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IN VITRO PLANT REGENERATION OF COFFEE SENNA (*SENNA OCCIDENTALIS*) FROM HYPOCOTYL-DERIVED CALLUS

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An efficient system for plant regeneration of *Senna occidentalis* from hypocotyl-derived callus was developed. Callus was induced from leaf and hypocotyl explants on MS medium amended with 9.04 μM 2,4-D + 2.22 μM BAP and 10.74 μM NAA + 2.22 μM BAP. Medium browning due to leaching of compounds from callus was encountered and ameliorated through incorporation of 2.84 μM ascorbic acid. Leaf-derived callus showed no shoot induction ability, while hypocotyl-derived callus produced shoots in all cytokinin-amended treatments and also in combination with 2.68 μM NAA. For shoot formation, BAP-augmented treatments were better than medium with Kin added. Rhizogenesis was better on 1/2 MS basal medium with IBA than in the NAA and IAA treatments. Regenerated plants were acclimatized with 94% survival and showed similar morphology to field-grown plants.

Key words: *Senna occidentalis*, callus, regeneration. shoot induction, rhizogenesis, acclimatization.

INTRODUCTION

Senna occidentalis (L.) Link (formerly placed in the genus *Cassia*) is an important ayurvedic medicinal legume of tropical American origin. The plant is a shrub with lanceolate or ovate-lanceolate leaves 15–20 cm long. The leaflets are in pairs, membranous, glaucous ovate or lanceolate. The flowers are yellow, in short racemes. The pods are recurved, glabrous, compressed and bearing hard smooth shiny seeds. The plant is bitter, sweet and acrid, and purgative, laxative, anti-inflammatory, expectorant, hepatoprotective, antimalarial, analgesic, vermifuge and febrifuge properties are attributed to it (Warrier, 1994). Societies around the globe employ various parts of the plant in traditional medicines for treating a variety of diseases. Many household preparations such as decoctions, powder, syrup, confections and infusions are made with parts of the plant. These preparations are used to treat gastrointestinal complaints like stomach ache, dyspepsia, flatulence and constipation (Sharma et al., 2012). The whole plant is generally considered to be anti-inflammatory, antipyretic, sudorific, diaphoretic and immune-stimulating, and is widely used to treat various forms of fever (Yadav et al., 2010). Poor seed set resulting in nonviable seeds and low germination are the major impediments limiting the availability of its seedlings for the pharmaceutical industry (Co-operative Regional Project, 1963). Micropropagation offers a way to overcome this limitation. In vitro regeneration of *S. occidentalis* might easily be integrated into the production of herbal and pharmaceutical medicines and especially production of its active compounds. It also offers a useful tool for genetic improvement of the species and for selection of somaclonal variants. To the best of our knowledge there is no published report of in vitro regeneration of the species. Here we describe in vitro regeneration of *S. occidentalis* from hypocotyl-derived callus and nonregeneration from leaf-derived callus. In this work we overcame the effect of medium browning due to leaching of compounds during in vitro culture by incorporating 2.84 μM ascorbic acid in the culture medium.

MATERIALS AND METHODS

SEED COLLECTION, CULTURE ESTABLISHMENT AND CONDITIONS

Seeds were collected from *S. occidentalis* plants of known identity growing in the herbal garden of the Botany Department, Hamdard University, New Delhi, India. The seeds were washed with cetrimide

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Abbreviations: MS – Murashige and Skoog (1962); PGR – plant growth regulators; BAP – 6-benzylaminopurine; NAA – 1-naphthaleneacetic acid; IAA – 3-indoleacetic acid; Kin – kinetin

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for 10–15 min, followed by washing under running tap water for 20–25 min before soaking in distilled water overnight to facilitate imbibition and dormancy breaking. This was followed by treatment with 70% ethanol for 5–10 min and then rinsing 3 or 4 times with distilled water. Further seed sterilization with 0.1% mercuric chloride for 3–4 min was carried out in a laminar airflow chamber followed by rinsing with autoclaved distilled water 3–4 times to remove traces of mercuric chloride prior to seed inoculation. Sterilized seeds were inoculated on solidified 1/2 MS basal medium with or without 2.60 µM GA_3 supplementation in culture flasks for seed germination (Parveen et al., 2010).

Hypocotyls (1.0–1.5 cm long) and pairs of leaf explants were excised from 12–18-day-old axenic seedlings and inoculated on callus induction MS medium fortified with 9.04 µM 2,4-D + 2.22 µM BAP or 10.74 µM NAA + 2.22 µM BAP. The MS medium had added 3% (w/v) sucrose and was solidified with 0.6% (w/v) agar (Agar Agar Microbiology, Mumbai, India). Medium pH was adjusted to 5.6–5.8 using NaOH or HCl prior to autoclaving at 121°C for 20 min. Cultures were maintained at 24±2°C and 50–60% relative humidity under a 16 h photoperiod (Phillips cool white fluorescent tubes, 40 W, 50 µmol m^{-2} s^{-1} flux).

ORGANOGENESIS

Hypocotyl and leaf-derived callus masses (~1.0 g) were inoculated on shoot induction MS medium amended with different concentrations of BAP (2.22, 3.33, 4.44, 8.88, 13.32, 17.76, 22.22 µM), Kin (2.32, 3.48, 4.64, 9.28, 13.92, 18.56, 23.2 µM), BAP (2.22, 3.33, 4.44, 8.88, 13.32, 17.76, 22.22 µM) + 2.68 µM NAA or Kin (2.32, 3.48, 4.64, 9.28, 13.92, 18.56, 23.2 µM) + 2.68 µM NAA in culture tubes (Borosil India, 25 × 150 mm). Data were recorded after eight weeks of culture.

For in vitro rooting, induced microshoots were excised and inoculated on 1/2 MS root induction medium augmented with different concentrations of NAA (2.68, 4.02, 5.37, 10.74, 16.11, 21.48, 26.85 µM), IAA (2.85, 4.28, 5.71, 11.4, 17.1, 22.8, 28.51 µM) or IBA (2.42, 3.63, 4.84, 9.68, 14.52, 19.36, 24.20 µM). The rooting response (percentage of explants rooting), number of roots formed and root length (cm) were recorded after 4 weeks of culture.

HARDENING AND ACCLIMATIZATION

In vitro-raised plants with well-developed roots and shoots were removed from the culture tubes and the medium adhering on their surface was washed off under running tap water prior to transfer to plastic pots containing 1:1 perlite and soilrite (Keltech Energies Mumbai, India) mixture. These were covered with polyethene bags to maintain higher RH in a culture room for two weeks. The bags were gradually removed and the plants were transferred to a mixture of sterilized sand, silt and farmyard manure (1:1:1). They were irrigated with 1/4 MS basal salt solution every two days. The plants were later transferred to greenhouse conditions under normal day length and their survival was scored.

TABLE 1. Seed germination of *S. occidentalis* on 1/2 MS basal medium with or without GA_3 supplementation

Culture flask (F)	Seeds germinated on 1/2MS+GA_3 (%)	Seeds germinated on 1/2MS (%)
F1	90	80
F2	100	80
F3	100	90
F4	80	100
F5	90	60
F6	100	80
F7	100	70
F8	90	80
F9	90	100
F10	90	60
	Σ 93%	Σ 80%

Data recorded two weeks after seed inoculation.

STATISTICAL ANALYSIS

The experiments were done in triplicate with twelve replicates per treatment. The results were expressed as means ±SE of triplicates of twelve replicates per treatment. The significance of differences between treatments was assessed by ANOVA followed by Tukey's range test at $P<0.05$ using SPSS ver. 17 (U.S.A.).

RESULTS AND DISCUSSIONS

After overnight soaking of seeds in distilled water, leaching of phenolic compounds was noted. Inoculated seeds germinated after 3–14 days. The response was earlier and greater on medium amended with 2.60 µM GA_3 (93% germination) than on GA_3-free medium (80%) (Tab. 1). Excised hypocotyls and pairs of leaf explants inoculated on callus induction medium swelled, followed by bursting/splitting and the appearance of callus at the cut end in the second week and later on various regions of the explant (Fig. 1a,b). The response was greater and faster for leaf explants than for hypocotyl explants. Leaf callus was initially yellow but later turned dark brown; hypocotyl-derived callus was soft, yellowish brown and translucent. Callus dif-

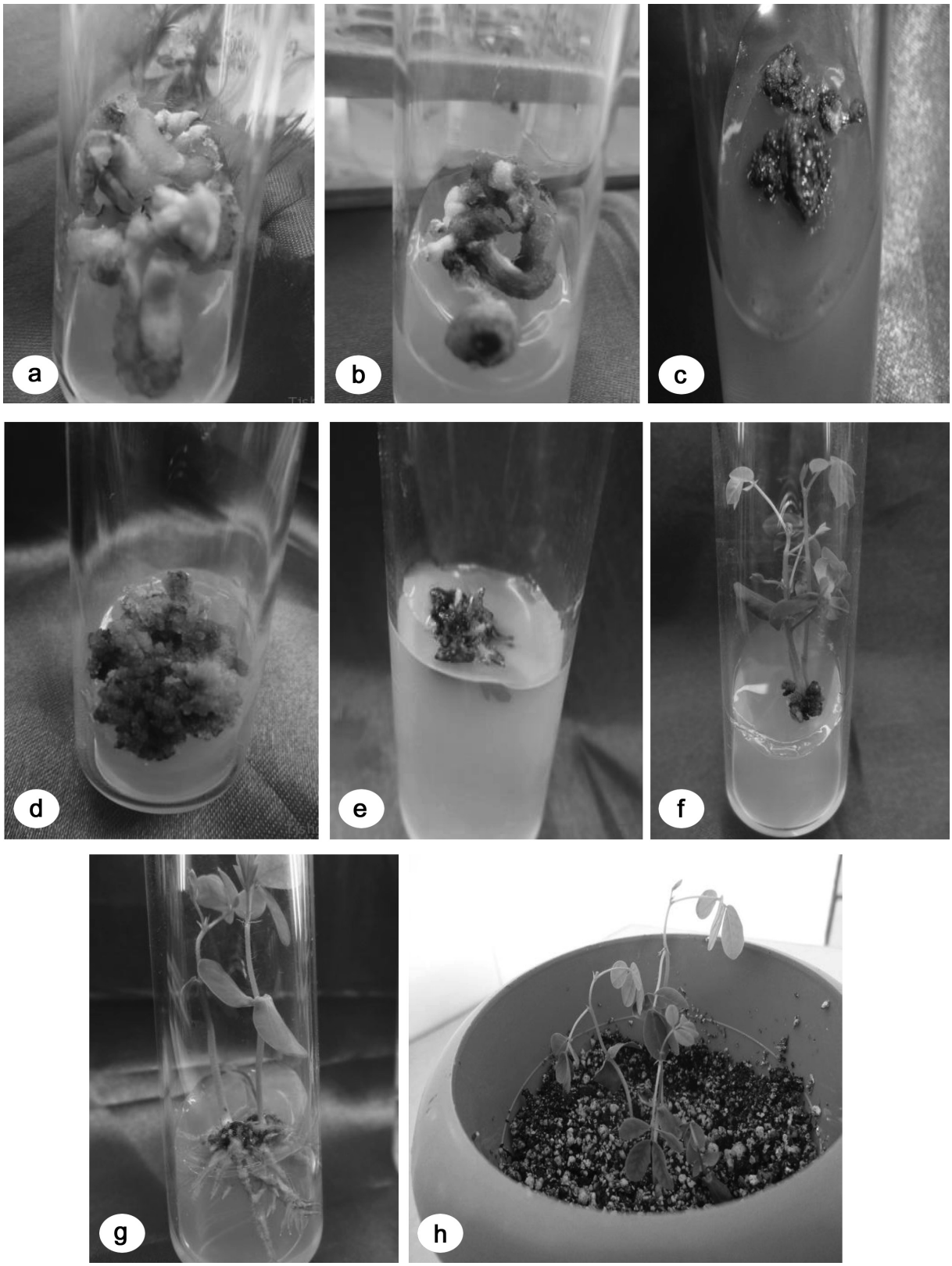

Fig 1. Callus and shoot induction from *Senna occidentalis* leaf and hypocotyl explants (**a, b**), medium browning and its amelioration (**c, d**), shoot induction from hypocotyl-derived callus (**e, f**), rhizogenesis (**g**) and acclimatization in potted mix (**h**).

TABLE 2. Effect of cytokinin concentration on shoot induction from hypocotyl-derived callus of *S. occidentalis*

PGRs (µM)				% response	No. of shoots	Shoot length (cm)
BAP	KIN	BAP+ NAA (2.68)	KIN+NAA (2.68)			
2.22				74.02	5.16±0.41^{bc}	4.31±0.26^{c}
4.44				98.00	6.86±0.71^{ab}	6.22±0.71^{ab}
8.88				96.06	5.93±0.15^{b}	5.81±0.33^{ab}
13.32				81.65	5.18±0.17^{bc}	4.71±0.92^{bc}
17.76				76.16	4.71±0.60^{c}	3.11±0.50^{cd}
22.22				70.13	4.31±0.11$^{c\ d}$	2.83±0.62^{cd}
	2.32			66.32	2.10±0.61^{d}	2.61±0.58^{cd}
	4.64			76.57	4.62±0.15^{c}	3.02±0.21^{cd}
	9.28			79.27	5.65±0.81^{b}	3.60±0.81^{c}
	13.92			58.67	4.33±0.77^{cd}	2.41±0.97^{d}
	18.56			49.34	3.78±0.99^{cd}	1.88±0.53^{d}
	23.20			41.56	3.36±0.08^{cd}	1.43±0.77^{d}
		4.44		99.02	7.77±0.51^{a}	6.87±0.90^{a}
		8.88		98.54	6.99±0.63^{ab}	6.62±0.68^{ab}
		13.32		91.11	6.34±0.71^{b}	5.52±0.21^{b}
		17.76		86.15	5.56±0.11^{b}	4.58±0.79^{bc}
		22.22		79.16	5.15±0.03^{bc}	3.43±0.37^{cd}
			4.64	83.81	5. 19±0.60^{bc}	4.11±0.62^{c}
			9.28	87.16	6.70±0.50^{ab}	5.24±0.78^{b}
			13.92	79.10	5.17±0.75^{bc}	4.77±0.15^{bc}
			18.56	69.55	4.30±0.74^{cd}	3.20±0.63^{cd}
			23.20	55.73	3.91±0.49^{cd}	2.46±0.65^{d}

Values are means ±SE of triplicate experiments with twelve replicates per treatment. Values bearing different letters within column differ significantly at P<0.05 by ANOVA followed by Tukey's range test.

fered in texture and response depending on the PGR type: it was compact on medium with 2,4-D and friable on medium with NAA. Induced friable leaf callus turned hard, nodular and compact on medium amended with 2,4-D + BAP upon subculture. Hypocotyl-derived callus became more friable without change in pigmentation on medium amended with NAA + BAP. Excessive leaching of compounds from leaf callus into the medium was observed in the third week of subculture; hypocotyl-derived callus showed less of this effect. Leaching resulted in callus death. Observation of the part of the callus in direct contact with medium during subculture showed that callus death began from these cells towards other regions. Callus death was greater and faster on medium with higher PGR concentrations. Goyal and Bhadauria (2008) overcame the effect of leaching during in vitro shoot proliferation of *Emblica officinalis* from nodal explants by incorporating antioxidants and frequent subculture. Jabeen et al. (2006) could not overcome the problem by using antioxidants and resorted to weekly subculture to overcome the problem during maintenance of *Aconitum heterophyllum* nodal segment-derived callus. In this study we overcame leaching by incorporating 2.84 µM ascorbic acid in the culture medi-

TABLE 3. Effect of auxin concentration on root induction from *S. occidentalis* shoots regenerated in vitro

Auxin (µM)	Percentage response (%)	Number of roots / shoot	Root length (cm)
2.68 NAA	36.04	3.21±0.35^{cd}	3.82±0.12^{bc}
5.37 NAA	48.23	3.68±0.31^{cd}	4.53±0.81^{ab}
10.74 NAA	53.18	3.83±0.97^{c}	4.15±0.60^{b}
16.11 NAA	61.09	4.48±0.60^{c}	4.51±0.53^{b}
21.48 NAA	67.56	4.93±0.80^{bc}	4.80±0.52^{ab}
26.85 NAA	75.32	5.76±0.53^{ab}	5.26±0.67^{ab}
2.85 IAA	27.41	2.40±0.54^{d}	2.12±0.17^{d}
5.71 IAA	34.82	2.97±0.87^{cd}	2.93±0.81^{cd}
11.4 IAA	56.52	4.15±0.80^{c}	3.83±0.39^{bc}
17.1 IAA	61.07	4.87±0.62^{bc}	4.12±0.44^{b}
22.8 IAA	68.22	5.03±0.66^{bc}	4.50±0.11^{b}
28.51 IAA	71.57	5.25±0.80^{b}	4.72±0.28^{ab}
2.42 IBA	72.44	5.35±0.11^{b}	3.30±0.22^{c}
4.84 IBA	98.61	6.33±0.31^{a}	5.57±0.17^{a}
9.68 IBA	88.33	5.89±0.22^{ab}	5.02±0.66^{ab}
14.52 IBA	85.35	5.67±0.13^{ab}	4.89±0.11^{ab}
19.36 IBA	82.22	5.44±0.55^{b}	4.49±0.88^{b}
24.20 IBA	79.29	5.28±0.28^{b}	4.12±0.80^{b}

Values are means±SE of triplicate experiments with twelve replicates per treatment. Values bearing different letters within column differ significantly at P<0.05 by ANOVA followed by Tukey's range test.

um (Fig. 1c,d). It was very difficult to transform the entire explant into callus, especially in leaf, in a few passages. Hypocotyl-derived callus rooted on medium with NAA + BAP in the third week.

Attempts to induce shoot formation from leaf callus proved unsuccessful on all the media tested. Callus proliferated on shoot induction medium through three passages. When hypocotyl-derived callus was inoculated on MS shoot induction medium amended with BAP or Kin, callus proliferation was observed in the first 2 weeks. After two passages, callus proliferation was followed by shoot induction in the third week in the form of green protuberances from callus and shoot emergence by the fourth week (Fig. 1e). The shoot formation response varied with the cytokinin type and concentration (Fig. 1f). The response was better on BAP-amended than on Kin-amended medium. After eight weeks of culture, the response, number of shoots per explant and shoot length were higher on 4.44 μM BAP-amended medium (98%, 6.86±0.71, 6.22±0.71 cm respectively) than on medium with 9.28 μM Kin (79.27%, 5.65±0.81, 3.60±0.81 cm) (Tab. 2).

Adding 2.68 μM NAA to medium amended with 4.44 μM BAP gave the highest response (99.02%), number of shoots (7.77±0.51) and shoot length (6.87±0.90 cm) after 8 weeks of culture. The corresponding values for medium amended with 9.28 μM Kin + NAA are 87.16%, 6.70±0.50 and 5.24±0.78 cm. BAP concentrations above 4.44 μM, alone or in combination with lower concentrations of NAA, inhibited shoot induction. The same was true for Kin concentrations above 9.28 μM (Tab. 2). BAP is the cytokinin most widely used for shoot induction in vitro. It is known to elicit plant growth and developmental responses through blossoming, via cell division stimulation. It gives higher shoot induction than other cytokinins in many taxa. In our experiment it gave superior shoot induction, better than Kin. This effect has been reported in *Santalum album* (Mujib, 2005), *Cichorium intybus* (Velayutham et al., 2006) and other taxa. In *Solanum nigrum*, Bhat et al. (2010) found that the response was better with Kin than with BAP.

Cytokinins and auxins interact in the control of developmental processes such as the formation and maintenance of the meristematic centers essential to morphogenesis. Their action is synergistic. Our experiments with *S. occidentalis* showed such an effect on shoot induction when BAP or Kin were combined with NAA at a lower concentration. Similar effects have been documented in a number of plant species, such as *Santolina canescens* (Casado et al., 2002), *Rhodiola rosea* (Dimitrov et al., 2003) and *Salvia nemorosa* (Skala and Wysokinska, 2004). The effect was not observed in *Cassia augustifolia* treated with Kin + NAA (Agrawal and Sardar, 2006).

Excised microshoots were transferred to 1/2 MS root induction medium augmented with various concentrations of IAA, IBA or NAA. Rhizogenesis was obtained after the first week. The response was exhibited with all tested auxin-amended treatments and differed in intensity between treatments (Fig. 1g). The response was better with IBA than IAA or NAA treatments. Among the concentrations tested, the rhizogenesis response after 4 weeks of culture was best on medium with 4.84 μM IBA added: 98.61% rooting, 6.33±0.31 roots per microshoot, and 5.57±0.17 cm root length. In the IAA and NAA treatments the rooting response and root number increased with the increase in concentration; at high IBA concentrations those parameters declined, and root formation was abnormal above 98.61 μM IBA. Root length increased with the increase in IBA and IAA concentration; the response to NAA concentrations was not unidirectional (Tab. 3). High IBA yielded thick branched roots. IBA has been reported to be more effective than NAA or IAA for rhizogenesis of *Centella asiatica* (Tiwari et al., 2000), *Cichorium intybus* (Rehman et al, 2003; Velayulam et al., 2006) and *Cassia augustifolia* (Agrawal and Sardar, 2006).

We removed the rooted plants (Fig. 1f) from the culture tube, washed the medium from their surfaces under running tap water and transferred them to plastic pots containing Soilrite and covered them with polyethene bags. The bags were gradually removed and the plants were transferred to a mixture of sand, silt and farmyard manure, kept in a culture room and irrigated with 1/4 MS basal solution every 2 days (Fig. 1h). Later they were transferred to a greenhouse under normal daylength, where 94% survived. The regenerated plants showed normal morphology comparable to field-grown plants.

Here we described efficient regeneration of *S. occidentalis* from hypocotyl-derived callus. This system offers a way of ensuring the availability of seedlings of this species for the pharmaceutical industry and traditional medicine. It also offers a tool for use in biotechnology, and specifically for genetic improvement of the species and for selecting somaclonal variants.

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