subculture times longer or shorter than 4 weeks resulted in a decline in number of shoot bud induction. When large number of \textit{de novo} shoots was regenerated in response to TDZ exposure and 4 weeks subculture. It was obviously found that the supplementation of TDZ in the culture media is important for direct organogenesis in \textit{A. lanata}. 

\textit{In vitro} culture studies on medicinal plant of \textit{Aerva lanata} (L.) Juss. Ex Schult.
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Figure 13: In vitro flowering and acclimatization. A) In vitro flowering on MS medium containing 1.0 mg L⁻¹ TDZ and 0.25 mg L⁻¹ NAA. B) In vitro flowering with inhibited shoot growth on MS medium containing 1.0 mg L⁻¹ TDZ and 0.5 mg L⁻¹ NAA. C, D) In vitro root induction in half strength MS medium containing 1.0 mg L⁻¹ IBA. E, F) An acclimatized plant survived ex vitro. Scale bars: 3 mm (A, B), 1 mm (C), 2 mm (D) and 4 mm (E, F).

An interesting feature of the present study is that the treatment of leaf explants on TDZ in combination with NAA has a positive effective on flowering in vitro (Fig. 13 A). Although 1.0 mg L⁻¹ TDZ with 0.5 mg L⁻¹ NAA, achieved the highest ratio of flowering (data not shown) (Fig. 13 B), it was not better for multiple shoot formation. Mean while, TDZ alone or in combination with IAA failed to induce floral bud formation.

In vitro culture studies on medicinal plant of *Aerva lanata* (L.) Juss. Ex Schult.
Figure 14: Effect of IBA on in vitro rooting.

Data represented mean of three replicates, each with 15 cultures.

The success of micropropagation relies on the rooting percentage and survival of plantlets upon transfer to the field condition. Regenerated shoots larger than 25 mm were selected and transferred to IBA media for rooting (Fig. 13 C). The maximum frequency of rooting (86.6 %) with highest number of (11.7 ± 0.15) roots per shoot was obtained in IBA at 1.0 mg L⁻¹ after 4 weeks (Fig. 13 D, 14). More than 200 plantlets with 4 to 5 fully expanded roots were successfully hardened off inside in the growth chamber within a period of 4 weeks (Fig. 13 E, F). Therefore these plantlets were transferred to soil and were maintained in a shade house with a survival rate of 92.0 %. Regenerated plants grew well and phenotypically similar to the parental stock. Histological analysis provided morphological details of the process of organogenesis from the leaf explants of A. lanata. One week after culture initiation, epidermal cells of the explants exhibited continuous cell division leading to formation of numerous dome shaped meristematic protrusions with high cytoplasmic content and prominent nuclei (Fig. 12 D, E).
4.2. Indirect Organogenesis

4.2.1. Callus induction and morphology

Effect of different concentration and combination of 2,4-D and NAA on callus induction from leaf and nodal explants of *A. lanata* is given in Table 8. The MS basal medium without growth regulators exhibited no callus formation. The explants cultured on the medium containing growth regulators showed callus formation from the cut edges. The leaf derived callus was yellowish green compact and fast growing. Among the different concentrations of 2,4-D tested, maximum callus induction response (67.0 %) was from leaf segments on the medium containing 0.5 mg L⁻¹ 2,4-D. This response increased to 90.0 % when 0.5 mg L⁻¹ 2,4-D was used in combination with 0.2 mg L⁻¹ NAA (Fig. 15 a, b). When nodal explants cultured on MS medium supplemented with 0.5 mg L⁻¹ 2,4-D in combination 0.2 mg L⁻¹ NAA, they induced 78.7 % brownish green compact callus after three weeks, whereas no callus formed in the presence of any concentrations of 2,4-D in combination with BAP (data not shown). Comparatively leaf segments responded better than nodal explants for callus induction.
Table 8: Effect of different concentrations and combinations of auxins on callus formation from leaf and nodal segments

<table>
<thead>
<tr>
<th>Plant growth regulators</th>
<th>Leaf</th>
<th>Node</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D NAA Callus induction frequency (%)</td>
<td>Nature of callus</td>
<td>Callus induction frequency (%)</td>
</tr>
<tr>
<td>0.0 0.0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>0.2 -</td>
<td>44.3</td>
<td>Yellowish green compact</td>
</tr>
<tr>
<td>0.3 -</td>
<td>49.9</td>
<td>White green compact</td>
</tr>
<tr>
<td>0.5 -</td>
<td>67.0</td>
<td>Yellowish green compact</td>
</tr>
<tr>
<td>1.0 -</td>
<td>63.6</td>
<td>Yellowish green compact</td>
</tr>
<tr>
<td>2.0 -</td>
<td>50.1</td>
<td>White green compact</td>
</tr>
<tr>
<td>0.2 0.1</td>
<td>52.3</td>
<td>Yellowish green compact</td>
</tr>
<tr>
<td>0.3 0.1</td>
<td>68.7</td>
<td>White green compact</td>
</tr>
<tr>
<td>0.5 0.2</td>
<td>90.0</td>
<td>Yellowish green compact</td>
</tr>
<tr>
<td>1.0 0.5</td>
<td>65.6</td>
<td>Yellowish green compact</td>
</tr>
<tr>
<td>2.0 1.0</td>
<td>48.4</td>
<td>White green compact</td>
</tr>
</tbody>
</table>

The values represent the means (±SE) of three independent experiments after 4 weeks. At least 20 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan’s multiple range test ($P \geq 0.05$)

4.2.2. Shoot proliferation from callus

The regenerative ability of yellowish green compact calli was studied by using cytokinins alone or in combination with NAA. After 2 weeks of culture, most of the calli turned green and produced shoot primordia. The MS medium containing 1.0 mg L\(^{-1}\) BAP and 0.5 mg L\(^{-1}\) NAA was significantly different compared to other concentration, as it induced highest number of shoots per callus ($7.2 \pm 0.2$) recorded after 7 weeks of culture (Table 9, Fig. 15 c, d). However, 1.0 mg L\(^{-1}\) KIN along with 0.5 mg L\(^{-1}\) NAA showed the lowest regeneration frequency (67.3 %) and produced 3.0 ± 0.1 shoots per callus.

*In vitro* culture studies on medicinal plant of *Aerva lanata* (L.) Juss. Ex Schult.
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Table 9: Effect of different concentrations and combinations of cytokinins and NAA on shoot regeneration from leaf derived callus

<table>
<thead>
<tr>
<th>Plant growth regulators (mg L⁻¹)</th>
<th>Regeneration frequency (%)</th>
<th>Mean number of shoots per callus</th>
<th>Mean number of shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP 0.0 KIN 0.0 NAA 0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.2 0.1</td>
<td>54.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.2 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.1 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5 0.2</td>
<td>67.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.6 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0 0.5</td>
<td>89.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.8 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.0 1.0</td>
<td>76.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.6 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2 0.1</td>
<td>52.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.1 ± 0.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.8 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5 0.2</td>
<td>59.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.8 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0 0.5</td>
<td>67.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.0 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.7 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.0 1.0</td>
<td>47.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.2 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values represent the means (±SE) of three independent experiments after 7 weeks. At least 20 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan’s multiple range test (P ≥ 0.05) level.
Figure 15: Callus induction and regeneration of plantlets from leaf derived callus. (A, B) Induction of calli from leaf segments on MS medium containing 0.5 mg L⁻¹ 2,4-D and 0.2 mg L⁻¹ NAA after 3 weeks of culture. (C) Initiation of multiple shoot from calli on MS medium containing 1.0 mg L⁻¹ BAP in combination of 0.5 mg L⁻¹ NAA after 4 weeks of culture. (D) Multiplication of shootlets on same medium after 7 weeks of culture. (E) Rooting of regenerated shoot with 1.0 mg L⁻¹ IBA after 4 weeks of culture. (F) An acclimatized plant survived in *ex vitro*. Scale bars: 3.3 mm (A, B), 3 mm (C), 1.5 cm (D, E).
4.2.3. **In vitro rooting and acclimatization**

*In vitro* derived shoots separated from multiple-shoot clusters started to root after 3 weeks of culture and well developed roots were recorded in 4-5 weeks on MS medium supplemented with 1.0 mg L$^{-1}$ IBA (Table 10). The highest rooting response (8.1 ± 0.3) was achieved in the MS solid medium supplemented with 1.0 mg L$^{-1}$ IBA alone (Fig. 15 e). The rooted plantlets were transferred to soil: perlite: vermiculate (1: 1: 1; v/v/v) mixture without damaging their root system and maintained in the shade house. Survival of 86.0 % of plantlets was achieved during hardening for the first 4 weeks under 85.0 % shading (Fig. 15 f). The *in vitro* derived plants were eventually transferred to natural habitat. The regenerated plants did not show detectable variation in morphology and growth characteristics when compared with that of mother plant.

**Table 10:** Effect of different concentrations of IBA on root formation from regenerated shoots

<table>
<thead>
<tr>
<th>IBA (mg L$^{-1}$)</th>
<th>Response of roots (%)</th>
<th>Mean number of roots per shoot</th>
<th>Mean number of root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.25</td>
<td>50.4</td>
<td>4.3 ± 0.2$^d$</td>
<td>2.1 ± 0.3$^c$</td>
</tr>
<tr>
<td>0.5</td>
<td>70.8</td>
<td>5.2 ± 0.2$^c$</td>
<td>3.3 ± 0.2$^b$</td>
</tr>
<tr>
<td>1.0</td>
<td>88.5</td>
<td>8.1 ± 0.3$^a$</td>
<td>4.4 ± 0.2$^a$</td>
</tr>
<tr>
<td>1.5</td>
<td>76.2</td>
<td>6.2 ± 0.2$^b$</td>
<td>3.6 ± 0.1$^b$</td>
</tr>
<tr>
<td>2.0</td>
<td>72.0</td>
<td>3.7 ± 0.4$^c$</td>
<td>2.7 ± 0.4$^c$</td>
</tr>
</tbody>
</table>

The values represent the means (±SE) of three independent experiments after 4 weeks. At least 20 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan’s multiple range test ($P \geq 0.05$) level.
4.2.4. Histological analysis

Leaf explants were used for initiation of callus and a histological study was carried out to confirm the origin of shoot tip. In this case, it was observed that the undifferentiated mass of cells, called primary callus was derived from the parenchymatous cells of the explants. A detailed study showed that small group of cells were surrounded by larger cells and these two types of cells together gave rise to shoot apical meristem.

![Histological analysis images](image)

**Figure 16: Histological analysis of indirect organogenesis.** (A) Meristemoid development within the callus mass. (B) Development of multiple shoot bud. (C, D) Development of shoot bud with leaf primordia. **Scale bars:** 150 μm (A-D).

The meristematic division and the details were shown in Fig. 16 A. The detailed histological analysis showed that the shoots regenerated from the leaf derived callus of *A. lanata* have no organized cellular connection with the original explants tissue, indicating an adventitious shoot origin (Fig. 16 B-D).
4.3. Synthetic seed technology

4.3.1. Effects of encapsulation matrix

In the present study, the nodal segments, excised from in vitro raised plantlets of *A. lanata* were used for synthetic seed production (Fig. 17 A). The morphology of calcium alginate beads with respect to shape, texture, transparency and rigidity varied with different concentrations of sodium alginate and CaCl₂·2H₂O with exposure time of 25 min. A gelling matrix of 3.0 % sodium alginate and 100 mM CaCl₂·2H₂O was found most suitable for formation (83.3 %) of ideal calcium alginate beads (Fig. 17 B) and subsequent regeneration of encapsulated nodal segments in to plantlets (Table 11). Lower concentrations of sodium alginate (1-2 % (w/v)) and CaCl₂·2H₂O (50 mM) resulted in beads without a defined shape and were too soft to handle. Whereas with a higher concentration (Fig. 17 E) of sodium alginate (4-5 %) or CaCl₂·2H₂O (150 mM), the beads were isodiametric and hard to cause considerable delay in shoot emergence. A significant improvement (83.3 %) in plantlet regeneration was observed when nodal segments were encapsulated in sodium alginate prepared in 1/2 strength MS liquid medium supplemented with BAP (0.5 mg L⁻¹) compared to the other strength of MS liquid medium (Table 12).
4. Results

Table 11: Effect of sodium alginate concentrations with 100 mM CaCl₂·2H₂O on regeneration from encapsulated nodal segments

<table>
<thead>
<tr>
<th>Sodium alginate (%)</th>
<th>Regeneration response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Fragile beads difficult to handle</td>
</tr>
<tr>
<td>2.0</td>
<td>Fragile beads difficult to handle</td>
</tr>
<tr>
<td>3.0</td>
<td>83.3±0.2</td>
</tr>
<tr>
<td>4.0</td>
<td>78.5±0.1</td>
</tr>
<tr>
<td>5.0</td>
<td>57.6±0.3</td>
</tr>
<tr>
<td>6.0</td>
<td>23.3±0.4</td>
</tr>
</tbody>
</table>

Data represent mean ± SE was carried out using Duncan’s multiple range (DMRT) test. $P < 0.05$

All experiments were carried out three times with at least 24 explants

Table 12: Effect of alginate matrix composition on regeneration of synseed

<table>
<thead>
<tr>
<th>Alginate matrix</th>
<th>Regeneration response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>½ strength MS</td>
<td>75.6±0.3</td>
</tr>
<tr>
<td>½ strength MS + 0.5 mg L⁻¹ BAP</td>
<td>83.3±0.5</td>
</tr>
<tr>
<td>Full strength MS</td>
<td>68.7±0.2</td>
</tr>
<tr>
<td>Full strength MS + 0.5 mg L⁻¹ BAP</td>
<td>73.3±0.4</td>
</tr>
</tbody>
</table>

Data represent mean ± SE was carried out using Duncan’s multiple range (DMRT) test. $P < 0.05$

All experiments were carried out three times with at least 24 explants

4.3.2. Effect of medium and growth regulators

In the present study, ideal beads produced by encapsulating nodal segments in 3% sodium alginate and 100 mM CaCl₂·2H₂O were cultured on MS basal medium without any plant growth regulators as well as with various concentrations of BAP alone or in combination with IBA. Alginate beads were cultured on MS basal medium exhibited 70% regeneration response and this occurred after 3 weeks of culture (Table 13). Addition of 0.5 mg L⁻¹ BAP, enhanced the regeneration potential of the beads and the shoots emerged
within 2 weeks of inoculation onto the regeneration medium (Fig. 17 C, D). An average number of (3.6 ± 0.2) shoots /beads was produced in the MS medium containing 0.5 mg L⁻¹ BAP in combination with 0.25 mg L⁻¹ IBA (86 % regeneration response) and also simultaneously root developed after 5 weeks of culture (Fig 17 F). MS liquid medium containing 0.5 mg L⁻¹ BAP in combination with 0.25 mg L⁻¹ IBA was found to be the best for multiple shoot formation from 2 weeks emerged encapsulation of nodal segments. Liquid medium was found to be the best (89 % responses) for the shoot multiplication (16.6 ± 0.2), shoot elongation (5.2 ± 0.1) and root length (4.2 ± 0.4) from encapsulated nodal segments (Table 13, Fig. 17 G)

**Table 13 : Effect of media and growth regulators on regeneration from encapsulated nodal segment**

<table>
<thead>
<tr>
<th>PGRs mg L⁻¹</th>
<th>Regeneration response (%)</th>
<th>No. of shoots/bead</th>
<th>Shoot length (cm)</th>
<th>Root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>70</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.3</td>
<td>-</td>
</tr>
<tr>
<td>MS solid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>73</td>
<td>1.3 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>82</td>
<td>2.5 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>0.75</td>
<td>72</td>
<td>1.7 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>59</td>
<td>1.2 ± 0.4</td>
<td>1.0 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>BAP+IBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5+0.25</td>
<td>86</td>
<td>3.6 ± 0.2</td>
<td>4.7 ± 0.3</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>MS liquid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAP+IBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5+0.25</td>
<td>89</td>
<td>16.6 ± 0.2</td>
<td>5.2 ± 0.1</td>
<td>4.2 ± 0.4</td>
</tr>
</tbody>
</table>

Data represent mean ± SE was carried out using Duncan’s multiple range (DMRT) test. P < 0.05 All experiments were carried out three times with at least 24 explants
**4. Results**

![Figure 17: Encapsulated nodal segments on MS solid and liquid medium. A. In vitro raised nodal segments. B) Calcium alginate beads placed on MS basal medium. C&D) Shoot bud sprouting on MS medium containing BAP 0.5 mg L\(^{-1}\) after two weeks. E) Stunted growth in 4 % sodium alginate. F) Well developed shoot and root on MS medium containing BAP 0.5 mg L\(^{-1}\) in combination with IBA 0.25mg L\(^{-1}\) after 4 week. G) Multiple shoots formation from encapsulated nodal segments on full strength MS liquid medium containing with BAP 0.5 mg L\(^{-1}\) in combination with IBA 0.25 mg L\(^{-1}\) after 5 weeks. H) Ex vitro shoot bud germination on potting mixture (S: S: C: V). I) 4 weeks old synthetic seed derived plantlet on potting mixture (S: S: C: V). Scale bars: 0.5 cm (A-F), 1.0 cm (G-I).**
4.3.3. Ex vitro germination of encapsulated seeds

Encapsulated nodal segments were sown into the sterilized potting mixture of soil: vermicompost (1:1), soil: sand: vermicompost (1:2:1), soil: sand: coconut fiber: vermicompost (1:1:1:1) for ex vitro regeneration of beads into the plantlets. They were moistened with 1/2 strength MS liquid medium. Sowing of synthetic seeds directly into the potting mixture facilitated the development of shoots as well as roots and production of complete plantlets with 40% survival rate after 5 weeks (Fig. 17 H).

4.3.4. Effect of storage condition

In the present study, longer storage of encapsulated nodal segments in low temperature (4°C) shows a significant reduction in plantlet regeneration. Encapsulated nodal segments of A. lanata could be stored at 4°C for 90 days. However, regeneration percentage decreased in comparison to control (Fig. 18). The experiment was carried out and observations showed that synthetic seed could be stored for 60 days that is sufficient for germplasm exchange. These findings suggested that storage of encapsulated explants for a considerable period of time allows the preservation of germplasm and could be used efficiently for regeneration of plantlets.
4. Results

Figure 18: Effect of low temperature storage (4°C) on regeneration of encapsulated nodal segments. Data represent mean ± SE, all experiments were carried out three times with at 24 explants

4.3.5. Acclimatization

Plantlets with six to eight fully expanded leaves and well-developed roots obtained from germinated/sprouted encapsulated nodal segments were successfully acclimatized in polyethylene cover containing potting mixture: soil: vermicompost (1:1), soil: sand: vermicompost (1:2:1), soil: sand: coconut fiber: vermicompost (1:1:1:1) were moistened with 1/2 strength MS liquid medium. soil: sand: coconut fiber: vermicompost (1:1:1:1) was found to be a superior potting substrate compared to other potting mixture (Fig. 19). The percentage of survival in soil: sand: coconut fiber: vermicompost (1:1:1:1) was recorded to be 85% (Fig. 17 I). These plantlets were successfully transplanted in field conditions where they grew normally without any discernable morphological variation.
Figure 19: Effect of potting mixture on plant establishment from encapsulated nodal segments. Data represent mean ± SE, all experiments were carried out three times with at 24 explants.

4.4. Adventitious root culture

4.4.1. Effect of auxins on adventitious root induction

The leaf segments were cultured on MS solid medium supplemented with different types of auxins (IBA, NAA and IAA) at different concentrations (0.5-3.0 mg L⁻¹) to scrutinize their ability of adventitious root induction. IBA was found to be more efficient when compared to both IAA and NAA (Table 14). Among the various concentrations, 2.0 mg L⁻¹ IBA (Fig. 20 A) showed best response (85 %), with optimum number of roots (7.46 ± 0.05). Increase and decrease in the concentration of IBA to this level showed, shorter and lower number of roots (Fig. 20 B-D). In this experiment, root within callus formation was observed when the medium containing at 2.0 mg L⁻¹ NAA (Fig. 20 E, F).
4. Results

Table 14: Effect of auxins on adventitious root induction from leaf explants

<table>
<thead>
<tr>
<th>Auxin (mg L⁻¹)</th>
<th>Explants response (%)</th>
<th>No. of adventitious roots/explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>59</td>
<td>$1.66 \pm 0.01^{d}$</td>
</tr>
<tr>
<td>1.0</td>
<td>72</td>
<td>$2.23 \pm 0.04^{b}$</td>
</tr>
<tr>
<td>2.0</td>
<td>85</td>
<td>$7.46 \pm 0.05^{a}$</td>
</tr>
<tr>
<td>3.0</td>
<td>66</td>
<td>$2.27 \pm 0.05^{bc}$</td>
</tr>
<tr>
<td>NAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>45</td>
<td>$1.57 \pm 0.03^{d}$</td>
</tr>
<tr>
<td>1.0</td>
<td>56</td>
<td>$2.46 \pm 0.05^{b}$</td>
</tr>
<tr>
<td>2.0</td>
<td>78</td>
<td>$4.54 \pm 0.04^{a}$</td>
</tr>
<tr>
<td>3.0</td>
<td>65</td>
<td>$2.17 \pm 0.03^{bc}$</td>
</tr>
<tr>
<td>IAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>41</td>
<td>$1.43 \pm 0.02^{bc}$</td>
</tr>
<tr>
<td>1.0</td>
<td>48</td>
<td>$1.55 \pm 0.03^{b}$</td>
</tr>
<tr>
<td>2.0</td>
<td>70</td>
<td>$2.66 \pm 0.05^{a}$</td>
</tr>
<tr>
<td>3.0</td>
<td>59</td>
<td>$1.37 \pm 0.04^{bcd}$</td>
</tr>
</tbody>
</table>

Data represent mean ± SE, all experiments were carried out three times with at 15 explants. Mean values within a column followed by the same letter are not significantly different by Duncan’s multiple range test $P \leq 0.05$
Figure 20: Adventitious root induction from leaf explant. A) Adventitious root development from leaf explants on MS solid medium with 2.0 mg L⁻¹ IBA. B&C) Adventitious root initiation from adaxial part of the leaf; D) Abaxial part of the leaf. (E, F) Adventitious root initiation along with callus on 2.0 mg L⁻¹ NAA. (G, H) Adventitious root culture in 1/2 strength MS medium containing 2.0 mg L⁻¹ IBA and 0.25 mg L⁻¹ IAA after 4-5 weeks of culture. Scale bars: 1.0 cm (A), 2 mm (B-F), and 1.0 cm (G, H).
4.4.2. Effect of auxins on biomass accumulation in liquid medium

In the present investigation, adventitious roots derived from the previous cultures were subcultured in the MS liquid medium containing IBA (0.5-2.0 mg L\(^{-1}\)) alone or IBA (2.0 mg L\(^{-1}\)) in combination with IAA (0.25 and 0.5 mg L\(^{-1}\)). Among the various treatments, IBA (2.0 mg L\(^{-1}\)) in combination with 0.25 mg L\(^{-1}\) of IAA showed the best response (88 %) with perfused (13.44) lateral root formation (Table 15, Fig. 20 G, H).

**Table 15: Adventitious root biomass production in 1/2 strength MS liquid medium.**

<table>
<thead>
<tr>
<th>PGRs mg L(^{-1})</th>
<th>% of response</th>
<th>No. of lateral roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>67</td>
<td>3.27 ± 1.05(^{b})</td>
</tr>
<tr>
<td>1.0</td>
<td>73</td>
<td>5.56 ± 0.05(^{a})</td>
</tr>
<tr>
<td>2.0</td>
<td>83</td>
<td>9.66 ± 0.04(^{bc})</td>
</tr>
<tr>
<td>IBA+IAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 + 0.25</td>
<td>88</td>
<td>13.44 ± 0.05(^{a})</td>
</tr>
<tr>
<td>2.0 + 0.50</td>
<td>77</td>
<td>6.63 ± 0.03(^{b})</td>
</tr>
</tbody>
</table>

Data represent mean ± SE, all experiments were carried out three times with at 15 explants. Mean values within a column followed by the same letter are not significantly different by Duncan’s multiple range test (\(P \leq 0.05\)).

4.4.3. Effect of inoculum density

Inoculum density depends on the volume of culture medium and vessel. In the present study, 150 ml Erlenmeyer flasks containing 30 ml medium were used to optimize the inoculum density for achieving maximum root biomass production. On the different initial inoculum density, maximum adventitious root biomass (6.42 ± 0.05 FW; 0.47 ± 0.05 DW) and growth ratio (8.20) were recorded at 50 mg FW of initial inoculum. Further, decrease or increase inoculum density led to decrease in the biomass production (Table 16).
Table 16: Effect of inoculum size on root biomass production

<table>
<thead>
<tr>
<th>Inoculum size (mg L⁻¹ FW)</th>
<th>Fresh weight (g L⁻¹)</th>
<th>Dry weight (g L⁻¹)</th>
<th>Growth ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>3.70 ± 0.02d</td>
<td>0.26 ± 0.03c</td>
<td>2.20</td>
</tr>
<tr>
<td>50</td>
<td>6.42 ± 0.05a</td>
<td>0.47 ± 0.05a</td>
<td>8.20</td>
</tr>
<tr>
<td>70</td>
<td>4.14 ± 0.04b</td>
<td>0.38 ± 0.02b</td>
<td>6.32</td>
</tr>
<tr>
<td>100</td>
<td>2.33 ± 0.04c</td>
<td>0.19 ± 0.01d</td>
<td>3.10</td>
</tr>
</tbody>
</table>

Data represent mean ± SE, all experiments were carried out three times with at 15 explants. Mean values within a column followed by the same letter are not significantly different by Duncan’s multiple range test (P ≤ 0.05)

4.4.4. Effect of MS salt strength on biomass accumulation

The present study reveals that the medium strength significantly influenced adventitious root formation in *Aerva lanata*. Among the various medium strengths, the higher root biomass (6.42 ± 0.05 g FW) production was observed in 1/2 strength MS medium supplemented with 3.0 % sucrose (Table 17). Root growth was inhibited when the medium strength was increased or decreased to this optimum level.
4. Results

Table 17: Effect of MS medium strength on biomass accumulation of adventitious root culture

<table>
<thead>
<tr>
<th>Medium strength</th>
<th>Fresh weight (g L⁻¹)</th>
<th>Dry weight (g L⁻¹)</th>
<th>Growth ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4 strength MS</td>
<td>1.70 ± 0.02d</td>
<td>0.16 ± 0.03c</td>
<td>2.30</td>
</tr>
<tr>
<td>1/2 strength MS</td>
<td>6.42 ± 0.05b</td>
<td>0.47 ± 0.05a</td>
<td>8.20</td>
</tr>
<tr>
<td>3/4 strength MS</td>
<td>3.14 ± 0.04b</td>
<td>0.28 ± 0.02b</td>
<td>4.12</td>
</tr>
<tr>
<td>Full strength MS</td>
<td>2.33 ± 0.04c</td>
<td>0.17 ± 0.01d</td>
<td>2.21</td>
</tr>
</tbody>
</table>

Data represent mean ± SE, all experiments were carried out three times with at 15 explants. Mean values within a column followed by the same letter are not significantly different by Duncan’s multiple range test (P ≤ 0.05)

4.5. Hairy root culture

4.5.1. Influence of bacterial strains on hairy root induction

In the present study, 30 days old in vitro raised leaf explants were used for transformation experiments. Initially, they were placed on 1/2 strength MS medium containing a range of cefotaxime concentrations 100, 250, 300, 350 and 450 mg L⁻¹ to examine their resistance to the antibiotic substance. All untransformed leaf segments survived cefotaxime concentrations up to 250 mg L⁻¹ (Fig. 22 A) and at higher concentrations, the survival frequency decreased and no leaf segments survived at 300 or 400 mg L⁻¹. On this basis, 250 mg L⁻¹ cefotaxime was used in the selection medium for further experiments. Three strains of *A. rhizogenes*, R1000, MTCC 2364 and MTCC 532 were tested for their ability to induce the formation of hairy roots on leaf segments. The transformation efficiency of R1000 was more efficient than that of MTCC 2364 and MTCC 532 for *A. lanata* transformation. R1000 had the highest transforming ability (72 %) and MTCC 532 had the lowest (55 %; Fig. 21; 22 B, C).
4. Results

Figure 21: Effect of *A. rhizogenes* strains on hairy root induction.

Data represented mean ± SE, of the three independent experiments.

Figure 22: Hairy root induction from leaf explants after co-cultivation

A) Leaf explants survived on 250 mg L⁻¹ cefotaxime. B-C) Hairy root initiation from leaf explants after co-cultivation with *A. rhizogenes* strain R1000 strain (after 2 weeks). *Scale bars*: 1.0 cm (A-C).
4. Results

4.5.2. Influence of sonication on hairy root induction

In the present study, the effect of sonication on transformation efficiency was evaluated using the *A. rhizogenes* strain R1000. Based on the available literature data, the exposure of wounded explants to ultra sonication was chosen as 10, 20, 30, 40, 50 and 60 s. Among the different time durations, 20 s sonication proved to be the best and produced 85 % of the transformation efficiency after 3 weeks (Fig. 23, 24 A, B). Beyond this level, the performance of transformation efficiency was reduced and necrosis of mother plant tissue also observed (Fig. 23)

![Graph showing influence of sonication treatment on transformation efficiency](image)

**Figure 23: Influence of sonication treatment on transformation efficiency**
4. Results

![Figure 24: Establishment of hairy root culture. A&B) Effect of sonication treatment (20s) for hairy root induction after 3 weeks. C, D) Biomass accumulation of hairy roots on 1/2 strength MS liquid medium after 3 to 5 weeks. Scale bars: 1.0cm (A, B), 1.5 cm (C, D).]

4.5.3. PCR analysis for confirmation of transgenic status of hairy root

The rolB gene is one of the major parts of T-DNA of *A. rhizogenes* strain R1000 and plays an important role in root induction. Integration of T-DNA in the genome of hairy roots of *A. lanata* was confirmed by PCR amplification of DNA fragment of 423 bp specific to *rolB* gene (Fig. 25). Genomic DNA from various lines of putative hairy roots showed amplification
of this fragment, indicating the presence of rolB gene. Amplification was not observed from untransformed roots (negative control). Size of DNA fragment amplified from the Ri plasmid (positive control) was similar to the hairy roots.

![Image of agarose gel](image.png)

Figure 25: PCR detection of rol B gene in hairy root. Lane M molecular weight marker (1000 bp); lane 1, rol B positive control (Ri plasmid); lane 2, rol B (423 bp) transformed hairy roots; lane 3, negative control (non-transformed roots); lane 4, water control

4.5.4. Establishment of hairy root culture and biomass accumulation

In the present study, the potential growth of hairy roots in MS liquid medium without hormone was successfully achieved. Different strength of medium (1/4, 1/2, 3/4 and full strength) were employed and the results revealed that 1/2 strength medium was found to be superior when compared with other strength medium for biomass accumulation (Table 18; Fig. 24 C, D). Highest accumulation of biomass (9.72 ± 0.05 FW and 0.76 ± 0.04 DW) was recorded in the 1/2 strength MS liquid medium with growth ratio of 12.20 and it was followed by 3/4 strength medium, which accumulated biomass of 4.14 ± 0.02 FW and 0.38 ± 0.03 DW with growth ratio of 7.12.
Table 18: Effect of MS liquid medium strength on biomass accumulation of hairy root culture

<table>
<thead>
<tr>
<th>Medium strength</th>
<th>Fresh weight (g L(^{-1}))</th>
<th>Dry weight (g L(^{-1}))</th>
<th>Growth ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4 MS</td>
<td>3.40 ± 0.02(^c)</td>
<td>0.28 ± 0.01(^c,d)</td>
<td>5.30</td>
</tr>
<tr>
<td>1/2 MS</td>
<td>9.72 ± 0.04(^a)</td>
<td>0.76 ± 0.05(^a)</td>
<td>12.20</td>
</tr>
<tr>
<td>3/4 MS</td>
<td>4.14 ± 0.02(^b)</td>
<td>0.38 ± 0.03(^b)</td>
<td>7.12</td>
</tr>
<tr>
<td>Full MS</td>
<td>2.83 ± 0.03(^d)</td>
<td>0.27 ± 0.02(^c)</td>
<td>4.21</td>
</tr>
</tbody>
</table>

Cultures were grown in 150 ml Erlenmeyer’s containing 30 ml medium for 5 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean values showed by different letter are significantly different at p < 0.05 according to Duncan’s multiple range test (DMRT).

4.5.6. Effect of sucrose concentration on biomass accumulation

Among the various concentrations of sucrose (1-6 % w/v) tested in the present study, 3 % sucrose concentration was found suitable for biomass accumulation (9.72 ± 0.04 FW; 0.76 ± 0.05 DW), followed by 4 % sucrose which accumulated biomass of 4.84 ± 0.02 FW and 0.33 ± 0.02 (Table 19). However, increase or decrease in the concentration of the sucrose leads to decrease in the accumulation of biomass.
### Table 19: Effect of sucrose concentration on biomass accumulation of hairy root culture

<table>
<thead>
<tr>
<th>Sucrose concentration (%)</th>
<th>Fresh weight (g L(^{-1}))</th>
<th>Dry weight (g L(^{-1}))</th>
<th>Growth ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.20 ± 0.02(^{ec})</td>
<td>0.10 ± 0.01(^{ef})</td>
<td>2.20</td>
</tr>
<tr>
<td>2</td>
<td>3.70 ± 0.03(^{c})</td>
<td>0.34 ± 0.04(^{b})</td>
<td>7.20</td>
</tr>
<tr>
<td>3</td>
<td>9.72 ± 0.04(^{a})</td>
<td>0.76 ± 0.05(^{a})</td>
<td>12.20</td>
</tr>
<tr>
<td>4</td>
<td>4.84 ± 0.02(^{b})</td>
<td>0.33 ± 0.02(^{bc})</td>
<td>5.30</td>
</tr>
<tr>
<td>5</td>
<td>2.13 ± 0.03(^{cd})</td>
<td>0.28 ± 0.03(^{cd})</td>
<td>2.10</td>
</tr>
<tr>
<td>6</td>
<td>1.31 ± 0.02(^{de})</td>
<td>0.17 ± 0.02(^{e})</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Cultures were grown in 150 ml Erlenmeyer’s containing 30 ml medium for 5 weeks. Data represents mean values ± SE of three replicates; each experiment was repeated twice. Mean values showed by different letter are significantly different at p < 0.05 according to Duncan’s multiple range test (DMRT).

#### 4.6. Biochemical analysis

#### 4.6.1 GC-MS composition of aerial part of ex vitro and in vitro plants

The components present in the methanol extract of ex vitro and in vitro aerial parts of *A. lanata* were identified by GC-MS analysis (Fig. 26 A, B). The active principles with their retention time (RT) and molecular weight (MW) in the methanol extract of aerial parts of *A. lanata* are shown in Table 20 and 21. Eleven compounds in the ex vitro aerial plant and ten compounds in the in vitro aerial plant parts were identified.
Table 20: Phytochemical profile of *ex vitro* aerial plant parts

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Rt</th>
<th>Compound name</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.080</td>
<td>3A-α, 4β, 7A β) -octahydro-4-methoxy-3A, 7A</td>
<td>C_{12}H_{20}O_{2}</td>
<td>196</td>
</tr>
<tr>
<td>2</td>
<td>14.402</td>
<td>(R)-(−) 4 methyl hexanoic acid</td>
<td>C_{7}H_{14}O_{2}</td>
<td>130</td>
</tr>
<tr>
<td>3</td>
<td>16.922</td>
<td>3-methyl-2-(2-butenyl)-furan</td>
<td>C_{10}H_{14}O*</td>
<td>150</td>
</tr>
<tr>
<td>4</td>
<td>19.394</td>
<td>Caryophyllene oxide</td>
<td>C_{15}H_{24}O</td>
<td>220</td>
</tr>
<tr>
<td>5</td>
<td>20.104</td>
<td>Gamma-cis-sesquicyclogeranol</td>
<td>C_{15}H_{26}O*</td>
<td>222</td>
</tr>
<tr>
<td>6</td>
<td>20.194</td>
<td>Alpha-cedrol</td>
<td>C_{15}H_{26}O*</td>
<td>222</td>
</tr>
<tr>
<td>7</td>
<td>20.404</td>
<td>Beta-bisbolene cyclohexene,1-methyl-4-(5-methyl)</td>
<td>C_{15}H_{24}*</td>
<td>222</td>
</tr>
<tr>
<td>8</td>
<td>22.946</td>
<td>2, 6, 10, 14, 18, 22-tetracosahexane</td>
<td>C_{30}H_{50}</td>
<td>410</td>
</tr>
<tr>
<td>9</td>
<td>23.867</td>
<td>1, 5-diphenyl-2H-1, 2, 4 trizoline-3-thione</td>
<td>C_{14}H_{11}N_{3}S</td>
<td>253</td>
</tr>
<tr>
<td>10</td>
<td>24.917</td>
<td>1, 3-diphenyl-1, 3, 5, 5-tetramethyl-cyclostiloxane</td>
<td>C_{16}H_{22}O_{3}</td>
<td>346</td>
</tr>
<tr>
<td>11</td>
<td>27.340</td>
<td>Tetradecanoic acid</td>
<td>C_{16}H_{32}O_{2}</td>
<td>256</td>
</tr>
</tbody>
</table>

The major compounds of field grown plant were 3A-α, 4β, 7A β) - octahydro-4-methoxy-3A, 7A, (R)- (−) 4 methyl hexanoic acid, 3-methyl-2-(2-butenyl)-furan, Caryophyllene oxide, Gamma-cis-sesquicyclogeranol, Alpha-cedrol, Beta-bisbolene cyclohexene, 1-methyl-4-(5-methyl), 2, 6, 10, 14, 18, 22-tetracosahexane, 1, 5-diphenyl-2H-1, 2, 4 trizoline-3-thione, 1, 3-diphenyl-1, 3, 5, 5-tetramethyl-cyclostiloxane and tetradecanoic acid.
Table 21: Phytochemical profile of *in vitro* raised aerial plant parts

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Rt</th>
<th>Compound name</th>
<th>Molecular formula</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.080</td>
<td>3A-α, 4β, 7A β) -octahydro-4-methoxy-3A, 7A</td>
<td>C_{12}H_{20}O_{2}</td>
<td>196</td>
</tr>
<tr>
<td>2</td>
<td>14.832</td>
<td>1, 2, 3, 6-tetra methyltricyclo [5.4.0.0(3.9)] undeca-5, 10-dien-2-ol</td>
<td>C_{13}H_{22}O_{*}</td>
<td>218</td>
</tr>
<tr>
<td>3</td>
<td>16.712</td>
<td>1-αAllyloxy-1-ethynyl-5-methylcyclohexane</td>
<td>C_{13}H_{24}O_{*}</td>
<td>220</td>
</tr>
<tr>
<td>4</td>
<td>17.502</td>
<td>Caryophyllene oxide</td>
<td>C_{15}H_{24}O</td>
<td>220</td>
</tr>
<tr>
<td>5</td>
<td>18.803</td>
<td>Nonyl methyl ether</td>
<td>C_{10}H_{22}O</td>
<td>158</td>
</tr>
<tr>
<td>6</td>
<td>18.873</td>
<td>Alpha-cedrol</td>
<td>C_{13}H_{26}O</td>
<td>222</td>
</tr>
<tr>
<td>7</td>
<td>19.584</td>
<td>6-Heptanolide</td>
<td>C_{7}H_{12}O_{2}</td>
<td>128</td>
</tr>
<tr>
<td>8</td>
<td>21.886</td>
<td>Butyl-9-butyl acetoacetate</td>
<td>C_{12}H_{22}O_{3}</td>
<td>214</td>
</tr>
<tr>
<td>9</td>
<td>23.546</td>
<td>Longifolenaldehyde</td>
<td>C_{15}H_{24}O</td>
<td>230</td>
</tr>
<tr>
<td>10</td>
<td>26.860</td>
<td>(+)-(1'R2'S)-2(2'-methyl cyclopropyl)aniline</td>
<td>C_{10}H_{14}{*}</td>
<td>147</td>
</tr>
</tbody>
</table>

On the other hand, the aerial part of *in vitro* plants were characterized by 3A-α, 4β, 7A β) -octahydro-4-methoxy-3A, 7A, 1, 2, 3, 6-tetra methyltricyclo [5.4.0.0(3.9)] undeca-5, 10-dien-2-ol, 1-αAllyloxy-1-ethynyl-5-methylcyclohexane, Caryophyllene oxide, Nonyl methyl ether, Alpha-cedrol, 6-Heptanolide, Butyl-9-butyl acetoacetate, Longifolenaldehyde, (+)-(1'R2'S)-2(2'-methyl Cyclopropyl) aniline. Similarly three compounds were present in the aerial parts of *ex vitro* and *in vitro* plants and showed qualitative similarity
but quantitative difference in the mono 3A-α, 4β, 7A β) -octahydro-4-methoxy-3A, 7A, Caryophyllene oxide and Alpha-cedrol constituents.

(A)

(B)

Figure 26: GC-MS spectrum from methanol extract of *A. lanata*. A) Aerial part of *ex vitro* plant  B) Aerial part of *in vitro* plant.
4.6.2. GC-MS composition of *ex vitro* root and *in vitro* root culture

The GC–MS technique is used to analyze the volatile secondary metabolite profile of the *in vivo* root and hairy root culture of *A. lanata*. n-hexadecanoic acid is an unique compound found both in the normal and hairy culture (Table 22, 23). In *ex vitro* raised root cultures indicates the presence of chemical profile (Fig. 27) such as 8-Hydroxy - 6, 7-epoxydendrolasin, Citronellyl tiglate, Methyl 16-hydroxy-3-3-dimethylhepatadecanoate, 1-x Allyloxy-1-ethynyl-5-methylcyclohexane, 1-x Allyloxy-1-ethynyl-5-methylcyclohexane, 2-(Methoxycarbonyl)-2-propargyl-1-cyclopentanol, 1,2 Benzene dicarboxylic acid, dicyclohexyl ester, 1-(R)-endo-Methylbornyl E-butenoate and n-hexadecanoic acid and hairy root culture showed the presence of Cyclopentasiloxane, benzene acetaldehyde, α-methyl, n-hexadecanoic acid, 8aH-2, 4a-methanaphthalene-7a-01, octahydro-1 and Triphenyl phosphate.
### Table 22: Phytochemical profile of ex vitro root

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Rt</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.080</td>
<td>8-Hydroxy-6, 7-epoxydendrolasin</td>
<td>C$<em>{13}$H$</em>{22}$O$_{3}$</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>2.400</td>
<td>Citronellyl tiglate</td>
<td>C$<em>{15}$H$</em>{26}$O$_{2}$</td>
<td>238</td>
</tr>
<tr>
<td>3</td>
<td>13.192</td>
<td>Methyl 16-hydroxy-3-3-dimethylhepatadecanoate</td>
<td>C$<em>{26}$H$</em>{40}$O$_{3}$</td>
<td>328</td>
</tr>
<tr>
<td>4</td>
<td>14.822</td>
<td>1-xAllyloxy-1-ethynyl-5-methylcyclohexane</td>
<td>C$<em>{15}$H$</em>{24}$O*</td>
<td>220</td>
</tr>
<tr>
<td>5</td>
<td>16.712</td>
<td>2-(Methoxycarbonyl)-2-propargyl-1-cyclopentanol</td>
<td>C$<em>{10}$H$</em>{14}$O$_{3}$</td>
<td>182</td>
</tr>
<tr>
<td>6</td>
<td>18.713</td>
<td>1, 2-Benzenedicarboxylic acid, dicyclohexyl ester</td>
<td>C$<em>{20}$H$</em>{26}$O$_{4}$</td>
<td>330</td>
</tr>
<tr>
<td>7</td>
<td>25.562</td>
<td>1-(-R)-endo-Methylbornyl E-butenoate</td>
<td>C$<em>{15}$H$</em>{24}$O$_{2}$</td>
<td>236</td>
</tr>
<tr>
<td>8</td>
<td>26.842</td>
<td>n-hexadecanoic acid</td>
<td>C$<em>{6}$H$</em>{32}$O$_{2}$</td>
<td>256</td>
</tr>
</tbody>
</table>

### Table 23: Phytochemical profile of hairy root culture

<table>
<thead>
<tr>
<th>S.No</th>
<th>Rt</th>
<th>Name of the compound</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.84</td>
<td>Cyclopentasiloxane</td>
<td>C$<em>{10}$H$</em>{30}$O$<em>{5}$Si$</em>{5}$</td>
<td>370</td>
</tr>
<tr>
<td>2</td>
<td>12.85</td>
<td>Benzene acetaldehyde, α-methyl</td>
<td>C$<em>{9}$H$</em>{10}$O</td>
<td>148</td>
</tr>
<tr>
<td>3</td>
<td>20.51</td>
<td>8aH-2, 4a-methanaphthalene-7a-01, octahydro-1</td>
<td>C$<em>{11}$H$</em>{18}$O</td>
<td>222</td>
</tr>
<tr>
<td>4</td>
<td>24.22</td>
<td>Triphenyl phosphate</td>
<td>C$<em>{14}$H$</em>{18}$O$_{4}$P</td>
<td>326</td>
</tr>
<tr>
<td>5</td>
<td>34.06</td>
<td>n-hexadecanoic acid</td>
<td>C$<em>{6}$H$</em>{32}$O$_{2}$</td>
<td>256</td>
</tr>
</tbody>
</table>
4. Results

(A)

(B)

Figure 27: GC-MS spectrum from methanol extract of *A. lanata*.

A) *Ex vitro* root. B) Hairy root