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

Dharmendra[1,*], M.S. Sudarshana[2] & M.H.Niranjan[2]

1. Department of Botany, Sir M. V. Govt. Science College, Bhadravathi-577 302.Shimoga dist, India
2. Department of studies in Botany, Manasagangotri, University of Mysore, Mysore-570 006.India
*dharambotany@gmail.com, dharam_ptc@rediffmail.com

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}). Individual elongated shoots were rooted on half strength MS liquid medium containing IAA (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 – dichlorophenoxy acetic acid; IAA – indole - 3- acetic acid; IBA – indole -3-butyric acid; Kn-kinetin; MS – Murashige and Skoog; NAA - ∝ - 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 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 atleast 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 Skoog, 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

at 1.06 kg cm^{-2} for 20 min. All cultures were maintained at 22 ± 2 ^{0} 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.

Table1. Effect of 2, 4 – D on callus induction from stem explants in MS medium after 3 weeks of culture.

Concentration (mg l^{-1})	% Response
0.5	63.0
1.0	70.0
1.5	94.0
2.0	88.0
3.0	67.0
5.0	45.0
8.0	32.0
10.0	20.0

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 *Asclepias* (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.secamone* in MS medium

Plant growth regulator(mg l^{-1})			Frequency of shoot regeneration	Mean No. of shoot /callus	Mean shoot length(cm)
BAP	Kn	NAA			
1.0	-	-	14	18.0 ± 14.1	1.74 ± 0.14
2.0	-	-	70	44.5 ± 2.41	3.92 ± 0.21
3.0	-	-	55	20.8 ± 1.05	3.28 ± 0.09
-	1.0	-	20	18.0 ± 1.41	1.74 ± 0.14
-	2.0	-	55	30.6 ± 1.20	2.98 ± 0.11
-	3.0	-	17	25.0 ± 0.70	2.46 ± 0.05
2.0	-	1.0	65	37.4 ± 1.32	3.58 ± 0.06
3.0	-	1.0	85	64.8 ± 0.74	4.50 ± 0.12
-	2.0	1.0	33.6	21.5 ± 2.36	2.25 ± 0.95
-	3.0	1.0	41.6	11.0 ± 1.82	2.00 ± 0.81

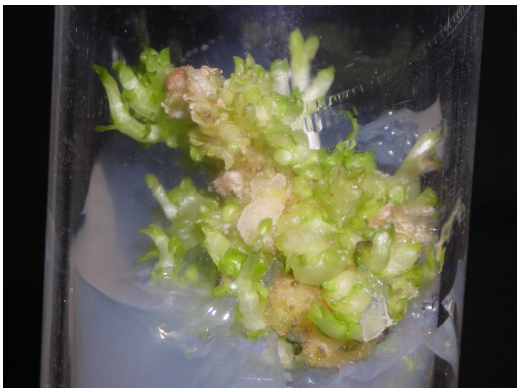

Fig.1. Various stages of embryoids differentiated from stem callus

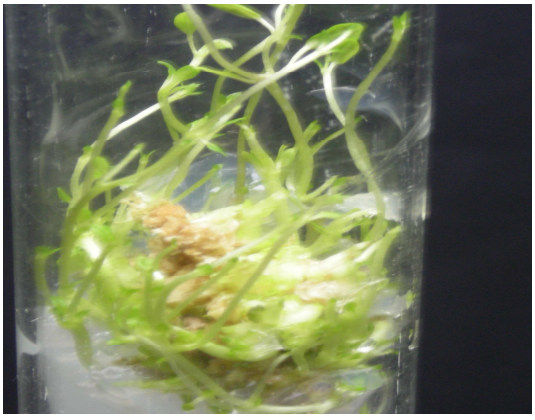

Fig.2.Regeneration of shoots on MS medium supplemented with 2.0 mg l^{-1} kinetin

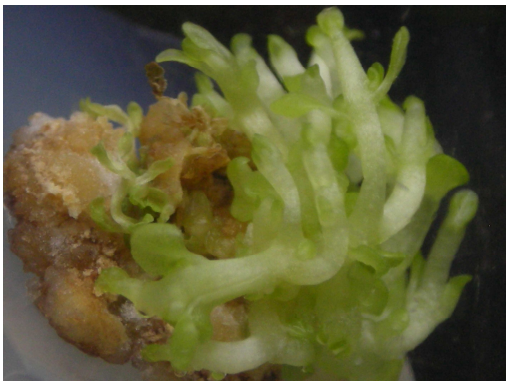

Fig.3.Multiple shoot induction on MS + BAP (3.0 mg l^{-1}) + NAA (1.0 mg l^{-1}).

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.

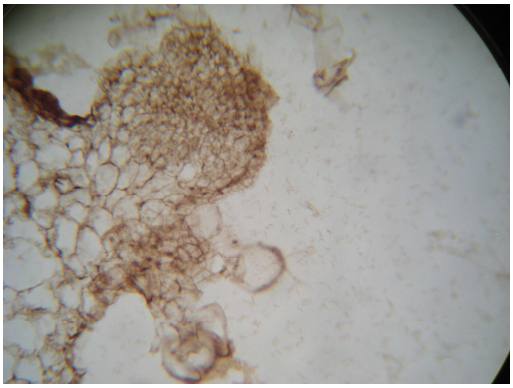

Fig.4. Developmental stages of a globular embryoid showing single celled and 4-celled stages with suspensor.

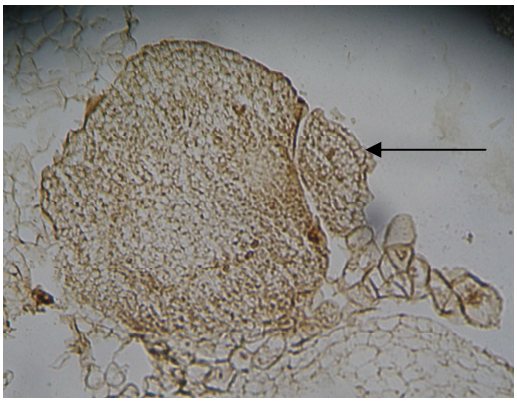

Fig.5. A section showing pre-embryonic complex and arrow point to a spindle shaped embryoid with a suspensor.

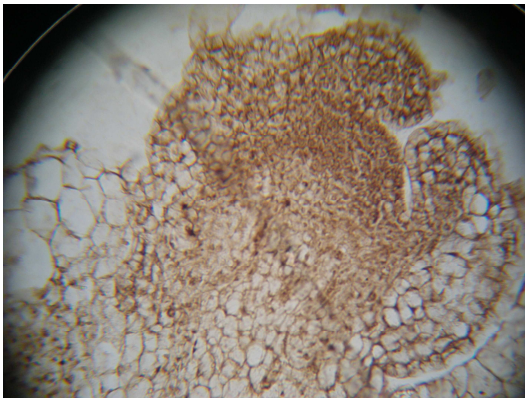

Fig.6. Shootbud regenerated from the callus after 5 – weeks of culture.

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 3. Effect of IAA on root induction in ½ MS basal medium

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

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.

Acknowledgement

Dharmendra is thankful to the special cell for SC/ST, University of Mysore, Mysore for providing the financial assistance.

Correspondence to:
Dharmendra
Assistant professor
Department of Botany,
Sir M.V.Govt. Science College,
Bommanakatte, Bhadravathi,
Shimoga dist, Karnataka-577 302, India
Contact No: +91-9844490643, +91-9449131751
E-mail: dharambotany@gmail.com, dharam_ptc@rediffmail.com.

References

1. Ahroni A, Zuker A, Rojen Y, Shejtman H, Vainstein. An efficient method of adventitious shoot regeneration from stem

explants of *Gypsophila,* Plant Cell Tiss. Org. Cult. 1997: 49: 101 – 106.

2. Anonymous. The Wealth of India. Raw Material, Publications and Information Directorate, CSIR New Delhi, India. 1966:7:200-201..

3. Casado JP, Navarro MC, Utrilla MP, Martinez A, Jimenez J. Micropropagation of *Santolina canescens* Lagasea and *in vitro* volatiles production by shoot explants. Plant Cell Tiss. Org. Cult. 2002:69: 147 – 153.

4. Chi Won L and John CT. Propagation of desert milkweed by shoot tip culture. Hort. Sci. 1985:20: 263 – 264.

5. Fraternale O, Giamperi L, Ricci D, Rocchi MBL (2002) Micropropagation of *Bupleurum fruticosum*: the effect of triacontanol. Plant Cell Tiss. Org. Cult. 2002:69: 135-140.

6. Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 1968:50: 151 – 158.

7. Gras MCS, Calvo MC. Micropropagation of *Lavandula latifolia* through nodal bud culture of mature plants. Plant Cell Tiss. Org. Cult. 1996:45: 259 – 261.

8. Kirtikar KR, Basu BD. Indian Med. Plants, II edition, Vol. III, International Book Distributors, Dehradun, India.1975.

9. Liu C, Xu Z, Chua NH. Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell. 1993:5: 621 – 630.

10. Lusia MAV, Rojas G. *In vitro* propagation of *Mimosa teniflora* (Willd.) Poiret, a Mexican medicinal tree. Plant Cell Rep. 1996:16: 80 -82.

11. Martin KP. Rapid propagation of *Holostemma ada – kodien* Schult., a rare medicinal plant, through axillary bud multiplication and indirect organogenesis. Plant Cell Rep. 2000:21: 112 – 117.

12. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 1962:15: 473 – 497.

13. Reddy SP, Gopal RG, Sita GL. *In vitro* multiplication of *Gymnema sylvestre*, R. Br. – An important medicinal plant. Curr. Sci.1998:75: 843- 845.

14. Reddy PS, Rodrigues R, Rajashekaran R. Shoot organogenesis and mass propagation of *Coleus forskholii* from leaf derived callus. Plant Cell Tiss. Org. Cult. 2001:66: 183 – 188.

15. Saini R, Jaiwal PK. *In vitro* multiplication of *Peganum harmala* an important medicinal plant. Indian J.Exp. Biol. 2000:38: 499- 503.

16. Sharma N, Chandel KPS, Srivastava VK. *In vitro* propagation of *Coleus forskholii* Briq. A threatened medicinal plant. Plant Cell Rep. 1991:10: 67 -70.

17. Srinivasan. Chemical investigation of certain South Indian medicinal plants. Ph.D. Thesis University of Mysore, Mysore, India.1985.

18. Tisserat B, DeMason DA. A histological study of development of adventive embryos in organ cultures of *Phoenix dactylifera* L. Ann. Bot. 1980:46: 465- 472.

19. Trivedi R, Khare A, Khare MP. A pregnane triglycoside from *Oxystelma esculentum*. Phytochemistry. 1990:29(12): 3967- 3970.

20. White PR. A Handbook of Plant Tissue Culture. Jacques Cattell Press, Lancaster PA.1963.

6/5/2010