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RESEARCH ARTICLE

An efficient and reproducible indirect shoot regeneration from female leaf explants of *Simmondsia chinensis*, a liquid-wax producing shrub

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Abstract *Simmondsia chinensis* (Link) Schneider is a perennial, dioecious, drought resistant and multipurpose seed oil crop grown in arid and semi-arid conditions throughout the world. A reproducible and more efficient method for indirect shoot organogenesis from female leaf explants has been standardized. The leaf explants cultured on Murashige and Skoog (MS) medium with 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) alone produced the highest frequency of callus compared with 1.5 mg l^{-1} IBA. Maximum proliferation of callus was observed on MS medium containing a combination of 1.0 mg l^{-1} 2,4-D with 0.5 mg l^{-1} BAP. For shoot differentiation, the proliferated callus was subcultured on MS medium supplemented with 6-benzylaminopurine (BAP) (1.0–4.0 mg l^{-1}) along with 40 mg l^{-1} adenine sulphate as additive or in combination with α-naphthalene acetic acid (NAA) or Indole-3-butyric acid (IBA). Optimum shoots differentiated from callus was obtained on MS medium supplemented with 2.0 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. On this medium, 100 % cultures were responded with an average number of 14.44 shoots per explant with their mean length of 4.78 cm. In vitro rooting (6.22 roots per explant) was achieved on half strength MS medium containing 2 % sucrose with 3.0 mg l^{-1} IBA and 300 mg l^{-1} activated charcoal (AC). Rooted plantlets were successfully hardened under control conditions and acclimatized under field conditions with 90 % success rate. The present protocol is highly efficient, reproducible and economically viable for large scale production of female plants.

Keywords 2,4-D · Callus · Differentiation · Liquid-wax · Shoot · *Simmondsia chinensis*

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Introduction

Simmondsia chinensis (Link) Schneider, commonly known as jojoba, is an oil-yielding plant, belongs to the family *Simmondsiaceae* (Kumar et al. 2012). *S. chinensis* is a dioecious shrub and only female plant bears acorn shaped seeds of dark brown color. The seed contains about 50–60 % of a colorless and odorless liquid-wax which is unique in plant kingdom. It is composed of long chain of fatty acids and fatty alcohols (Zaher et al. 2004). The liquid-wax generated from jojoba has potential applications in cosmetics (Ayerza 2001), pharmaceutical industry (Canoira et al. 2006), plastic industry (Reddy and Chikara 2010), leather industry (Radwan et al. 2007) and bio-fuel industry (Le Dréau et al. 2009). The physical properties of liquid-wax involve high viscosity, high flash and fire point, high dielectric constant, high oxidative stability and low volatility which makes it usable as lubricant in high pressure machinery and electric insulators (Agrawal et al. 2007). Liquid-wax is generally used in folk remedies for renal colic, sunburn, hair loss, headache, sore throat and wound healing properties (Ranzato et al. 2011). Interestingly, liquid wax possesses anti-inflammatory activity (Habashy et al. 2005), anti-microbial activity (Guirguis et al. 2013), insecticidal, antifeedant and antifungal activities (Abbassy et al. 2007). It has similar chemical and rheological properties to the sperm whale oil which is listed as one of the endangered species (Low and Hackett 1981), therefore; it can be used as a best substitute for sperm whale oil. This plant is considered to

be drought resistant because it has an extensive and deep root system (Naqvi and Ting 1990). Also, it can be grown on marginal and wasteland due to tolerance of extreme range of temperature from −5 to 54 °C (Bhardwaj et al. 2010). All these features signify the importance of this commercial crop.

Conventionally, *S. chinensis* is propagated through seeds, seedlings and stem cuttings. Plantation raised through seeds has slow and non-uniform growth; male and female plants cannot be distinguished morphologically before the flowering stage which occurs atleast 1½years after planting (Prakash et al. 2003; Ince et al. 2010) and male biased ratio is 5:1 (Sharma et al. 2008). However, for a successful plantation male to female ratio of 1:9 is sufficient and it results in higher seed yield. Moreover, commercial plantation established through cuttings is difficult (Naqvi and Ting 1990), slow growing (Lee and Thomas 1985) and season dependent process (Benzioni and Ventura 1998). Therefore, there is need of non-conventional methods for the large scale propagation of female plants for establishment of commercial plantation to fulfil the demand of jojoba liquid wax in various industries. Plant regeneration through tissue culture is a prerequisite tool of biotechnology for successful establishment of this crop (Kumar et al. 2012). In the recent past, in vitro plant regeneration through nodal segment explants of *S. chinensis* has been done by few researchers both from seedling explants (Roussos et al. 1999; Gao and Cao 2001), and mature plants (Mills et al. 1997; Llorente and Apostolo 1998; Agrawal et al. 2002; Singh et al. 2008; Kumar et al. 2009). Mohammed et al. (2008) stated that maturation and germination of somatic embryos derived from leaf explants were not observed successfully. However, to our knowledge there is no report on shoot regeneration from callus of leaf explants. The present investigation was performed to develop a high efficient and female sex specific protocol to increase the female propagules for successful establishment of this commercial crop. A difference between male and female plant regeneration and differential requirement of hormones have been reported by Agrawal et al. (2002), Prakash et al. (2003) and Rai et al. (2012). It is proposed that a specific genotype tends to maintain characteristic endogenous levels of growth regulators, even when subjected to tissue culture (Champault et al. 1985). The literature also revealed that callus mediated regeneration from in vivo grown female *Momordica dioica* produced female plants of similar morphology to the mother plant (Thiruvengadam et al. 2012; Debnath et al. 2013). Somaclonal variations arising due to tissue culture have no effects on the sex of dioecious plants regenerated from callus. Evidence from earlier works shows that sexual fidelity is likely to be maintained in dioecious plants through callus mediated regeneration (lrish and Nelson 1989; Gorelicku and Osborneb 2002). Therefore, it is feasible to produce female plants from female explants maintaining the sex fidelity. To the best of our knowledge, this is the first report on in vitro regeneration of plantlets through callus using leaf of female *S. chinensis*.

Materials and methods

Plant material and preparation of explants

About 2 years old female plants of *S. chinensis* were obtained from Thar Nursery, Bikaner, India (Latitude 27°97′43.11″N; Longitude 73°11′43.33″E) during the month of September, 2011. The plants were transplanted in Screen house of the Department of Environmental Sciences, Maharshi Dayanand University, Rohtak, India (Latitude 28°52′48.87″N; Longitude 76°37′09.97″E). Leaf explants were taken from about 3 years old female plant. Explants were washed with a few drop of liquid detergent for 5 min and then in running tap water to remove the superficial dust particle followed by surface sterilization with 0.1 % (*w/v*) $HgCl_2$ for 2–3 min and washing in sterilized distilled water for 5 times. All steps of surface sterilization of explants were performed in a laminar flow under aseptic conditions.

Culture media and growth conditions

The culture medium consisted of MS (Murashige and Skoog 1962) basal media with 3 % (*w/v*) sucrose and 0.8 % (*w/v*) agar. The pH of the media was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl and 40 ml media dispensed into 250 ml culture flask. The culture flasks were autoclaved at 121 °C for 20 min. All cultures were maintained at 25±2 °C temperature under 16 h photoperiod conditions and light intensity of 50 μmol m^{-2} s^{-1} was provided by cool white fluorescent tubes (Philips, India).

Induction and proliferation of callus

For callus induction, about 1–1.5 cm^2 of leaf explants were inoculated on MS medium supplemented with 2,4-D or IBA (0.5, 1.0, 1.5, 2.0 mgl^{-1}) alone. Further, induced callus were sub cultured on MS medium containing 2,4-D, 1.0 mg l^{-1} (optimum concentration) with different concentration of BAP (0.1, 0.5, 1.0, 1.5 mg l^{-1}) for proliferation of callus.

Shoot differentiation

For shoot differentiation, proliferated callus was sub cultured on MS medium supplemented with adenine sulphate, 40 mgl^{-1} as an additive with different concentrations of BAP (1.0, 2.0, 3.0, 4.0 mgl^{-1}). Further, for shoot multiplication, regenerated shoots were transferred into MS medium containing BAP, 2.0 mgl^{-1} (optimum concentration) with different concentrations of NAA or IBA (0.1, 0.2, 0.5, 1.0 mg l^{-1}) along with additive.

In vitro rhizogenesis

The elongated shoots about 3–5 cm length were transferred on half-strength MS medium supplemented with different concentrations of IBA (1.0, 2.0, 3.0, 4.0 mgl^{-1}) with AC (150 and 300 mgl^{-1}). Rooting media contained sucrose 2 and 0.7% (*w/v*) agar-agar as gelling agent and 15 ml nutrient media was poured into 50 ml test tubes.

Hardening and acclimatization

Plantlets with well-developed roots were removed from test tubes and washed with sterilized distilled water to remove adhering medium. Subsequently, plantlets were transferred to thermocol cups containing sterilized soilrite. Initially, the plantlets were maintained in the controlled environment for 15 days and then shifted to green house in pots containing sand and soil mixture in 1:2 ratios. After 1 month, the plantlets were transferred to the field.

Data analysis

Each treatment had 20 replicates and all experiments were performed three times. The experimental observations were recorded after an interval of 1 month. All data are expressed as mean value ± standard error (Mean ± SE). The differences

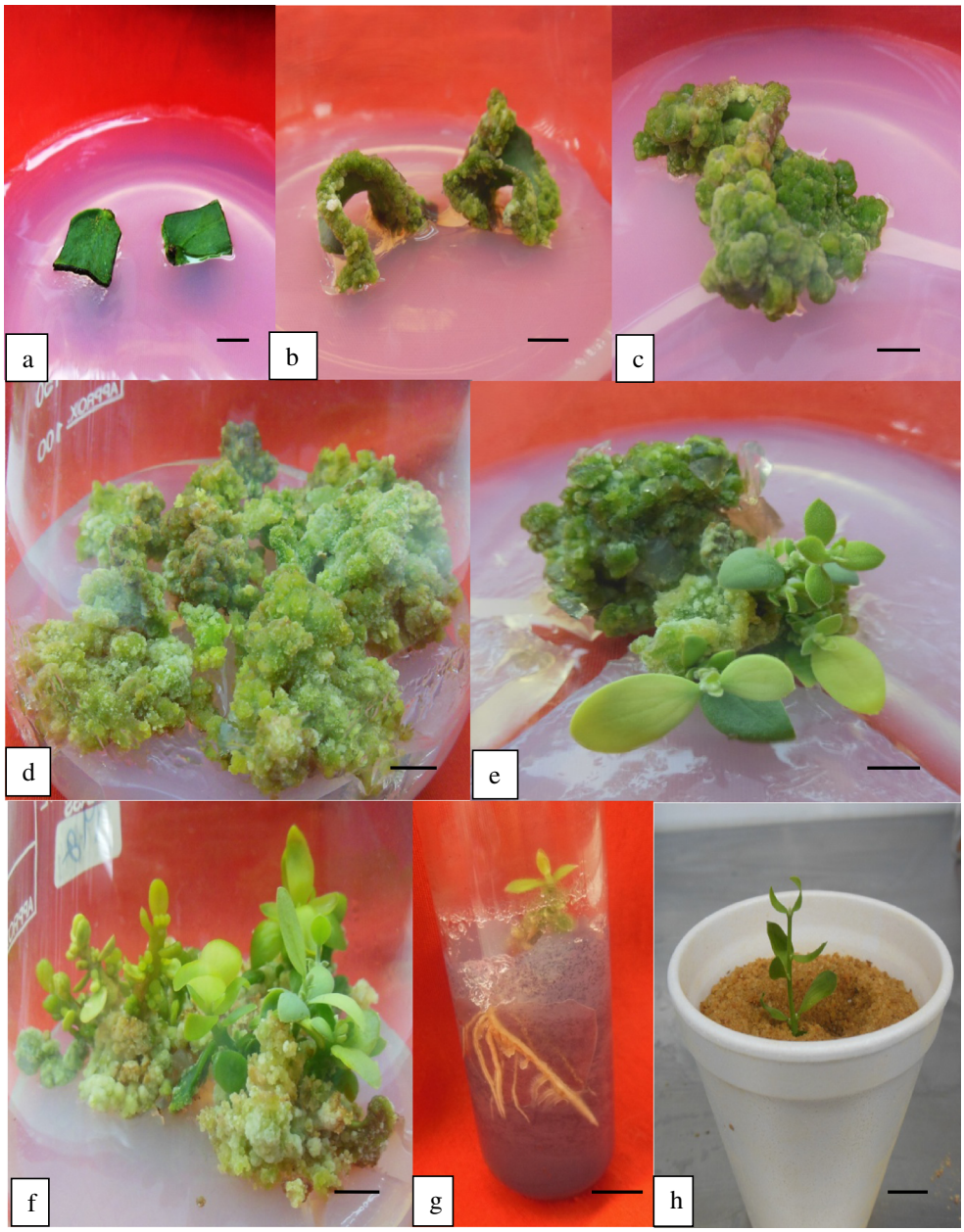

Fig. 1 Plant regeneration through callus induction from female leaf explants of *S. chinensis*. **a** Establishment of leaf explant. **b** Callus initiation on leaf explant. **c** Callus induction on MS + 1.0 mgl^{-1} 2,4-D. **d** Callus proliferation on MS + 1.0 mgl^{-1} 2–4, D + 0.5 mgl^{-1} BAP. **e** Shoot differentiation from callus on MS + 2.0 mgl^{-1} BAP. **f** Multiple shoots induction on MS + 2.0 mgl^{-1} BAP + 0.2 mgl^{-1} NAA. **g** In vitro root induction on half strength MS + 3 mgl^{-1} IBA + 300 mgl^{-1} AC. **h** Hardened plantlet in vermiculite medium. (Scale bar = 1.0 cm)

Table 1 Effect of auxins (2,4-D, IBA) alone and combination with cytokinin (BAP) on induction and proliferation of callus of *S. chinensis*

2,4-D (mgl^{-1})	IBA (mgl^{-1})	BAP (mgl^{-1})	Callus induction (%)	Growth and morphology of callus
0.5	–	–	54.2±0.46^{d*}	Light green, friable callus
1.0	–	–	76.8±1.22^{b}	Light green, soft callus
1.5	–	–	70.2±1.04^{c}	Green yellowish, hard callus
2.0	–	–	68.4±0.36^{c}	Dark green, hard callus
–	0.5	–	32.8±0.14^{d}	White, very hard callus
–	1.0	–	45.5±0.34^{d}	Creamy white, very hard callus
–	1.5	–	52.8±1.21^{d}	Greenish white, hard callus
–	2.0	–	47.4±0.42^{d}	Greenish white, hard callus
1.0	–	0.1	74.2±1.23^{b}	Green white, friable and fast growing callus
1.0	–	0.5	98.7±1.28^{a}	Yellowish green, soft and fast growing callus
1.0	–	1.0	76.5±0.62^{b}	Light green, granular and slow growing callus
1.0	–	1.5	72.3±0.46^{b}	Dark green, friable and slow growing callus

*Mean values within the column followed by the same letters in superscripts are not significantly different according to SNK test at $P<0.05$

among means were determined by Student-Newman-Keuls (SNK) test at 5 % significance level.

Results and discussion

Callus induction and proliferation

Leaf explants from mature female plants of *S. chinensis* were cultured on MS medium supplemented with different concentration of 2,4-D and IBA (0.5, 1.0, 1.5, 2.0 mgl^{-1}) alone for induction of callus (Fig. 1a). After 1 week of incubation, initiation of callus at cut sides from the leaf explants appeared (Fig. 1b). Out of different concentrations of 2,4-D, 1.0 mgl^{-1} concentration of 2,4-D was found optimum for callus induction. On this medium, the percentage of callus induction was 76.8 % and the morphogenic response of observed callus was light green in color with soft texture (Fig. 1c). IBA 1.5 mgl^{-1} concentration produced 52.8 % induction of callus with greenish white and hard texture whereas other concentrations of IBA gave low percentage response of callus induction. Further, results of proliferation of callus with 1.0 mgl^{-1} 2,4-D (optimum concentration) supplemented with different concentrations of BAP is depicted in Table 1. Highest percentage of callus i.e., 98.7 % proliferated on 1.0 mgl^{-1} 2,4-D fortified with 0.5 mgl^{-1} BAP and morphogenic response of this callus was yellowish green and soft texture with fast growing nature

Table 2 Effect of cytokinin (BAP) alone and combination with auxins (NAA, IBA) on shoot differentiation from leaf callus of *S. chinensis*

BAP (mgl^{-1})	NAA (mgl^{-1})	IBA (mgl^{-1})	Shoot number per explant (Mean ± SE)	Shoot height (cm) (Mean ± SE)
1.0	–	–	3.32±0.56^{d*}	1.58±0.24^{d}
2.0	–	–	6.12±1.24^{c}	2.62±0.68^{c}
3.0	–	–	4.28±1.02^{d}	2.14±0.56^{d}
4.0	–	–	2.42±0.39^{d}	2.02±0.22^{d}
2.0	0.1	–	10.24±0.68^{b}	3.06±0.12^{b}
2.0	0.2	–	14.44±1.21^{a}	4.78±0.43^{a}
2.0	0.5	–	10.74±0.46^{b}	3.24±0.32^{c}
2.0	1.0	–	9.26±1.22^{c}	2.68±0.32^{b}
2.0	–	0.1	7.46±0.52^{c}	2.12±0.39^{d}
2.0	–	0.2	10.24±0.24^{b}	2.14±0.23^{d}
2.0	–	0.5	10.72±0.32^{b}	2.68±0.14^{c}
2.0	–	1.0	9.34±0.24^{c}	2.20±0.45^{d}

*Mean values within the column followed by the same letters in superscripts are not significantly different according to SNK test at $P<0.05$

Table 3 Effect of auxin (IBA) combination with activated charcoal (AC) on in vitro rooting of regenerated shoots

IBA (mgl^{-1})	AC (mgl^{-1})	Rooting response (%)	Number of roots per explant (Mean±SE)	Root length (cm) (Mean±SE)
1.0	150	29.8±0.42^{d}	1.02±0.22^{d*}	2.50±1.25^{d}
2.0	150	38.2±1.24^{d}	1.46±0.34^{d}	2.92±0.42^{c}
3.0	150	45.7±1.12^{d}	2.81±1.68^{c}	3.87±0.16^{b}
4.0	150	54.1±0.65^{c}	1.08±1.16^{d}	3.52±1.45^{c}
1.0	300	68.3±1.08^{c}	3.02±1.02^{c}	2.02±1.67^{c}
2.0	300	78.5±1.42^{b}	4.14±0.68^{b}	4.18±1.23^{b}
3.0	300	92.8±1.28^{a}	6.22±0.46^{a}	5.80±0.34^{a}
4.0	300	85.6±1.34^{b}	3.64±1.32^{c}	3.84±0.24^{b}

*Mean values within the column followed by the same letters in superscripts are not significantly different according to SNK test at $P<0.05$

(Fig. 1d). Synthetic auxin 2,4-D was found to be more effective for callus induction; and medium without 2,4-D or combination of 2,4-D with low concentrations of cytokinin was necessary for callus proliferation (Phulwaria and Shekhawat 2013). It appeared that auxins cause DNA to become more methylated than usual and this might be necessary for the reprogramming of differentiated cells and to make them initiate division (George et al. 2008). The combination of 2,4-D with lower concentration of BAP has been found to be effective for the proliferation of callus in *Zoysia matrella* (Chai et al. 2011) and *Desmodium gangeticum* (Cheruvathur et al. 2013).

Shoot differentiation

For shoot differentiation from callus, different concentrations of BAP along with 40 mgl^{-1} adenine sulphate as additive were tested. The optimum concentration of BAP; 2.0 mgl^{-1} produced 6.12 shoots with greater length 2.62 cm (Fig. 1e). Further, shoot multiplication was investigated with 2.0 mgl^{-1} BAP in combination with different concentrations of NAA and IBA (Table 2). Maximum number of shoots 14.44 with their greatest length 4.78 cm was observed at 2.0 mgl^{-1} BAP plus 0.2 mgl^{-1} NAA (Fig. 1f). BAP 2.0 mgl^{-1} fortified with IBA 0.5 mgl^{-1} concentration gave 10.72 shoots having 2.68 cm length. Whereas, 1.0 mgl^{-1} IBA concentration with BAP 2.0 mgl^{-1} produced callus at the base of shoots. The cytokinins alone play a significant role in shoot regeneration and the synergistic combination of auxin and cytokinin promoted shoot regeneration (Thomas and Philip 2005; Thomas and Yoichiro 2010). It is proposed that cytokinins are derivatives of adenine and were required to regulate the synthesis of proteins which are involved for the formation and functioning of mitotic spindle. They are also required for adventitious shoot formation and stimulation of cell division (Chawla 2002; George et al. 2008). The role of BAP alone and the synergetic effect of BAP with NAA for shoot regeneration from callus cultures has been reported in *Echinacea purpurea* (Koroch et al. 2002) and *Cyamopsis tetragonoloba* (Prem et al. 2005).

In vitro rhizogenesis

Half strength MS medium with AC (150 and 300 mg l^{-1}) tested for in vitro root induction. Half strength MS medium containing 2 % sucrose with 3.0 mgl^{-1} IBA and 300 mgl^{-1} AC was found to be best for in vitro rooting (92.8 %). On this medium, maximum numbers of roots (6.22) with greatest length (5.80 cm) were obtained within 1 month of incubation period (Table 3, Fig. 1g). Out of the two concentrations of AC, 300 mgl^{-1} AC was found to be prominent in terms of root number and length. Incorporation of AC in rooting medium resulted in a number of stimulatory and inhibitory activities including the release of substances naturally present in AC which promote the growth and helps in balancing the pH level as well as enhances the nitrogen uptake by shoots and induces in vitro rooting (Thomas 2008). The synergistic effect of AC with IBA was found to play significant role in other plant species like *Cinnamomum camphora* (Nirmal Babu et al. 2003), *Thapsia garganica* (Makunga et al. 2006) and *Jatropha curcas* (Sharma et al. 2011).

Hardening and acclimatization

In vitro rooted shoots were hardened successfully under the control conditions with 90 % survival rates (Fig. 1g). For hardening, plantlets were exposed to green house conditions from high humidity (75–85 % RH) and low temperature (25–27 °C) to low humidity (40–50 % RH) and high temperature (30–35 °C) conditions. The small pots containing hardened plantlets survived (90 %) successfully under field conditions. During the present study 240 hardened plants were acclimatized and transplanted within 4–5 weeks. The acclimatized plants exhibited similar growth and vegetative morphology to the mother plant. There are a few reports where in vitro raised plantlets were acclimatized successfully under control conditions in some plant species *Phyllanthus urinaria* (Catapan et al. 2002), *Jatropha curcas* (Deore and Johnson 2008), *Clitoria ternatea* (Singh and Tiwari 2010) and *Sarcostemma acidum* (Rathore and Shekhawat 2013).

Conclusion

A reproducible and efficient protocol for in vitro regeneration of female *S. chinensis* using leaf explants has been developed. Indirect (callus intermediated) shoot organogenesis achieved from female leaf explants could be useful for establishment and rapid propagation of this multipurpose industrial crop. To our knowledge, this is the first report on indirect in vitro shoot regeneration from leaf explants in *S. chinensis*. The present protocol is highly efficient for large scale production of female plants and minimizing male over female ratio at commercial level.

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