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Rapid micropropagation for *Mucuna pruriens* f. *pruriens* L.

Sharmila Chattopadhyay [1], S. K. Datta [1], and S. B. Mahato [2]

[1] Department of Botany, Visva Bharati University, Santiniketan – 731235, India
[2] Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Calcutta – 700032, India

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Abstract. A rapid micropropagation system was developed for Mucuna pruriens f. pruriens using explants from 1-week-old aseptically grown seedlings. Multiple shoot regeneration occurred following an initial callus growth on Revised Tobacco (RT) medium supplemented with 2.7 µM NAA and 9.8 µM 2iP. Maximum number of shoot regeneration was achieved only from seedling explant 6 to 7 days old. More than 90% of the regenerated shoots could be rooted on half-strength liquid RT medium supplemented with 2.7 µM NAA. Plantlets readily adopted to greenhouse conditions. This system provides a new tool for micropropagation of Mucuna pruriens f. pruriens, an important medicinal plant.

Abbreviations

BAP, 6-benzylaminopurine; Kn, kinetin; 2iP, 2-isopentenyladenine; AdS, adenine sulphate; NAA, alpha-naphthalene acetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog (1962) medium; RT, Revised Tobacco medium (Kaul and Staba, 1968).

Introduction

Mucuna pruriens f. pruriens, commonly known as cowhage, is a herbaceous twining annual legume found throughout the greater part of India. The plant has long been used in traditional Indian medicine (The Wealth of India 1962; Nadkarni 1976). The report on the occurrence of the catecholic amino acid 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) attracted attention for utilization of the plant for L-DOPA production (Daxenbichler et al. 1971). L-DOPA, a neurotransmitter precursor, has found wide application for symptomatic relief of Parkinson's disease and mental disorder (British Pharma. 1973). Synthesis of L-DOPA by callus cultures of M. pruriens was first reported in 1979 (Brain, 1979). The presence of L-DOPA in several cell lines of suspension-grown cells of M. pruriens has been demonstrated (Huizing et al. 1985; Wichers et al. 1989). Production of L-DOPA by two-stage cell suspension culture of M. pruriens f. pruriens has also been reported (Chattopadhyay et al. 1994). However, the great demand of L-DOPA is largely met by the pharmaceutical industry through extraction of the compound from wild populations. But commercial exploitation for production is hampered due to its limited availability. Mucuna is a seed-grown annual herbaceous plant and is not propagated by cuttings. Micropropagation can provide the opportunity to obtain a rapid and large-scale multiplication of the plant. Moreover, conventional seed legume breeding programmes can be improved and complemented with in vitro genetic manipulation methods if an efficient plant regeneration system is available. No attempt has yet been made for development of a rapid method for propagation of the plant. In this communication we describe an efficient and rapid method of propagation of M. pruriens f. pruriens from seedling explants.

Materials and Methods

Seeds obtained from the Sutton Seeds Co. (Calcutta, India) were surface-sterilized in 80% ethanol for 1 min, immersed in 1% sodium hypochlorite containing 0.01% Triton X-100 for 20 min, and then rinsed with sterile distilled water (5 washes, each for 5 min). Seeds were sown in Petri dishes (100 x 15 mm) containing 25 ml medium consisting of 3% sucrose and 0.9% bacteriological agar (Himedia Laboratories, Bombay, India). The plates were sealed with Parafilm and incubated in continuous darkness at 28±1°C. Explants (epicotyl, hypocotyl and hypocotyl-containing cotyledon) from 1-week-old seedlings were cut into approximately 1-cm segments and cultured in tubes (10 x 1.2 cm) containing solidified MS (Murashige and Skoog, 1962) or RT (Kaul and Staba, 1968) medium supplemented with auxins, NAA (0.54, 2.7 or 5.4 µM), IAA (0.57, 2.85 or 5.7 µM) or cytokinins, BAP (0.44, 4.4 or 8.8 µM), 2iP (0.49, 4.9 or 9.8 µM), Kn (0.46, 4.6 or 9.2 µM) or with combinations of auxins and cytokinins at several concentrations. Only the effective treatments which resulted in either callus or organogenesis were

Correspondence to: S. B. Mahato

included in the tables. All media contained 3% sucrose and 0.8% agar. The pH was adjusted to 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving at 108 kPa at 120°C for 15 min. Cultures were maintained at 22±1°C in 16/8 h light/dark cycles (daylight fluorescent tubes, 35-40 μ mol m^{-2} s^{-1}). Each treatment consisted of a minimum of five replicates for each type of explant, and all experiments were repeated five times.

The morphogenetic response of different explants at the end of two to three weeks from the day of culture was recorded as percentage of explants responding. In order to investigate the influence of seedling age on shoot, root or callus formation, 3 to 18 day-old explants were cultured, and only effective data were presented in the tables.

Regenerated shoots were rooted in 25-ml tubes containing half-strength liquid RT medium supplemented with 2.7 μM NAA. Rooted plantlets were washed thoroughly with distilled water, transferred to pots containing a mixture of autoclaved peat and perlite (2:1), and after two more weeks of growth in a growth chamber at 25°C under 16-h photoperiod (25-40 umol.m^{-2} s^{-1}), were transferred to soil in the greenhouse.

Results and Discussion

Explants from seedlings of M. pruriens f. pruriens cultured in MS medium supplemented with different growth regulators produced callus and roots after 15-20 days of culture. However the callus showed a differential response according to the growth regulators used. Cytokinins (Kn, 2iP, BAP) alone produced compact hard callus, and auxins (NAA, IAA, IBA) induced rooting, whereas a combination of auxin and cytokinin (2.7 μM NAA + 4.4 μM BAP) promoted the growth of friable callus (data not shown). Similar observations were also noted with explants from mature plants of M. pruriens f. pruriens (Chattopadhyay and Datta, 1985). No organogenic response has so far been obtained from these callus or cell suspensions.

When seedling explants (epicotyl, hypocotyl and hypocotyl with cotyledons) were cultured in RT medium supplemented with auxins and cytokinins, shoots, roots and calli were obtained. Shoot apices were removed prior to inoculation from epicotyl explants, and these generally differentiated into shoots. Hypocotyl explants produced mainly roots. Hypocotyl explants containing cotyledons produced multiple shoots (Fig.1) and the frequency of multiple shoot production, as well as the average number of shoots, were highest on RT medium containing 2.7 μM NAA and 9.8 μM 2iP, while other concentrations as well as other combinations of auxin and cytokinins were ineffective (data not shown). Every explant when cultured on RT medium formed small amounts of callus from the cut ends in contact with medium before regeneration. The effects of different combinations of auxin and cytokinin for inducing morphogenetic responses from seedling explants is shown in Table 1. It was observed that the combination 2.7 μM NAA + 9.8 μM 2iP produced different results according to the nature of explants and was the most effective combination in terms of organogenesis for hypocotyl with cotyledon explants. Another combination (2.7 μM NAA + 4.4 μM BAP) also produced shoots from epicotyl explant and callus from hypocotyl explant. Thus the present study revealed that morphogenetic responses varied to a great extent according to the nature of the exogenously supplied plant growth regulators and the differences in morphogenetic potential that exist among different parts of the same plant.

Multiple shoot regeneration was markedly affected by the seedling age. Hypocotyl explants with cotyledons derived from 6 to 7 days old seedlings gave rise to shoots at the highest frequency (96-100%) and also produced the highest number of shoots per explant (4-5). Explants obtained from seedlings, younger or older than 6-7 days old, showed a reduction of shoot regeneration in terms of the number of explants producing shoots and the number of shoots per explant (Table 2).

Table 1 : Morphogenetic response of seedling explants of M. pruriens f. pruriens grown on RT medium suplemented with different growth regulators.

Nature of explants	Growth regulators μM	Nature of response: callus	Nature of response: organogenesis	Days to response	No.of explants responding / No.of explants cultured	% of response
Epicotyl	NAA+2iP,2.7+9.8	-	SH	15	13/24	55±2.22
	NAA+BAP,2.7+4.4	-	SH	20	5/25	20±1.26
	Kn+2iP,2.3+9.8	-	SH	22	6/24	25±0.89
Hypocotyl	NAA+2iP,2.7+9.8	-	RT	21	13/25	52±2.05
	NAA+BAP,2.7+4.4	+	-	18	10/25	40±1.71
	NAA+AdS,2.7+40.5	-	RT	21	15/25	60±2.32
Hypocotyl with cotyledon	NAA+2iP,2.7+9.8	-	MSH	21	25/25	100±0.63

SH = shooting; RT = Rooting; MSH = multiple shooting.
Values are mean±SE of five replications each with at least 10 explants.

Table 2 : Effect of seedling age on shoot formations from hypocotyl with cotyledon explants of M. pruriens on RT medium containing 2.7 µM NAA and 9.8 µM 2iP.

Seedling age (days)	No. of explants cultured	No. of explants producing shoots	Shoot* regeneration frequency	Total no. of shoots produced	No.of shoots per explant
4	17	3	18±0.28	10±0.56	0.6±0.35
5	22	15	68±0.8	44±1.26	2.0±0.31
6	22	21	96±0.63	78±1.67	3.6±0.21
7	20	20	100±0.56	100±0.44	5.0±0.31
9	20	11	55±0.45	15±0.77	0.8±0.24
12	20	13	65±0.75	20±0.63	1.0±0.29
14	20	12	60±0.8	20±0.89	1.0±0.27
18	15	9	60±0.98	10±1.09	0.7±0.22

* % of explants producing shoot; Data scored after 30 days of culture. Values are mean±SE of five replications each with at least 10 explants.

Formation of shoots occurred after 15-20 days of culture from hypocotyl with cotyledon explants, while they took another 20 days to regenerate plantlets. To obtain plantlets, rooting experiments were carried out with modified RT medium containing 2.7 µM NAA under aseptic conditions. The frequency of rooting from excised shoot was 90% within 20 days. Healthy plantlets were successfully transferred to pots with a mixture of peat and perlite. The survival of plants after normal acclimatization to soil was 70%. All surviving plants displayed normal phenotype, and flowering started six months after transfer to soil. There was no variation in flower colour, size and shape, compared to seed-derived plants.

In conclusion, this study clearly demonstrated that rapid in vitro propagation of M. pruriens f. pruriens can be obtained by proper combination of explant age, and nature and concentrations of plant growth regulators. The system presented here is potentially useful in the future genetic manipulation studies of this species.

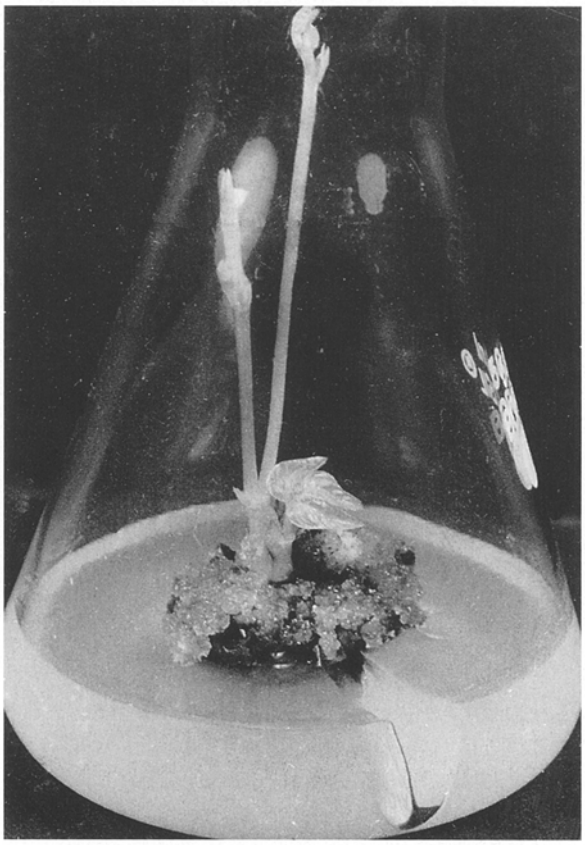

Fig.1. Multiple shoot formation from hypocotyl with cotyledon explant after three weeks of culture.

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