In vitro Micropropagation of Oxystelma secamone (L) Karst-A Medicinal Plant

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Abstract: An efficient protocol has been developed for rapid mass propagation of Oxystelma secamone from stem derived callus. Optimum callus was developed from the cut ends of stem explants on Murashige and Skoog (MS) basal medium supplemented with 2, 4-D (0.5-2.0 mg l⁻¹). Subculturing the callus on BAP (2.5 and 5.0 mg l⁻¹) fortified medium resulted in embryogenic cultures and somatic embryos. Adventitious shoots were regenerated (85%) from the surface of the callus on MS medium supplemented with BAP (3.0 mg l⁻¹) and NAA (1.0 mg l⁻¹). Regenerated plantlets with well developed shoots and roots were successfully transferred to soil. Histology confirmed different stages of development. The study demonstrated a dedifferentiated callogenic propagation route via adventitious shoot development in O. secamone, which could be useful for large scale multiplication of this medicinal plant. [Nature and Science 2010; 8(11):15-19]. (ISSN: 1545-0740).

Keywords: callus culture, plant regeneration, somatic embryogenesis, Oxystelma secamone

Abbreviations: BAP – benzyl amino purine; CH – casein hydrolysate; CM – coconut milk; 2, 4-D – 2,4 – dichlorophenoxacyetic acid; IAA – indole - 3- acetic acid; IBA – indole -3-butyric acid; Kn-kinetin; MS – Murashige and Skooog; NAA - α - naphthalene acetic acid

1. Introduction

Oxystelma secamone (L) Karst. (Asclepiadaceae) is a slender laticiferous climber. It is a potent medicinal plant possessing antiseptic, depurative, antihelmintic and galactogogue properties. A decoction of the plant is used as a gargle in ulcerations of the mouth and in sore throat. It also acts as an astringent and its roots are useful to cure jaundice (Anonymous, 1966; Kirtikar and Basu, 1975).

Biotechnology has offered a non-conventional method of plant propagation and has been intensively applied as a conservation strategy for sustaining plant biodiversity. It has been shown that shoot organogenesis via callus culture can be used as an effective method for multiplication of medicinal plants (Reddy et al., 2001; Lusia and Rajas, 1996; Ahroni et al., 1997). Tissue culture studies on O. secamone has been done for the first time. In the present study we have investigated callus mediated shoot organogenesis as an alternative method to achieve a higher rate of shoot multiplication.

Phytochemical analysis of this species (Srinivasan, 1985) and of O. esculentum in vivo has been carried out by Trivedi et al., (1990).

2. Materials and Methods

2.1. Plant Material and Explant Source

Mature pods were collected from an elite vine (1-year-old) at Kukkarahalli Lake, Mysore, India. The seeds were washed in running tap water for at least 30 min followed by soaking in 5% (v/v) liquid detergent labolene for 5 min and finally rinsed with 5-6 changes of sterile water. They were germinated on MS basal medium (Murashige and Skooog, 1962).

2.2. Bud Break and Shoot Multiplication

Nodal and internodal explants obtained from 20-day-old seedlings were cultured on various media; namely MS (1962), White (1963) and Gamborg’s B-5 (Gamborg et al., 1968) supplemented with 2, 4-D. The callus subcultured on MS medium fortified with various concentrations of cytokinins (BAP and Kn) either individually or in combination with auxins (IAA, IBA and NAA) were investigated to optimise hormonal requirements for embryogenesis and multiple shoot induction besides using complex extracts such as coconut milk (CM) (10 & 15%) and casein hydrolysate (CH) (50 & 100 mg l⁻¹). The morphogenic responses of nodes and internodes from the seedlings were investigated for effective, efficient and reproducible multiplication. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH prior to the addition of 0.8% (W/V) agar and autoclaving.

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at 1.06 kg cm\(^{-2}\) for 20 min. All cultures were maintained at 22 ± 2 °C with 16-h light / 8-h dark photoperiods, 80-85 % relative humidity and light intensity of 50 μ mol m\(^{-2}\) s\(^{-1}\).

2.3. Rooting and Transfer of plantlets to Soil

In vitro differentiated shoots measuring 4-5 cm in length were excised from both stem derived callus and adventitious bud elongation and cultured on half strength MS liquid medium supplemented with IAA and NAA (0.5 – 2.0 mg l\(^{-1}\)). After 4-5 weeks in rooting medium, the rooted microshoots were removed from the culture medium and the roots were washed in sterile distilled water. The plantlets were then transferred to plastic pots containing garden soil mixed with vermiculite and sand (1:1:1) under controlled growth chamber conditions. After two weeks of acclimatization they were placed outdoors under the sun. Development of embryogenic cultures was followed histologically. Tissues were fixed in FAA (Formalin: acetic acid: alcohol; 1:1:18) sections were taken at 11 μ thickness using a rotary microtome. The slides were processed and stained in Heidenhain’s haematoxylin following the customary methods and mounted in canada balsam.

3. Results and Discussion

Stem segments were cultured on MS, B-5 and White’s media supplemented with 2, 4-D. Among media tested, MS was found to be effective both for callus induction and shoot sprouting. Within 4 weeks of inoculation, callus was proliferated from cut edges of the explants besides sprouting of axillary buds. The axillary buds grew vigorously and 90 % of stem explants produced shoots within 4 weeks. The shoot buds sprouted on Gamborg’s B-5 medium showed only limited development even if they were maintained for longer period in culture.

Morphology and growth of the callus varied with different levels of 2, 4 – D (Table 1). At lower concentration of 2, 4-D (0.5 – 1.0 mg l\(^{-1}\)), 65% cultures formed callus. When 2, 4-D concentration was increased from 1.5-3.0 mg l\(^{-1}\), almost all the cultures showed callusing. At still higher concentration (5.0 mg l\(^{-1}\)) there is decline in the amount of callus formation. Callus formation at the proximal ends of the node explants in the present study is in line with Holostemma ada-kodien (Martin, 2000) and Peganum harmala (Saini and Jaiwal, 2000), who suggested that callus formation may be due to the action of accumulated auxin at the basal cut ends, which stimulates cell proliferation especially in the presence of cytokinins. Embryogenic culture induction, somatic embryo maturation and germination required different auxin and cytokinin combinations.

<table>
<thead>
<tr>
<th>Concentration (mg l(^{-1}))</th>
<th>% Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>63.0</td>
</tr>
<tr>
<td>1.0</td>
<td>70.0</td>
</tr>
<tr>
<td>1.5</td>
<td>94.0</td>
</tr>
<tr>
<td>2.0</td>
<td>88.0</td>
</tr>
<tr>
<td>3.0</td>
<td>67.0</td>
</tr>
<tr>
<td>5.0</td>
<td>45.0</td>
</tr>
<tr>
<td>8.0</td>
<td>32.0</td>
</tr>
<tr>
<td>10.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

The influence of growth regulators on plant regeneration is summarised in table 2. Friable callus was subcultured on MS medium supplemented with either BAP or Kn alone. Within 4 weeks of culture adventitious shoot buds were differentiated from the surface of the callus (Fig.1). Shoot buds were elongated within 5 weeks of culture. Among various concentrations of Kn tested, the highest shoot regeneration frequency (55%) was recorded at 2.0 mg l\(^{-1}\) concentration (Fig.2). Increasing the concentrations of Kn from 4.0-8.0 mg l\(^{-1}\) (data not shown), resulted in a decrease in the rate of shoot regeneration capacity. It is in contrast to the report of Sharma et al., (1991) on Coleus forskholii where the higher concentration enhanced the shoot multiplication.

The callus after subculture onto the MS medium, having various concentrations of BAP (1.0-3.0 mg l\(^{-1}\)) showed that highest shoot regeneration frequency (70%) per callus mass was obtained at 2.0 mg l\(^{-1}\) BAP. Medium supplemented with Kn resulted in a reduced number of shoots with shorter internodes. Similar response was also observed in the propagation of Asclepia (Chi Won and John, 1985) and Gymnema sylvestre (Reddy et al., 1998). Shoot multiplication required BAP and was found to be more effective than Kn. The combination of BAP (3.0 mg l\(^{-1}\)) with 1.0 mg l\(^{-1}\) NAA was the most effective (85%) for multiple shoot induction (Fig.3). This synergistic effect of BAP and NAA has been demonstrated in Santolina canescens (Casado et al., 2002) and in Bupleurum fruticosum (Fraternale et al., 2002), determining the optimum cytokinins and auxin levels for shoot regeneration, synergistic effect of complex extracts was studied to improve the frequency of regeneration. CM (10 & 15%) & CH (50 & 100 mg l\(^{-1}\)) did not significantly improve the regeneration ability. Treatment with CM & CH resulted in callusing and suppression of shoot bud proliferation. In contrast, CH & CM have been reported to have promotive effect in Lavandula latifolia (Gras and Calvo, 1996).
Table 2. Effect of growth regulators on shoot regeneration from stem derived callus of *O. secomone* in MS medium

<table>
<thead>
<tr>
<th>Plant growth regulator (mg l(^{-1}))</th>
<th>Frequency of shoot regeneration</th>
<th>Mean No. of shoots / callus</th>
<th>Mean shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP (mg l(^{-1}))</td>
<td>Kn</td>
<td>NAA</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>70</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>55</td>
</tr>
<tr>
<td>-</td>
<td>1.0</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>-</td>
<td>2.0</td>
<td>-</td>
<td>55</td>
</tr>
<tr>
<td>-</td>
<td>3.0</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>1.0</td>
<td>65</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>1.0</td>
<td>85</td>
</tr>
<tr>
<td>-</td>
<td>2.0</td>
<td>1.0</td>
<td>33.6</td>
</tr>
<tr>
<td>-</td>
<td>3.0</td>
<td>1.0</td>
<td>41.6</td>
</tr>
</tbody>
</table>

Fig. 1. Various stages of embryoids differentiated from stem callus

Fig. 2. Regeneration of shoots on MS medium supplemented with 2.0 mg l\(^{-1}\) kinetin

Histologically the embryogenic callus cells showed small size, dense cytoplasmic contents, large nuclei with prominent nucleoli, which started to divide themselves forming meristematic zone. These zones are pre-embryonic complexes as reported by Tisserat & DeMason (1980) in *Phoenix dactylifera*. After 4-weeks, somatic embryos of globular (Fig.4) and spindle shapes (Fig.5) were originated from the superficial cells of the pre-embryonic complexes. The callus that developed on MS medium fortified with BAP and NAA in section showed shoot apex having a pair of primordial leaves (Fig.6). Similar shoot apex formation was described by Liu et al., (1993) as juvenile apical meristem.

Fig. 3. Multiple shoot induction on MS + BAP (3.0 mg l\(^{-1}\)) + NAA (1.0 mg l\(^{-1}\)).
3.1. Rooting of in vitro Derived Shoots and Plantlet Acclimatization

In vitro shoots (4-5 cm long) were rooted only upon transfer to half strength MS medium containing IAA, whereas low frequency of rooting was noted in full strength MS medium. Of the two auxins tested, IAA (1.0 mg l\(^{-1}\)) was most effective for root induction (Table 3) and survival in the field. Rooted plantlets (5-6 cms height) with fully expanded leaves and well developed roots were successfully transferred to soil. Normal growth of the potted plantlets was visible after transfer to field conditions and the success was about 80%.

<table>
<thead>
<tr>
<th>Concentration (mg l(^{-1}))</th>
<th>% Response</th>
<th>Average No. of roots/shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>60</td>
<td>2.0±0.02</td>
</tr>
<tr>
<td>1.0</td>
<td>80</td>
<td>3.6±0.12</td>
</tr>
<tr>
<td>1.5</td>
<td>50</td>
<td>2.8±0.24</td>
</tr>
<tr>
<td>2.0</td>
<td>30</td>
<td>1.5±0.08</td>
</tr>
</tbody>
</table>

To conclude, the protocol described offers a potential system for improvement, conservation and mass propagation of *O. secamone* from stem explants. However, there are no published reports on the micropropagation of *O. secamone*. The results show that *O. secamone* can be propagated by somatic embryogenesis besides organogenesis. Plant cell cultures provide an excellent system for studying biosynthesis of secondary metabolites. Further work related to secondary metabolite production and its qualitative analysis is in progress.

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