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BRIEF COMMUNICATION

An improved protocol for micropropagation of elite genotypes of *Simmondsia chinensis* (Link) Schneider

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Abstract

An efficient micropropagation protocol was developed for elite male and female genotypes of *Simmondsia chinensis* using nodal segments. Bud initiation was found to be best on Murashige and Skoog's (MS) medium supplemented with 4.44 μM 6-benzylaminopurine (BAP) and 88.8 μM adenine. Upon sub-culture, 10 - 15 shoots per explant were obtained when 4.44 μM BAP and 74.0 μM adenine were incorporated in the medium. Increase in KNO_3 concentration in the medium improved shoot multiplication rate and *in vitro* flowering in 20 % of male cultures. Elongated shoots were harvested, pulse treated for 48 h on liquid medium supplemented with 49.0 μM indole-3-butyric acid, 5.40 μM α-naphthaleneacetic acid and 5.71 μM indole-3-acetic acid for root induction and rooting (92 %) was achieved on hormonal free half-strength MS medium supplemented with 1.37 μM chlorogenic acid, 1 % activated charcoal and 2 % sucrose. After successful hardening, plantlets were transferred to greenhouse with 99 % establishment.

Additional key words: female, flowering, jojoba, male, multiplication, vitrification.

Jojoba (*Simmondsia chinensis*) is a dioecious desert economic plant which has an immense economic potential and can be grown on wasteland/coastal sand dunes. Intense interest has developed on its cultivation due to high percentage of seed oil and potential application of oil in cosmetic, petroleum, pharmaceutical and plastic industries (Jacoboni and Standarti 1987). Propagation of the species is mainly through seeds, due to wind pollination, there is a high degree of variation in seed yield and oil content. In a heterogonous population, identification of male and female plants is not possible until the plant flowers. Vegetative propagation could be the alternative for the generation of desired sex specific clones but it was reported to be difficult (Yermanos 1979). *In vitro* approaches have been proved handy in establishing plants that are genetically uniform and enriched in selected characters (Alcaraz and Ayla-Rocha 1982, Lee *et al.* 1984). The *in vitro* protocols reported in the literature showed low percentage of culture survival (Chaturvedi and Sharma 1989), multiplication rate (Tyagi and Prakash 2004), rooting (Roussos 1999, Tyagi and Prakash 2004) and establishment (Tyagi and Prakash 2004). Genotypic variation affected bud initiation, induction of multiple shoots and rooting (Tyagi and Prakash 2004). From the literature it is evident that there is no single protocol for micropropagation of different genotypes. The aim of this paper was to find a protocol for large scale micropropagation of selected male and female genotypes of jojoba.

Depending upon seed yield, 5 female and 1 male genotype of *Simmondsia chinensis* (Link) Schneider were selected from the plantation at CSMCRI plantation, at Zanjmer, Gujarat. Actively growing shoots (5 - 10 internodes) were collected early in the morning. After removing the leaves, nodal segments were dissected (2.0 - 3.0 cm), surface sterilized in 0.1 % mercury chloride for 15 min and rinsed in sterile distilled water thrice. Explants were inoculated in to 25 × 150 mm culture tubes containing 10 cm^3 of culture medium. Hormones used in different concentrations and

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Abbreviations: BAP - 6-benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog's medium; NAA - α-naphthaleneacetic acid.

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combination were incorporated into the medium before autoclaving. The pH of medium was adjusted to 5.8 before adding 0.62 % agar (*Qualigens*, Mumbai, India) and sterilized at 121 °C for 20 min. Firstly, the nodal segments were inoculated on Murashige and Skoog (1962; MS) medium fortified with 4.44 µM 6-benzyl-aminopurine (BAP) and 88.8 µM adenine. Cultures were grown at 25 ± 1 °C under 16-h photoperiod. Irradiance of 50µmol m^{-2} s^{-1} was provided by cool white florescent tubes. Cultures were generated every month to observe seasonal variation in establishing sterile cultures and their *in vitro* response. After 30 - 40 d of inoculation, explants were sub-cultured on modified MS medium supplemented with 19.30 mM KNO_3, 4.44 µM BAP and 70.0 µM adenine. Elongated shoots (7.0 cm or with 2 - 3 internodes), were harvested by cutting below the base of a node and used for rooting. Root induction was initiated through pulse treatment in test tubes containing 10 cm^3 of half-strength MS liquid medium fortified with 49.0 - 73.5 µM indole-3-butyric acid, 5.40 - 10.80 µM α-naphthaleneacetic acid (NAA) and 5.71 - 11.42 µM indole-3-acetic (IAA) alone or in combinations for 48 h. After pulse treatment the shoots were transferred to half-strength hormones free MS medium, supplemented with 1.37 µM chlorogenic acid, 0.1 % activated charcoal and 2 % sucrose. Medium lacking additives served as control. Basal mass remained after harvesting the shoots for rooting were sub-cultured onto a fresh shoot proliferation medium for induction of multiple shoots.

Rooted plantlets were transferred to culture bottles filled with sterile sand or *Soilrite* or sand + *Soilrite* (50:50) and moistened with half-strength MS liquid medium. The bottles were covered with transparent polyethylene bags and allowed to harden for 15 d under conditions described for *in vitro* culture. Hardened plantlets were subsequently transferred to greenhouse with ambient humidity and temperature, by gradually increasing the aperture size of the plastic bag until the bag could be removed entirely. Rooted plantlets in the greenhouse were subjected to different temperature (25 - 40 °C) and relative humidity (45 - 80 %). At least 200 explants per treatment were used at every stage. The experiments were conducted for consecutive five years. All the results were statistically analyzed using *SPSS* version *7.5*.

Infection free cultures (90 - 99 %) could be established, when the explants were collected from the field during October to May, whereas only 20 to 50 % cultures were infection free when collected during June to October (rainy season). In the present protocol sterile cultures could be established by a more simple method than those reported in the literature (Roussos *et al.* 1999, Chaturvedi and Sharma 1989, Tyagi and Prakash 2004). Survival rate of primary cultures reported in the literature was quite low (Chaturvedi and Sharma 1989) as compared to 99 % reported in the present paper. Bud initiation was observed on the 7th day, when MS medium was supplemented with 4.44 µM BAP and 88.8 µM adenine, which was 7 times earlier than reported previously (Chaturvedi and Sharma 1989, Tyagi and Prakash 2004) and the response was independent of season. The early bud initiation in the present studies may be due to the differences in the concentration and combination of hormones and nutrients incorporated in to the medium. After 30 - 40 d of inoculation explants were sub-cultured on MS medium supplemented with 4.44 µM BAP and 74.0 µM adenine, which was less than half of the time reported for sub-culture by Tyagi and Prakash (2004). In the 1st sub culture, 5 -8 shoots per explant, in subsequent second and third sub-cultures, 8 - 12 and 10 - 15 shoots were obtained. Increase in the concentration of KNO_3 to 19.30 mM in culture medium increased number of shoots to 15 - 20 (Fig. 1) and induced flowering in 20 % of male cultures (Fig. 2) which indicated the requirement of more potassium and nitrate for increasing the multiple shoots. In repeated sub-cultures also 15 - 20 shoots per culture were obtained. Though Llorente and Apostolo (1998) reported sub-culture after 30 d, the cultures were vitrified and callus formed in the base. In the present protocol only in few occasions vitrification was observed (0.5 - 0.7 %) and the same could be overcome by growing the vitrified shoots on half-strength hormone free MS medium supplemented with 0.1 % activated charcoal for 10 d. The number of shoots (15 - 20) obtained with the present protocol was significantly higher than reported in the literature (Llorente and Apostolo 1998, Chaturvedi and Sharma 1989, Mills *et al*. 1997, Driver and Kuniyuki 1984).

Elongated shoots were pulse treated for 48-h in 73.5 µM IBA and then transferred to hormones free medium, root initiation was observed after 25 d, roots appeared in clusters more like callus and did not elongate even after 30 - 45 d. Since IBA alone was found insufficient for rooting, IAA and NAA were also incorporated into the medium, but in *Harpagophytum procumbens* IBA alone was found to be best for rooting (Kaliamoorthy *et al*. 2008). Pulse treatment in 49.0 µM IBA, 5.40 µM NAA and 5.71 µM IAA for 48 h and

Table 1. Effect of IBA, NAA and IAA on root induction of *Simmondsia chinensis.* Mean ± SD of 5 independent experiments.

Hormones [µM]	Root initiation [d]	Type of roots	Number of roots	Root length [cm]
IBA-73.5	20 ± 0.23	clump	38 ± 0.61	0.5 ± 0.11
IBA-73.5 + IAA-5.71	25 ± 0.26	thick and short	15 ± 0.69	0.9 ± 0.21
IBA-49.0 + NAA-5.40 + IAA-5.71	10 ± 0.56	desirable	7 ± 0.69	5.0 ± 1.01
IBA-49.0 + NAA-5.40 + IAA-11.42	15 ± 0.95	long brittle	7 ± 0.98	1.2 ± 0.31

Figs. 1 - 4. Micropropagation of *Simmondsia chinensis*: *1* - multiple shoots, *2* - flowering in male culture, *3* - rooted shoot, *4* - hardened plants in the nursery.

subsequent transfer to hormone free half-strength medium resulted in higher percentage of rooting (92 %) within 30 d (Table 1, Fig. 3), as compared to earlier reports (Chaturvedi and Sharma 1989, Tyagi and Prakash 2004). Only 24 - 32 % rooting with IAA pulse treatment and 50 - 60 % rooting with IAA and IBA combinations were reported in the same species (Miller *et al.* (1997). NAA increased root length in soft wood cutting of apple and plum (Peare 1938) According to Chaturvedi and Sharma (1989) combination of IBA and NAA were more effective in inducing rhizogenesis than either of alone. Chaturvedi and Sharma (1989) reported rooting through 3 step protocol and we could achieve in 2 steps only. It is an established fact that although auxins are essential for root induction, but they are not required for root growth, their continued presence may even inhibit the root growth (Mills *et al.* 1997, Torrey 1976, Babic and Nescovic 1984, Collet 1988). Therefore, after pulse treatment for 48-h shoots were subsequently placed on hormone free medium for rooting. Additional advantage of this protocol was repeated use (3 - 4 times) of liquid medium for root induction without compromising the results. 80 % humidity was found to be effective for hardening. Similar results were reported for other plants (Rao and Purohit 2006). Rooted plantlets were transferred to culture bottles and hardened as described in materials and methods. 15 d after hardening plantlets were subjected to different temperature and humidity regimes and temperature of 25 - 30 °C and relative humidity 80 % were found to be ideal for plant establishment (99 %). Minimum survival (2 %) was observed when subjected to 45 - 50 °C and 40 % humidity. Plantlets transferred from

Table 2. Effect of genotype on micropropagation of *Simmondsia chinensis*: comparative data of three stages (bud initiation within 20 days, rooting and survival of plantlet in greenhouse within 30 d). Mean ± SD of 5 independent experiments.

Year	Genotype	Bud initiation [%]	Rooting [%]	Survival [%]
2001	CSMCRI 1-1	99 ± 0.11	92 ± 0.13	99 ± 0.96
	CSMCRI 12-8	99 ± 0.13	92 ± 0.15	99 ± 0.86
	CSMCRI 10-4	99 ± 0.12	92 ± 0.11	99 ± 0.56
	CSMCRI 20-3	99 ± 0.11	92 ± 0.12	99 ± 0.26
	male	98 ± 0.13	92 ± 0.15	99 ± 0.96
	Israel	99 ± 0.17	92 ± 0.19	99 ± 0.99
2002	CSMCRI 1-1	98 ± 0.12	92 ± 0.14	99 ± 0.92
	CSMCRI 12-8	99 ± 0.11	92 ± 0.12	99 ± 0.62
	CSMCRI 10-4	98 ± 0.12	92 ± 0.12	99 ± 0.76
	CSMCRI 20-3	99 ± 0.11	92 ± 0.14	99 ± 0.38
	male	98 ± 0.12	92 ± 0.12	99 ± 0.69
	Israel	98 ± 0.19	92 ± 0.19	99 ± 0.16
2003	CSMCRI 1-1	99 ± 0.11	92 ± 0.12	99 ± 0.14
	CSMCRI 12-8	98 ± 0.11	92 ± 0.12	99 ± 0.11
	CSMCRI 10-4	99 ± 0.16	92 ± 0.12	99 ± 0.13
	CSMCRI 20-3	98 ± 0.14	92 ± 0.15	99 ± 0.15
	male	98 ± 0.11	92 ± 0.12	99 ± 0.11
	Israel	99 ± 0.12	92 ± 0.13	99 ± 0.13
2004	CSMCRI 1-1	98 ± 0.11	92 ± 0.16	99 ± 0.99
	CSMCRI 12-8	99 ± 0.13	92 ± 0.18	99 ± 0.66
	CSMCRI 10-4	99 ± 0.16	92 ± 0.13	99 ± 0.73
	CSMCRI 20-3	98 ± 0.14	92 ± 0.19	99 ± 0.34
	male	99 ± 0.16	92 ± 0.11	99 ± 0.61
	Israel	98 ± 0.19	92 ± 0.13	99 ± 0.69
2005	CSMCRI 1-1	99 ± 0.16	92 ± 0.11	99 ± 0.92
	CSMCRI 12-8	98 ± 0.11	92 ± 0.11	99 ± 0.62
	CSMCRI 10-4	99 ± 0.18	92 ± 0.14	99 ± 0.76
	CSMCRI 20-3	98 ± 0.14	92 ± 0.14	99 ± 0.38
	male	99 ± 0.11	92 ± 0.11	99 ± 0.69
	Israel	98 ± 0.16	92 ± 0.12	99 ± 0.53

culture bottles filled with sand established better as compared to *Soilrite* alone and *Soilrite* + sand (Fig. 4). The possible reason may be that as *Soilrite* has too soft texture which does not enable good aeration. No significant difference in bud initiation, rooting and survival in greenhouse was observed in all the selected genotypes (5 female and 1 male) studied (Table 2). The experiments were repeated for all genotype for 5 years, passed through 30 - 40 sub-cultures and found no significant difference in their response. Tyagi and Prakash (2004) reported differences in rooting behavior in male, female plants and female genotypes were more responsive compared to male genotypes studied.

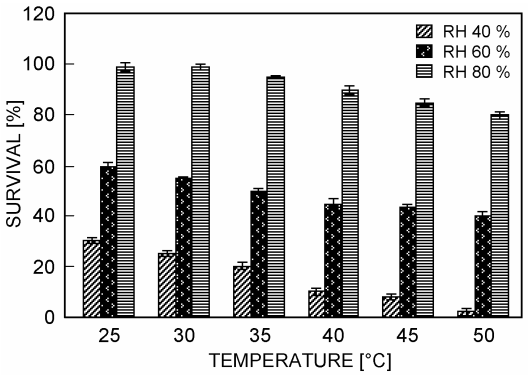

Fig. 5. Effect of temperature and humidity on survival of *Simmondsia chinensis* plantlets. Mean ± SD of 5 independent experiments.

This is the first report on micropropagation of selected high yielding 20 years old genotypes of jojoba with 99 % infection free cultures, reuse of vitrified shoots and 99 % survival during hardening and in nursery. Male, female explants and different genotypes responded similarly, hence this protocol will be useful for production of large number of elite male and female genotypes in a short time.

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