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BRIEF COMMUNICATION

Effective protocol for *in vitro* shoot production through nodal explants of *Simmondsia chinensis*

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Abstract

Nodal explants excised from 18 to 20-year-old female plants of *Simmondsia chinensis* if cultured on Murashige and Skoog's medium supplemented with 20 μM N^6-benzyladenine (BA) differentiated an average of 2.7 ± 0.4 shoots in 11.5 % explants. The percentage of nodal explants inducing multiple shoots enhanced significantly if *in vitro* raised shoots were used as source of explants. Nearly 100 % cultures differentiated an average of 4.7 ± 2.0 shoots per explant on the same medium. Nearly 85 % of the shoots induced roots when a pulse treatment of 50 μM indole-3-butyric acid (IBA) was given prior to their transfer to semi-solid MS medium containing 10 μM IBA + 0.5 % activated charcoal + 1 μM BA. Plantlets were gradually hardened in *Soilrite* and acclimatized to soil.

Additional key words: activated charcoal, benzyladenine, indole-3-butyric acid, jojoba, multiple shoots.

Simmondsia chinensis (jojoba), a promising dioecious shrub of arid zones, is commercially valuable for its seeds. Non-availability of elite germplasm, poor seed germination and lack of any marker for identification of male and female seedlings prior to flowering are serious constraints for large scale production of plants of known sexuality. Though several attempts were made earlier for *in vitro* organogenesis on jojoba employing seedling explants (Rost and Hinchee 1980, Scaramuzzi and D'Ambrosio 1988, Roussos *et al.* 1999) as well as explants from field grown plants (Rost and Hinchee 1980, Jacoboni and Standardi 1987, Mills *et al.* 1997, Llorente and Apostolo 1998), an efficient and repeatable protocol for clonal propagation is still lacking. In an earlier report Chaturvedi and Sharma (1989) described that only 10 % of aseptic cultures could be established successfully from field grown male and female plants. Further, they mentioned that both male and female plants behaved alike morphogenically under a defined culture conditions. However, differential hormonal requirements for *in vitro* clonal propagation of male and female plants have been mentioned in our earlier communication (Agrawal *et al.* 1999). Mills *et al.* (1997) established some male and female clones of jojoba, employing excised axillary buds, but they have not described the individual requirements or responses of different clones or plants belonging to different sexes and moreover, the rate of multiplication they obtained was very low. Thus, with a view to develop efficient protocols for large scale propagation of jojoba plants of known sexuality, the present investigation has been carried out.

Healthy twigs (20 - 30 cm long) excised from 18 to 20-year-old female plants belonging to clone EC 33198 (originally procured from USA and showing high productivity) of *Simmondsia chinensis* (Link) Schneid. growing at NBPGR Research Station, Jodhpur, were excised and their cut ends immediately dipped in freshly prepared 1 % (m/v) citric acid solution till the time they

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Abbreviations: AC - activated charcoal; BA - N^6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; B5 - medium of Gamborg *et al.*; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; 2iP - N^6-(2-isopentenyl) adenine; Kn - kinetin; MS - medium of Murashige and Skoog; NAA - α-naphthaleneacetic acid; SH - medium of Schenk and Hildebrandt; WPM - woody plant medium of Lloyd and McCown.

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were brought to the laboratory at the University of Delhi for further processing. These twigs were thoroughly washed under running tap water for 30 min. After defoliating, they were cut into nodal and internodal explants and surface sterilized. The nodal explants were immersed in 0.5 % (m/v) *Bavistin* solution (fungicide) contained in 250 cm^3 flasks and kept on table top rotary shaker at 200 r.p.m. for 10 min. After pouring off the *Bavistin* solution, the explants were rewashed thoroughly under running tap water 6 or 7 times. These were then surface sterilized with freshly prepared 0.5 % $HgCl_2$ for 10 min on a rotary shaker and finally washed 4 or 5 times with sterile distilled water in a laminar flow and stored there till inoculation. Both ends of explants, turning brown, were trimmed prior to inoculating them on semi-solid medium.

Initially, four basal media like B5 (Gamborg *et al.* 1968), Knop (Knop 1865), MS (Murashige and Skoog 1962) or WPM (Lloyd and McCown 1980) were employed. They contained 3 % sucrose (*DCM*, Daurala, India) as carbon source and were gelled with 0.8 % agar (*Qualigens*, Bombay, India). The media were either used alone or were supplemented with different growth regulators: N^6-(2-isopentenyl)adenine (2iP), N^6-benzyl-adenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or α-naphthaleneacetic acid (NAA). The pH of the media was set at 5.8 with 1 M HCl or 1 M NaOH. 20 cm^3 of molten medium was poured into 150 × 25 mm glass test tube (*Borosil Glass Works*, Mumbai, India), plugged with non-absorbent cotton wrapped in two-layered muslin cloth. For axillary shoot proliferation, the medium was poured into jam bottles covered with autoclavable *Kasablanka* (Mumbai, India) caps. The glass vessels containing media were autoclaved at 121 °C for 15 min. All chemicals used were of analytical grade (*British Drug House*, *Sigma-Aldrich* and *E. Merck*). The cultures were incubated in a culture room at 25 ± 2 °C under continuous irradiance 45 - 46 W m^{-2} provided by cool white fluorescent tubes (40 W; *Philips India Ltd.*, Calcutta, India) and relative humidity 55 - 65 %. Explants were subcultured after 30 d on fresh medium.

Three months after initial inoculation, the shoots developed in the axils of the nodal segments were excised where each shoot comprised four nodes, each of which were again subcultured on the same but fresh medium (maintenance medium) at an interval of 30 d. The jam jars proved better for axillary shoot bud proliferation as well as for maintaining the cultures up to six months. Four or five explants per vessel were inoculated. For inducing rhizogenesis, some of the aforesaid shoots were excised and directly transferred to auxin (IBA, IAA or NAA) supplemented medium wherein they formed callus at the basal cut end with concomitant decay of shoot tip. In another experiment basal ends of the shoots were dipped in filter sterilized IBA of different molarity for variable durations and then transferred to semisolid MS medium containing 1 μM BA + 10 μM IBA + 0.5 % activated charcoal for 15 d.

After removing the plantlets from semisolid medium, they were washed thoroughly with autoclaved water and dipped for 15 min in 0.1 % (m/v) *Bavistin*. They were initially transplanted in plastic pots with *Soilrite* for one month. These pots were covered with polythene sheets and were maintained under controlled conditions (45 - 46 W m^{-2}, 25 ± 2 °C, 55 - 65 % RH). The plantlets were irrigated with ¼-strength MS solution without sucrose for one week and then with tap water. After one month, they were transferred to soil. 24 explants were inoculated per treatment and the experiments were repeated twice. Data were analysed statistically employing *ANOVA*.

Establishment of primary aseptic cultures of explants excised from field-grown plants of known sexuality has been difficult in this taxon due to high incidence of microbial contamination (Chaturvedi and Sharma 1989, Mills *et al.* 1997). This has been overcome during the present investigations by using *Bavistin* and mercuric chloride as described earlier and nearly 98 % aseptic cultures were procured.

Nodal explants (Fig. 1*A*) cultured on four basal media *viz.* B5, Knop, MS or WPM, did not respond at all and turned brown within a month. Even the axillary buds failed to grow. Explants cultured on 0, 1.0, 5.0, 10.0, and 20.0 μM BA augmented MS medium, elicited very poor response. The emergence of axillary buds took place after 30 d (Fig. 1*B*). Only 11.5 % of the segments developed shoots (2.8 % single axillary shoots + 8.7 % multiple shoots), with an average of 2.7 ± 0.4 shoots per explant having an average length of 1.00 ± 0.40 cm on 20 μM BA, within three months of inoculation (Fig. 1*C*). Thereafter, regeneration potential of shoots developed in axils of explants was assessed. The nodal explants of such excised from *in vitro* raised shoots cultured on MS medium containing cytokinins (BA or 2iP) responded differentially. Of these, 20 μM BA proved optimum both for eliciting maximum percentage of morphogenic cultures (100 %) as well as the highest average number of 4.74 ± 2.00 shoots per explants (Table 1, Fig. 1*D*). At higher concentrations of BA, both the percentage of explants forming multiple shoots as well as the average shoot number declined significantly. A significantly low response (both, in the percentage of morphogenic cultures as well as the average number of shoots per explant) was observed on MS medium with 2iP. Only 19 % of the explants induced multiple shoots with an average of 1.70 ± 0.95 shoots per explant at 50 μM 2iP, while at its lower levels, the response was even poorer. However, average shoot length was maximum (5.38 ± 4.41 cm) on 20 μM 2iP (Table 1). After harvesting the axillary shoots (2.0 - 2.5 cm long), the original explants as well as nodal micro-

cuttings of these shoots were repeatedly subcultured on the same but fresh maintenance medium (MS + 20 μM BA), where they again produced an average of 4 adventitious shoots. Such explants retained the shoot producing competence for over four years. The number of shoots enhanced significantly (25 - 30 per explant) if the mother explants were subcultured in jam jars or 250 cm^3 conical flasks in lieu of test tubes (Fig. 1*G*).

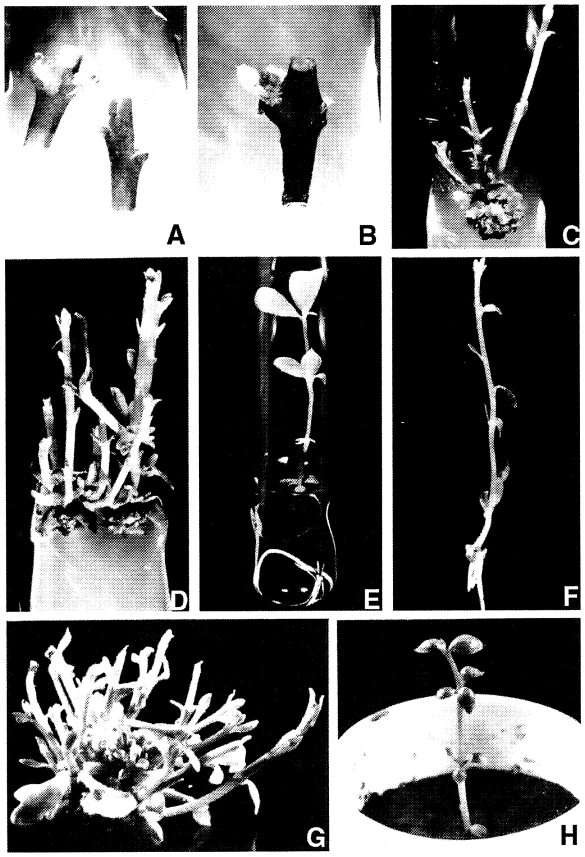

Fig. 1. *In vitro* morphogenic response of nodal explants excised from *in vivo* grown 18 to 20-year-old plants and then *in vitro* multiplied shoots, used as micro-cuttings of *Simmondsia chinensis*: *A*,*B* - nodal explants from field grown plant at the time of inoculation on MS medium (*A*, ×1.5) and response after 30 d of culture (*B*, ×2); *C* - multiple shoots developed *in vitro* in the nodal explant reared on MS + 20 μM BA after 90 d of culture (×0.9); *D* - multiple shoots differentiated in the nodal explants, excised from *in vitro* shoots, on MS + 20 μM BA adjuvanted medium, after 60 d (×0.9); *E* - induction of roots in the excised shoots on MS + 0.5 % activated charcoal + 10 μM BA + 1 μM IBA (×0.8); *F* - a tissue culture raised plantlet (×0.9); *G* - profuse differentiation of multiple shoots on nodal explant after second subculture in jam bottle (×0.5); *H* - plantlet thriving in soil (×0.7).

Influence of different auxins (IAA, 2,4-D or NAA) alongwith optimum level of BA (20 μM) was also assessed with a view to improve the response, but none of the combinations tried proved beneficial (data not shown). Only single axillary shoots developed on almost all the combinations. Contrary to this, in an earlier report of Chaturvedi and Sharma (1989) a combination of IAA and BA supplemented to SH medium favoured the best caulogenic response. In the present investigation, however, addition of IAA to BA proved inhibitory forming calluses at the basal cut ends as well as in leaf axils along with reduced average number of shoots (data not given). In another study, Mills *et al.* (1997) mentioned that a combination of 0.3 mg dm^{-3} IAA and 30.5 mg dm^{-3} 2iP was optimum for differentiating an average of 1.83 ± 0.11 shoots per nodal explant, which is very low in comparison to our work (Table 1). Moreover, after 7 months they obtained only 63 propagules per explant whereas in present study 512 shoots per nodal explant can be obtained during the same period.

The induction of roots in the excised shoots was another serious problem. The *in vitro* raised shoots cultured on MS medium supplemented with various auxins like IAA, IBA or NAA alone formed calluses at the basal cut ends, which finally led to the death of the shoot tip and shoots in a month. The excised shoots rooted in MS medium containing 1 μM BA and 10 μM IBA along with 0.5 % activated charcoal (Fig. 1*E*). A

prior pulse treatment of higher dose of 50 μM IBA for 20 min duration improved the response (Table 2). By giving dip treatment to shoots in filter sterilized IBA the duration of root induction was reduced from 45 d to 15 d. Addition of AC proved helpful both for preventing callus and inducing roots in the shoots as seen earlier in *Populus* (Agrawal and Gupta 1991) and *Abies numidica* (Vooková and Kormuťák 2001). The influence of charcoal in absorbing toxic substances present in the medium or ingredients produced as a results of autoclaving or exuded by the cultured tissue has already been well documented (Fridborg *et al.* 1978, Weatherhead *et al.* 1979). Contrary to this a negative effect of activated charcoal has been reported on rooting of *Anacardium occidentale* by Boggetti *et al.* (2001). However, in the present system two step method, *i.e. 1*) dipping in IBA, and *2*) transfer

Table 1. Morphogenic response of nodal explants of *S. chinensis* female plants cultured for 60 d on MS medium supplemented with BA or 2iP alone. Means of 2 replicates of 24 explants ± SD Means within the column followed by the same superscript are not significantly different at $P < 0.05$.

Cytokinin	Conc. [μM]	Explants developing single shoots [%]	multiple shoots [%]	Number of shoots [explant^{-1}]	Shoot length [cm]	Length of tallest shoot [cm]
BA	0	37^{f}	0^{j}	0.41 ± 0.64^{j}	1.08 ± 0.42^{i}	0.92 ± 0.41^{k}
	1	62^{de}	38^{d}	2.47 ± 0.57^{d}	3.21 ± 1.54^{bc}	4.53 ± 1.23^{d}
	5	39^{f}	57^{c}	2.70 ± 0.67^{c}	2.26 ± 1.02^{c}	3.36 ± 0.78^{fg}
	10	12^{g}	88^{b}	3.03 ± 0.66^{b}	2.12 ± 1.29^{d}	3.54 ± 1.29^{f}
	20	0^{h}	100^{a}	4.74 ± 2.00^{a}	1.90 ± 1.14^{e}	3.74 ± 0.73^{f}
	30	56^{de}	43^{d}	2.74 ± 0.75^{c}	1.73 ± 1.16^{f}	2.73 ± 0.78^{g}
	40	71^{c}	29^{e}	2.20 ± 0.57^{e}	1.77 ± 0.88^{f}	2.43 ± 0.80^{g}
	50	73^{d}	20^{ef}	1.76 ± 0.91^{f}	1.48 ± 0.88^{g}	2.03 ± 0.70^{i}
2iP	0	64^{d}	0^{j}	0.68 ± 0.48^{i}	1.24 ± 1.07^{h}	1.24 ± 1.07^{j}
	1	79^{bc}	0^{j}	0.83 ± 0.43^{h}	2.55 ± 1.19^{c}	2.62 ± 1.20^{h}
	5	90^{ab}	2^{hi}	1.02 ± 0.40^{gh}	3.77 ± 1.47^{b}	3.95 ± 1.42^{e}
	10	98^{a}	0^{j}	1.50 ± 0.70^{g}	4.73 ± 2.82^{ab}	5.87 ± 2.49^{c}
	20	83^{bc}	8^{h}	1.79 ± 0.95^{f}	5.38 ± 4.41^{a}	7.44 ± 5.19^{a}
	30	94^{a}	4^{hi}	1.72 ± 0.98^{f}	4.62 ± 3.20^{ab}	7.17 ± 3.29^{ab}
	40	85^{bc}	8^{h}	1.55 ± 0.76^{g}	4.65 ± 3.19^{ab}	6.53 ± 2.97^{bc}
	50	75^{c}	19^{g}	1.70 ± 0.95^{f}	4.65 ± 3.29^{ab}	6.36 ± 3.34^{bc}

Table 2. Induction of roots in the *in vitro* raised shoots on MS medium containing 10 μM IBA + 1 μM BA + 0.5 % AC after pulse treatment of IBA, in 15 d. Mean of 2 replicates of 24 explants ± SD. Means within the column followed by the same superscript are not significantly different at $P < 0.05$.

IBA [μM]	Time [min]	Shoots developing roots [%]	Number of roots [shoot^{-1}]	Root length [cm]
25	1	38^{f}	3.00 ± 1.00^{c}	2.40 ± 0.80^{cd}
25	10	36^{f}	3.60 ± 1.20^{bc}	3.60 ± 1.20^{ab}
25	20	57^{d}	3.20 ± 1.20^{c}	3.20 ± 1.20^{b}
25	40	50^{e}	3.10 ± 1.00^{c}	2.70 ± 1.50^{c}
50	1	46^{e}	3.10 ± 0.90^{c}	1.90 ± 0.70^{d}
50	10	62^{c}	3.80 ± 1.50^{b}	2.90 ± 1.70^{bc}
50	20	85^{a}	4.60 ± 1.70^{a}	3.80 ± 1.80^{a}
50	40	73^{b}	4.00 ± 1.70^{a}	3.70 ± 1.90^{a}

to semi-solid medium proved beneficial both for improving the percentage of shoots inducing roots and minimizing the period of root induction.

In yet another report by Mills *et al.* (1997) for inducing roots, a significantly higher doze of IBA (120 mg dm^{-3}) was used where only 80 % shoots organised roots. In the present case, however, only 10 μM IBA (2 mg dm^{-3}) was required for rooting. Thus, the rooting medium used by us was simpler and cost-effective in comparison to those used earlier (Chaturvedi and Sharma 1989, Mills *et al.* 1997). After induction of roots the plantlets (Fig. 1*F*) were transferred to auxin and AC lacking medium for further 15 d to improve their growth. The plantlets, thus raised, were taken out of the medium after two months, dipped in 0.1 % *Bavistin* for half an hour prior to their transfer to sterile *Soilrite*. They were maintained there for *in vitro* hardening and subsequently transferred to soil for further acclimatization (Fig. 1*H*).

The aforesaid regeneration method of plantlets from explants of known sexuality holds great potential for large scale exploitation of this immensely valuable crop. This will be of great help in saving labour and cost over raising plants through seeds, where the ratio of male and female

seedlings remains unequal (5:1) or males are more in number in comparison to females (Gentry 1958, Mills *et al.* 1997). The multiple shoots once raised on nodes of the original explants have retained the regeneration potential for over four years.

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