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Studies on *In vitro* Somatic Embryogenesis of *Psoralea corylifolia* Linn. – An Endangered Medicinal Plant

Untersuchungen zur somatischen *in vitro* Embryogenese von *Psoralea corylifolia* Linn. – einer gefährdeten Arzneipflanze

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Summary

Plant regeneration via somatic embryogenesis was achieved in callus derived from leaf and stem explants of *Psoralea corylifolia* L. on modified Murashige and Skoog's medium containing 2300 mg l^{-1} potassium nitrate (KNO_3) supplemented with 1.5 mg l^{-1} kinetin, 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% (w/v) sucrose. Somatic embryos proliferated rapidly by secondary somatic embryogenesis after transfer to MS medium supplemented with kinetin and 2,4-D in subsequent subcultures. A high percentage (70.4%) of cultures of somatic embryos developed from stem derived calluses as compared to leaf derived calli (58.6%). Maturation and germination of somatic embryos were achieved on half strength MS basal medium supplemented with 0.1 mg l^{-1} indole-3-butyric acid (IBA) and 2% (w/v) sucrose. Somatic embryo derived plants were acclimatised and grown in the greenhouse.

Zusammenfassung

Es ist auf dem Wege somatischer Embryogenese gelungen, Pflanzen aus Kallus von Blatt- und Sproßexplantaten von *Psoralea corylifolia* L. zu regenerieren. Die Kultur erfolgte auf modifiziertem Murashige and Skoog-Medium (MS), dem 2300 mg l^{-1} Kaliumnitrat (KNO_3) sowie 1.5 mg l^{-1} Kinetin, 0.5 mg l^{-1} 2,4-D und 3% (w/v) Saccharose zugefügt worden waren. Nach dem Umsetzen auf MS-Medium, angereichert mit Kinetin und 2,4-D, vermehrten sich die somatischen Embryonen durch sekundäre somatische Embryogenese schnell weiter. Es entwickelten sich im Vergleich mehr somatische Embryonen aus Kalli von Sproßexplantaten (70.4%) als aus Kalli von Blattexplantaten (58.6%) Die Reifung und Wurzelbildung der somatischen Embryonen erfolgte auf halbkonzentriertem MS-Grundmedium, dem 0.1 mg l^{-1} (IBA) und 2% (w/v) Saccharose zugefügt wurden. Pflanzen, die sich aus somatischen Embryonen entwickelt hatten, wurden akklimatisiert und im Gewächshaus herangezogen.

Introduction

Psoralea corylifolia L. is a rare endangered herbaceous medicinal plant, distributed in the tropical and subtropical regions of the world (JAIN 1994). It is used as a laxative, aphrodisiac, diuretic and diaphoretic in febrile conditions. It has been specially recommended in the treatment of leucoderma, leprosy, psoriasis and inflammatory diseases of the skin and has been prescribed both for oral administration and external application in the form of a paste or ointment (ANONYMOUS 1988). Seed germination is unreliable due to poor germination and the death of young seedlings under natural condition. Pharmaceutical industries largely depend upon material procured from naturally occurring stands which are being depleted rapidly, raising concern about possible extinction and providing justification for development of *in vitro* techniques for this species. *In vitro* plant regeneration via organogenesis has been achieved in *Psoralea corylifolia* (SAXENA et al. 1997). Somatic embryogenesis potentially offers an alternative and efficient system for plant multiplication (AMMIRATO 1987; ROUT et al. 1995). Plant regeneration via somatic embryogenesis from single cells that can be induced to produce an embryo and then a complete plant has been demonstrated in many medicinal plant species (PUROHIT et al. 1994; ZHOU et al. 1994; JOHRI and AMINUDDIN PAL 1996; GASTALDO et al. 1996; CHOI et al. 1997; SU et al. 1997). This paper is the first report on induction of somatic embryogenesis and multiplication of somatic embryos of *Psoralea corylifolia*, a rare and endangered medicinal plant.

Materials and methods

Plant material

Plant material was collected from Chandaka Reserve Forest, Orrisa, India. Leaf and stem explants were washed in 2% (v/v) detergent solution "Teepol" (Qualigen, India) and surface sterilised in 0.1% (w/v) aqueous mercuric chloride solution for 15 min. After three rinses in sterile distilled water, the leaf (5 x 10 mm) and stem (0.25 – 0.5 cm) segments were used as explants.

Culture medium

Both leaf and stem explants were placed on semi-solid basal MS (MURASHIGE and SKOOG 1962) medium supplemented with different concentrations and combinations of 6-benzyladenine (BA: 0.0, 0.5, 1.0, 2.0 and 3.0 mg l^{-1}), kinetin (Kn: 0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg l^{-1}), 1-naphthaleneacetic acid (NAA: 0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg l^{-1}) and 2,4-dichlorophenoxyacetic acid (2,4-D: 0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg l^{-1}) for callus formation. The pH of the media was adjusted to 5.7 using 0.1N NaOH or 0.1N HCl prior to adding 0.8% (w/v) agar (Qualigen, India). Routinely, 20 ml of molten medium was dispensed into 25 x 150 mm glass culture tubes (Borosil, India) capped with non absorbent cotton plugs wrapped in one layer of cheese cloth. The cultures were sterilised at 121°C at 104 kPa for 15 min. To induce somatic embryogenesis, pieces of callus (200 ± 20 mg fresh weight) initiated from MS + 0.5 mg l^{-1} kinetin + 2.0 mg l^{-1} 2,4-D, were subcultured on modified MS (mMS) medium with varying levels of ammonium nitrate (1200, 1400, 1600, 1800 and 2000 mg l^{-1}) and potassium nitrate (1700, 1900, 2100, 2300 and 2500 mg l^{-1}), supplemented with various concentrations of kinetin (0.0, 0.5, 1.0, 1.5 and 2.0 mg l^{-1}) or BA (0.0, 0.5, 1.0, 1.5 and 2.0 mg l^{-1}) along with NAA or 2,4-D (0.0, 0.25, 0.50, 1.0 and 1.5 mg l^{-1}). All cultures were incubated under 16 h photoperiod with light intensity of 55 μmol $m^{-2}s^{-2}$ provided by cool white fluorescent lamps (Phillips, India) at 25 ± 2°C. Morphological changes were recorded on the basis of visual observations at 4-week intervals. The effects of different treatments were quantified as percentage of callus producing somatic embryos and average number of somatic embryos/culture. There were 20 replications/treatments and experiments were repeated thrice.

Maturation and germination of somatic embryos

Somatic embryos were isolated and transferred to the maturation and germination medium containing half-strength MS basal salts without growth regulator or supplemented with various concentrations of indole-3-acetic acid (IAA) or IBA (0.0, 0.1, 0.25 and 0.50 mg l^{-1}) and 2% (w/v) sucrose and incubated under the same culture conditions as mentioned earlier.

Establishment in soil

Germinated propagules were thoroughly washed to remove the adhering gel and planted in 2.5 cm earthen pots containing sterile garden soil : sand : cow dung manure (2 : 1 : 1) (v/v) and kept in a climate-controlled greenhouse for establishment.

Observations of cultures and presentation of results

Usually, 20 cultures were maintained per treatment and each experiment was repeated thrice. The data pertaining to mean percentage of callus growth, mean percentage of somatic embryogenesis and mean average number of somatic embryos/ culture was statistically analysed by the Post Hoc Multiple Comparison test (MARASCUILO and MCSWEENCY 1977). Between the treatments the average figures followed by the same letter were not significantly different at the level of $P<0.05$.

Results and Discussion

Stem and leaf explants expanded and developed callus at the cut surfaces within 9–10 days of inoculation which subsequently covered the entire surface of the explant within 15–16 days of culture. There was no sign of callus formation when explants were cultured in media without auxin or cytokinin. The intensity of callus proliferation was greater in the media with kinetin in combination with 2,4-D as compared to the media with Kn + NAA, BA + NAA and BA + NAA (data not shown). Most of the media containing 0.5–1.5 mg l^{-1} Kn and 2.0–3.0 mg l^{-1} 2,4-D stimulated rapid callus proliferation (Table 1). An analysis of variance for callus production is presented in Table 2. The frequency of callus production was maximum 80.2% and 68.4% in stem and leaf explants respectively in the medium containing 0.5 mg l^{-1} Kn and 3.0 mg l^{-1} 2,4-D (Table 1). The initial primary callus was yellowish white but grew

rapidly into pale-yellow, nodular, friable callus upon subsequent culture (Fig. 1a). Higher concentrations of 2,4-D or NAA induced friable calli; but overall 2,4-D promoted a high rate of callus growth. Of the two explant types used, callus was formed on the stem explants earler and this callus proliferated very rapidly. Leaf explants produced callus at relatively slow rates. The appearance of the calli varied with both the explant types and the media but the response was similar.

After a 4-week period on callus-induction media (MS + 0.5 mg l^{-1}Kn + 2.0 mg l^{-1} 2,4-D), callus was subcultured on modified MS media (mMS) containing different concentrations of BA, Kn, NAA and 2,4-D and varying levels of ammonium nitrate (1200–2000 mg l^{-1}) and potassium nitrate (1700–2500 mg l^{-1}) for induction of somatic embryos. Embryogenic calli were observed on mMS containing 2300 mg l^{-1} potassium nitrate and supplemented with 1.0–1.5 mg l^{-1} Kn + 0.25–0.5 mg l^{-1} 2,4-D + 3% sucrose within 2 weeks of subculture (Fig. 1b). Increase in the concentration of potassium nitrate (2500 mg l^{-1}) in the culture medium reduced the embryogenic callus. Somatic embryogenesis did not occur in the media having ammonium nitrate supplemented with NAA or in the absence of growth regulators (Table 3). Different concentrations of 2,4-D and kinetin produced embryogenic calli, but 1.5 mg l^{-1} kinetin and 0.5 mg l^{-1} 2,4-D appeared to be optimal. The embryogenic calli were nodular and greenish yellow in colour. On medium supplemented with 1.0–1.5 mg l^{-1} Kn, 0.25–0.5 mg l^{-1} 2,4-D and 3% (w/v) sucrose, numerous (globular and cotyledonary) somatic embryos developed which exhibited distinct bipolar organization without

Table 1. Effect of various media on callus formation from leaf and stem explants of *Psoralea corylifolia* on MS media containing kinetin in combination with 2,4-D at different concentrations after 4 weeks of culture.
Wirkung von unterschiedlichen Medien (MS-Medium mit Kinetin in Kombination mit 2,4-D in unterschiedlichen Konzentrationen) auf die Kallusbildung von Blatt- und Sproßexplantate von Psoralea corylifolia nach einer Kulturdauer von 4 Wochen.

MS + Growth regulators (mg l^{-1})		Callus induction (%) (Mean ± S.E)* Explant source	
Kn	2,4-D	Stem	Leaf
0.0	0.0	0.0	0.0
0.5	1.0	12.5±0.6 a	8.2±0.5 a
0.5	1.5	30.6±0.8 b	17.7±0.4 b
0.5	2.0	44.2±0.6 e	30.4±0.5 d
0.5	2.5	60.8±0.7 h	44.2±0.6 h
0.5	3.0	80.2±0.8 k	68.4±0.5 k
1.0	2.0	56.8±0.7 g	43.8±0.7 h
1.0	2.5	67.4±0.6 i	54.6±0.4 i
1.0	3.0	74.5±0.5 j	62.8±0.3 j
1.5	2.0	41.8±0.6 d	36.4±0.6 f
1.5	2.5	46.7±0.7 f	40.2±0.4 g
1.5	3.0	40.6±0.8 d	32.7±0.8 e
2.0	2.0	32.4±0.6 b	27.6±0.6 c
2.0	2.5	36.2±0.7 c	31.7±0.4 d
2.0	3.0	40.5±0.4 d	32.8±0.7 e

* Data represent means of 20 replicates/treatment; repeated three times.
a-k Means having the same letter in a column were not significantly different by Post-
Hoc Multiple Comparison test $p < 0.05$ level.

Table 2. Analysis of variance for the effects of kinetin and 2,4-D on production of callus from leaf and stem explants of *Psoralea corylifolia*.
Varianzanalyse der Wirkung von Kinetin und 2,4-D auf die Kallusbildung von Blatt- und Sproßexplantaten von Psoralea corylifolia.

Source of variation	df	Mean Squares	
		Stem	Leaf
Replication	19	4.29	2.04
Treatment	14	9626.52**	8822.47**
Error	266	2.88	1.82

*, ** significant at $p < 0.01$, 0.05 respectively as determined by the F-test.

Table 3. Effect of modified MS (mMS) medium supplemented with 2300 mg/l potassium nitrate and various concentrations of kinetin, NAA , 2,4-D and 2% (w/v) sucrose on embryogenic response of calli derived from stem (A) and leaf (B) explants of *Psoralea corylifolia* after 4 weeks of subculture. All cultures were initiated from friable calli (200 ± 20 mg).
Wirkung von modifiziertem MS-Medium (mMS) in Kombination mit 2300 mg/l Kaliumnitrat und unterschiedlichen Konzentrationen von Kinetin, NAA, 2,4-D und 2% (w/v) auf die Bildung von embryoidem Gewebe aus Kallus von Sproß- (A) und Blattexplantaten (B) von Psoralea corylifolia nach 4 Wochen Kulturdauer.

mMS + Growth regulators (mg l^{-1})			% of cultures forming embryogenic callus (% ± S.E*)		No. of somatic embryos / culture (Mean ± S.E*)	
Kn	NAA	2,4-D	A	B	A	B
0	0	0	0	0	0	0
0.5	0.25	0	0	0	0	0
1.0	0.25	0	0	0	0	0
1.0	0	0.25	46.4 ± 0.7 a	31.8 ± 0.5 a	26.8 ± 0.6 a	14.6 ± 0.7 a
1.5	0	0.25	54.3 ± 0.8 b	40.2 ± 0.4 b	32.2 ± 0.7 c	24.2 ± 0.5 b
2.0	0	0.25	0	0	0	0
1.0	0	0.50	61.6 ± 0.6 c	45.7 ± 0.4 c	30.8 ± 0.4 b	25.6 ± 0.4 b
1.5	0	0.50	70.4 ± 0.3 d	58.6 ± 0.6 d	37.2 ± 0.6 d	30.4 ± 0.7 c
1.0	0.50	0	0	0	0	0
1.5	0.50	0	0	0	0	0

* a–d Means having the same letter in a column were not significantly different by Post- Hoc Multiple Comparison test $p < 0.05$ level.

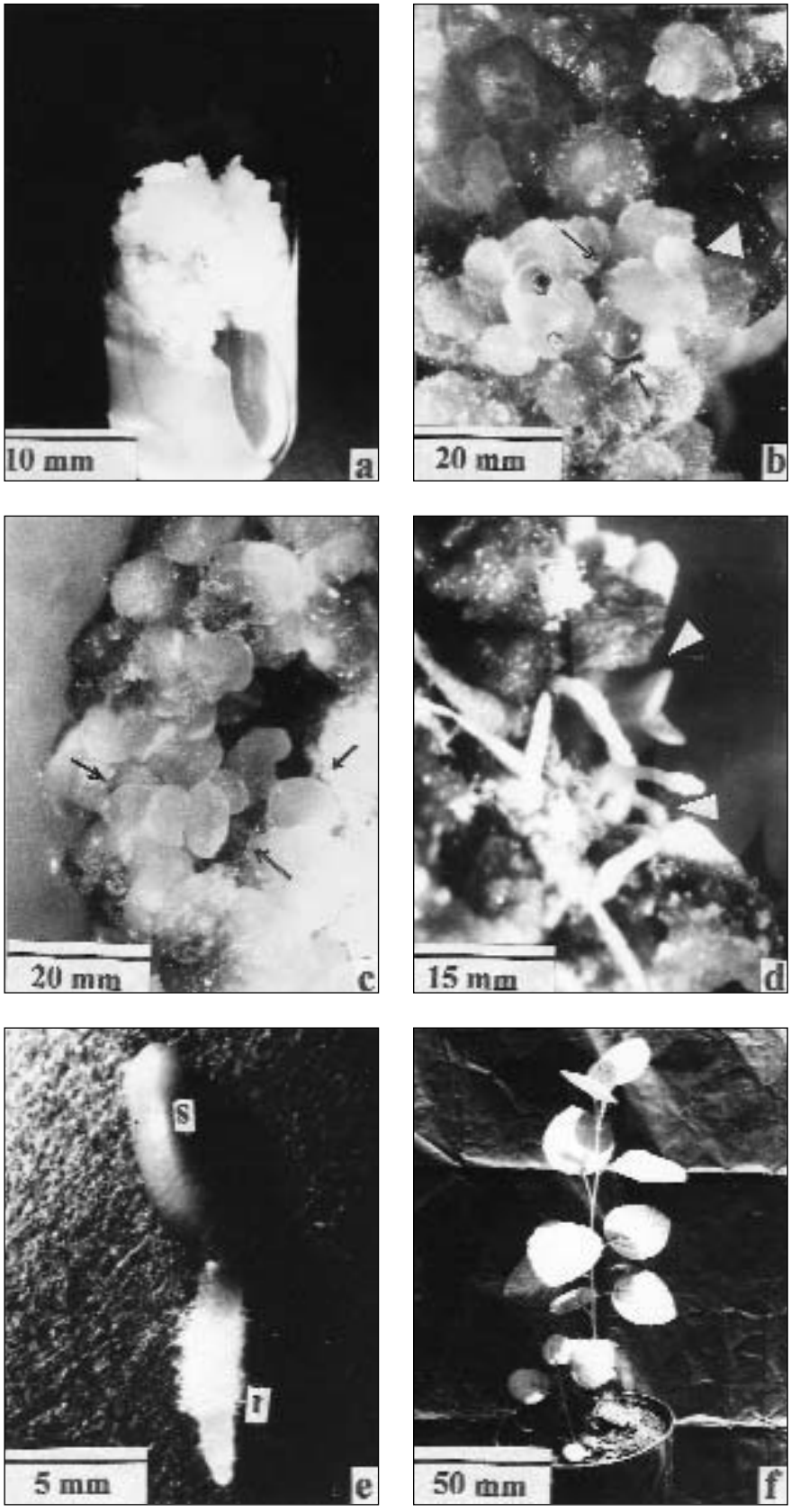

Fig. 1a–f. Somatic embryogenesis in *Psoralea corylifolia*. a. Callus initiation from stem explants cultured on MS basal salts supplemented with 0.5 mg/l kinetin, 3.0 mg/l 2,4-D after 15 days of culture (Bar= 10 mm). b. Induction of embryogenic callus derived from friable calli of P.corylifolia cultured on modified MS (mMS) medium supplemented with 2300 mg/l potassium nitrate, 1.5 mg/l kinetin, 0.5 mg/l 2,4-D after 2 weeks of subculture (Bar = 20 mm). c. Development of globular somatic embryos (arrow heads) after 3-weeks of culture (Bar = 20 mm). d. Development of cotyledonary somatic embryos (arrow heads) after 4-weeks of culture (Bar = 5 mm). e. Germinated somatic embryo with distinct shoot (s) and root (t) (Bar = 5 mm). f. Somatic embryo-derived plant established in soil (Bar = 50 mm).

Somatische Embryogeneses bei Psoralea corylifolia. a. Kallusbildung bei Sproßexplantaten nach 15 Tagen Kulturdauer (Kulturmedium: MS-Grundmedium mit 0.5 mg/l Kinetin und 3.0 mg/l 2,4-D (Maßstab = 10 mm). b. Induktion von embryoidem Kallus nach zwei Wochen Kulturdauer auf mMS-Medium angereichert mit 2300 mg/l Kaliumnitrat, 1.5 mg/l Kinetin und 0.5 mg/l 2,4-D (Maßstab = 20 mm). c. Entwicklung von globulären somatischen Embryonen (siehe Pfeilspitze) nach drei Wochen Kultur (Maßstab = 20 mm). d. Entwicklung von blattähnlichen somatischen Embryonen (siehe Pfeilspitze) nach vier Wochen Kulturdauer (Maßstab = 5 mm). e. Keimender somatischer Embryo mit Sproß (s) und Wurzel (t) (Maßstab = 5 mm). f. In Erde etablierte Pflanze aus somatischem Embryo (Maßstab = 50 mm).

any vascular connection with the callus (Figs. 1c and 1d). Medium containing high concentrations of 2,4-D (0.5–1.0 mg l^{-1}) helped in the induction of callus rather than embryo development. A very high percentage (70.4%) of cultures showing somatic embryos derived from stem derived calli as compared to leaf derived calli (58.6%) (Table 3). Similar embryogenic potential of calli subjected to high levels of cytokinins and low levels of auxins was reported in *Typhonium trilobatum* (DAS et al.1999) and *Cephaelis ipecacuanha* (ROUT et al. 2000). The number of somatic embryos/culture varied from 26.8–37.2 in stem derived calli and from 14.6–30.4 in leaf-derived calli (Table 3). At higher concentration of kinetin (2.0 mg l^{-1}) with low concentration of 2,4-D (0.25 mg l^{-1}) did not respond the formation of somatic embryo. The differential response could be due to the varying concentrations of the growth regulators used in the medium and to the explant types (WAKHLU et al.1990, FUENTES et al. 1993; ARUMUGAM and BHOJWANI 1990). After 8–9 days of inoculation under 16 h photoperiod, the somatic embryos turned greenish and developed into complete plantlets on medium containing half-strength basal MS salts supplemented with 0.1–0.25 mg l^{-1} IBA + 2% sucrose. About 55 to 60% of the somatic embryos were morphologically normal showing distinct shoot and root systems (Fig. 1e). An average of 60–70 plantlets were obtained from 100 mg of embryogenic callus.

Germinated propagules were transferred into pots containing soil:sand:cowdung manure in the ratio of 2:1:1 of the 50 plantlets that were subjected to acclimatization, 76% survived under greenhouse conditions. The plants grew well in the pots and looked similar to plants in the natural environment (Fig. 1f). There were no differences among plantlets in morphology.

In conclusion, an efficient protocol was developed for successful *in vitro* somatic embryogenesis in callus culture of *Psoralea corylifolia* on a modified Murashige and Skoog's medium containing 2300 mg l^{-1} potassium nitrate along with 1.5 mg l^{-1} kinetin and 0.5 mg l^{-1} 2,4-D. Long-term maintenance of embryogenic calli was achieved on the induction medium by subculturing at 4-week intervals. This investigation will help in the conservation of the endangered medicinal plant species for commercial exploitation.

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