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Clonal Multiplication of *Syzygium alternifolium* (WIGHT.) WALP., Through Mature Nodal Segments

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Abstract

Clonal multiplication of *Syzygium alternifolium* (WIGHT.) WALP., (Myrtaceae), a rare medicinal tree was achieved through mature nodal segments on modified MURASHIGE and SKOOG's (MS) medium. Shoot initiation was dependent on cytokinin supply, but the synergistic combination of 4.0 mg/l N^6 benzyladenine and 0.5 mg/l 1-naphthaleneacetic acid induced the highest percentage of nodal segments sprouting (69.5 ± 2.9%), number of shoots per node (3.9 ± 0.1), shoot length (2.9 ± 0.2 cm), the number of new nodal segments generated per active explant (2.2 ± 0.1) and the multiplication co-efficient (1.5) within 6 weeks. Explants harvest period influenced the shoot initiation in nodal segments. Repeated subculturing through five passages of nodes of shoot cultures enabled continuous production of healthy shoots. At the end of the 5th passage, 72% to 73% of nodal segments produced 8 to 9 shoots, each having 4 cm height and 3 to 4 nodes. Multiplication co-efficient was also increased from the 1st subculture (1.5) to the 5th subculture (2.3). Rooting involved a two step method: root initiation on solidified half-strength MS medium supplemented with 1.0 mg/l indole-3-butyric acid (IBA), and root elongation following transfer to half-strength MS medium devoid of growth regulators. Repeating this two step sequence yielded up to 70% of rooted shoots. Shoots derived from subcultures exhibited better rooting response than those from cultures of shoot initiation. About 70% of the rooted plants were established in 20 cm diameter pots containing a mixture (1:1) of soil and sand after 3 weeks of hardening.

Key words: in vitro, medicinal plant, micropropagation, Myrtaceae, nodal segments, *Syzygium alternifolium.*

FDC: 165.441; 161.4; 232.328.1; 176.1 *Syzygium alternifolium;* (540).

Abbreviations: BA: N^6 benzyladenine; KN: kinetin; IAA: indole-3-acetic acid; NAA: 1-naphthaleneacetic acid; MS: MURASHIGE and SKOOG's medium (1962); IBA: indole-3-butyric acid; PAR: Photosynthetically Active Radiation; RI: root initiation; RE: root elongation.

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Introduction

Syzygium alternifolium (WIGHT.) WALP., (Myrtaceae), popularly known as 'Mogi', is a rare fruit tree of great medicinal and economical importance. It's distribution is restricted to certain forested areas in the Southern part of India (MADHAVACHETTY and RAO, 1990). This species yields nutritious, edible fruits and durable timber. The ripe fruits are also used in making squashes and jellies. Timber is used for making furniture and agricultural implements (The Wealth of India, 1976). Blossoms yield honey and possess antibiotic properties. An alcoholic extract of seeds posses antidiabetic activity and tender shoots have been traditionally used by villagers and tribal folks to treat bacillary dysentery (RAJA REDDY *et al.,* 1989; NAGARAJU and RAO, 1990).

S. alternifolium is out breeder and routinely propagated by seeds. As seed progenies are not uniform, no standard varieties are available. Seed production is sporadic, the seeds cannot be stored for long periods due to short viability and insect attack. Vegetative propagation has not been successful. To date only one report has been published on micropropagation of *S. alternifolium* using seed-derived explants (SHA VALLI KHAN *et al.,* 1997). Considerable progress has been made for the *in vitro* micropropagation of other species of the genus *Syzygium* like *S. aromaticum* (MATHEW and HARIHARAN, 1990) and *S.cuminii* (YADAV et al., 1990) using seed-derived explants. The use of mature explants was fraught with difficulties like dark-brown phenolic-exudation and severe contamination (YADAV *et al.,* 1990). The present study was undertaken to establish a method for clonal multiplication of *S. alternifolium* using mature nodal segments.

Materials and Methods

Plant material

A 10-year-old tree *(S. alternifolium)* with a large trunk (~ 40 cm diameter), well-spread canopy and regular flowering and fruiting characteristics, growing in the protected forest area of Tirumala hills of Chittoor district, India served as the source for explants. Stem cuttings with the youngest four to

five leaves were collected from the crown of the tree during November to January, wrapped in wet white cloth and brought to the laboratory. After leaf excision, the cuttings were washed in 1.0% (v/v) teepol detergent for 15 min and in running tap water for 30 min. They were then immersed in 1.0% (v/v) Cetavelon® (an antiseptic plus detergent, Alakali and Chemical group, India) for 15 min, and surface-sterilized by passage through 70% (v/v) ethanol for 2 min and 0.05% (w/v) aqueous mercuric chloride with 0.1% of Tween-20 for 7 min. After 3 washes of 5 min each in sterile distilled water, nodal segments (1.0 cm to 1.5 cm) bearing a pair of axillary meristems were aseptically dissected, and incubated in a solution containing 500 mg/l ascorbic acid for 3 h to check phenolic-exudation. Finally, the cut edges of nodal segments were coated with paraffin wax and then implanted vertically in nutrient medium for the shoot initiation.

Shoot initiation

Three sets of experiments were carried out concerning the shoot initiation. In the first set, different concentrations (1.0 mg/l to 5.0 mg/l) of cytokinins like N^6-benzyladenine (BA) or kinetin (KN) were incorporated into modified MS medium to determine shoot forming ability. In the second set of experiments, the effect of BA (4.0 mg/l) in combination with different concentrations (0.1 mg/l to 1.0 mg/l) of auxins like indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) was tested on shoot forming potential. In the third set of experiments, influence of the explants harvest period (November to January, February to April, May to July and August to October) was studied on the shoot initiation.

Shoot multiplication

Multiple shoots emanating from the nodes in 6 weeks of shoot initiation were dissected into nodal segments (1.0 cm to 1.5 cm) and recultured for 6 weeks on nutrient medium containing 4.0 mg/l BA and 0.5 mg/l NAA. This process was repeated for 5 transfers (each 6 weeks) to increase the multiplication of propagules and to study the effect of subculturing.

Rooting

Shoots (2 cm or longer) harvested from the cultures of shoot initiation (0) as well as from the 1st to the 5th subcultures of shoot multiplication were used for rooting. Rooting involved a two step procedure: root initiation (RI) and root elongation (RE). Root initiation (RI) was carried out on a solidified half-strength MS medium containing 1.0 mg/l indole-3-butyric acid (IBA) for 3 weeks and root elongation (RE) took place after transfer to half-strength MS medium devoid of auxin for another 3 weeks.

Culture conditions

The nutrient medium consisted of half-strength Murashige and Skoog (MS) (1962) mineral salts, B_5 vitamins (Gamborg *et al.,* 1968), 2 mg/l glycine, 250 mg/l ascorbic acid, 500 mg/l activated charcoal, 2% (w/v) sucrose and 0.8% (w/v) agar (Qualigens, India) used for shoot initiation as well as shoot multiplication. The MS medium at half-strength was used for rooting experiments. The pH of the medium was adjusted to 5.7 before adding agar. The medium was dispensed in 20 ml aliquots into 25 mm x 150 mm culture tubes and autoclaved at 120 °C and 1.06 kg cm^{-2} pressure for 20 min. All the cultures were incubated in a culture room at a temperature of 25° C ± 2 °C with 55% relative humidity under 16 h light /8 h dark photoperiod with irradiance of 50 µ $Em^{-2}sec^{-1}$ photosynthetically active radiation (PAR) provided by cool-white fluorescent tubes in combination with incandescent bulbs.

Hardening

Rooted shoots were removed from the culture tubes, washed well to remove remnants of agar from roots and transplanted in 10 cm diameter plastic pots containing autoclaved vermiculite. Plants were then covered with polythene bags to ensure high humidity and kept in the culture room at a temperature of 25 °C ± 2 °C and 16 h light / 8 h dark photoperiod under 100 µmol m^{-2} sec^{-1} PAR provided by cool-white fluorescent lamps and incandescent bulbs. Plants were well irrigated with half-strength MS salt solution and hardened for 3 weeks. Afterwards the hardened plants were repotted in 20 cm diameter plastic pots containing a mixture (1:1) of soil and sand, and kept in the field for developing into mature plants.

Data collection and statistical analysis

During shoot initiation as well as shoot multiplication experiments, observations on percentage of nodal segments sprouting, number of shoots per nodal segment, shoot length, the number of new nodal segments generated per active explant and the multiplication co-efficient were recorded after 6 weeks. The multiplication coefficient is defined as the proportion of explants forming axillary shoots x the mean number of new nodal segments per explant forming shoots. During rooting, data collected were percentage of rooting, number of roots per shoot and root length after 3 weeks of RI. Twelve explants were cultured per treatment and all experiments were repeated three times. The effects of different treatments were quantified and the data subjected to statistical analysis using 'standard error of the mean'.

Results and Discussion

Shoot initiation

Under the conditions employed, the mature nodal segments of *S. alternifolium* were free from contamination and phenolic-exudation. The pre-existing axillary meristems of nodal segments induced to proliferate into 2 to 3 shoot buds after 2 weeks of inoculation on modified MS medium supplemented with either BA or KN. However, the percentage of nodal segments sprouting, mean number of shoots per nodal segment, shoot length, the number of new nodal segments generated per active explant and the multiplication co-efficient varied with the type and concentration of cytokinin *(Table 1)*. Increasing the concentration of cytokinins (BA or KN) from 1.0 mg/l to 4.0 mg/l enhanced the response of shoot initiation. Higher concentration of cytokinins (5.0 mg/l) decreased the overall pattern of shoot initiation. Shoots produced on media containing a higher concentration of cytokinins also appeared glassy, compacted with short nodes and small leaves.

BA was found to be more effective than KN for the shoot formation from mature nodal segments *(Table 1)*. KN produced an average of 2 shoots per node in 47.2% ± 2.2% to 63.8% ± 2.7% explants with maximum 1.6 cm shoot length. Whereas BA stimulated sprouting in 61.0% ± 2.7% to 69.4% ± 2.8% of explants with an average of 3 to 4 shoots, each having 1.5 cm to 2.5 cm length. The number of new nodal segments generated per active explant and the multiplication co-efficient were also better on BA as compared to KN *(Table 1).* The superiority of BA over KN has been well demonstrated in tissue cultures of Myrtaceae for the induction of adventitious shoot induction (Speer, 1993; List *et al.,* 1996). Of all the concentrations of BA tested, the best values were obtained using 4.0 mg/l BA within 6 weeks.

A synergistic influence was evident, when combination of 4.0 mg/l BA and selected concentrations of auxins (IAA or NAA)

Table 1. – Effect of cytokinins on shoot initiation from mature nodal segments of *S. alternifolium* (after 6 weeks of culture).

Cytokinins (mg/l)	% Nodal segments sprouting ± S.E.[a]	Mean number of shoots ± S.E.[a]	Mean shoot length (cm) ± S.E.[a]	Mean number of nodal segments ± S.E.[a]	Multiplication co-efficient
0.0[b]	0.0	0.0	0.0	0.0	0.0
BA					
1.0	61.0 ± 2.7	2.7 ± 0.1	1.6 ± 0.1	1.0 ± 0.1	0.6
2.0	61.0 ± 5.5	2.3 ± 0.2	1.8 ± 0.1	1.1 ± 0.1	0.7
3.0	63.8 ± 5.5	2.8 ± 0.1	2.0 ± 0.1	1.3 ± 0.1	0.8
4.0	69.4 ± 2.8	3.8 ± 0.1	2.3 ± 0.2	1.7 ± 0.1	1.1
5.0	63.8 ± 2.7	3.5 ± 0.1	2.0 ± 0.1	1.4 ± 0.1	0.8
KN					
1.0	47.2 ± 2.2	1.6 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.4
2.0	55.5 ± 2.7	1.6 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	0.5
3.0	55.5 ± 2.7	1.7 ± 0.1	1.6 ± 0.1	1.0 ± 0.1	0.6
4.0	63.8 ± 2.7	1.8 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	0.6
5.0	61.2 ± 2.6	1.8 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	0.6

[a]) = Mean ± S.E. for three replications (12 cultures for each replication)
[b]) = Medium lacking cytokinins served as control

Table 2. – Effect of 4.0 mg/l BA and auxins on shoot initiation from mature nodal segments of *S. alternifolium* (after 6 weeks of culture).

4.0 mg/l BA + auxins (mg/l)	% Nodal segments sprouting ± S.E.[a]	Mean number of shoots ± S.E.[a]	Mean shoot length (cm) ± S.E.[a]	Mean number of nodal segments ± S.E.[a]	Multiplication co-efficient
IAA					
0.1	69.4 ± 7.3	3.8 ± 0.1	2.3 ± 0.1	1.9 ± 0.1	1.3
0.5	69.5 ± 2.9	3.9 ± 0.1	2.5 ± 0.1	2.0 ± 0.1	1.3
1.0	61.0 ± 2.7	3.3 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	0.7
NAA					
0.1	69.4 ± 7.3	3.9 ± 0.2	2.7 ± 0.1	2.1 ± 0.1	1.5
0.5	69.5 ± 2.9	3.9 ± 0.1	2.9 ± 0.2	2.2 ± 0.1	1.5
1.0	63.8 ± 2.2	3.4 ± 0.2	2.5 ± 0.1	1.8 ± 0.1	1.1

[a]) = Mean ± S.E. for three replications (12 cultures for each replication)

were tested *(Table 2)*. Addition of lower concentrations (0.1 mg/l or 0.5 mg/l) of auxins (IAA or NAA) to the medium containing 4.0 mg/l BA enhanced shoot elongation as well as the multiplication co-efficient. A low concentration of auxins can promote growth of axillary shoots by counteracting the inhibitory effects of high cytokinin concentrations on shoot elongation (NEHRA and KARTHA, 1994). Shoot formation was slightly reduced in the presence of 4.0 mg/l BA and 1.0 mg/l of auxins (IAA or NAA). The optimum growth regulator combination for shoot formation from nodal segments was found to be 4.0 mg/l BA and 0.5 mg/l NAA, where the highest percentage of nodal segments sprouting (69.5% ± 2.9%), number of shoots per node (3.9 ±0.1), shoot length (2.9 cm ± 0.2 cm), the number of new nodal segments generated per active explant (2.2 ± 0.1) and the multiplication co-efficient (1.5) were achieved within 6 weeks *(Table 2)*.

Table 3. – Effect of the explant harvest period on shoot initiation from mature nodal segments of *S. alternifolium* (after subculture period of 6 weeks).

Harvest period (months)	% Nodal segments sprouting ± S.E.[a]	Mean number of shoots ± S.E.[a]	Mean shoot length (cm) ± S.E.[a]	Mean number of nodal segments ± S.E.[a]	Multiplication co-efficient
Nov.-Jan.	69.5 ± 2.9	3.9 ± 0.1	2.9 ± 0.2	2.2 ± 0.2	1.5
Feb.-Apr.	61.0 ± 2.7	3.4 ± 0.2	2.4 ± 0.1	1.7 ± 0.2	1.0
May-July	55.6 ± 2.6	3.2 ± 0.1	2.0 ± 0.1	1.4 ± 0.2	0.7
Aug.-Oct.	58.3 ± 4.7	3.3 ± 0.2	2.3 ± 0.2	1.2 ± 0.2	0.7

[a]) = Mean ± S.E. for three replications (12 cultures for each replication)

Table 4. – Effect of the subculture on shoot multiplication of *S. alternifolium* (after 6 weeks of root initiation).

Subculture	% Nodal segments sprouting ± S.E.[a]	Mean number of shoots ± S.E.[a]	Mean shoot length (cm) ± S.E.[a]	Mean number of nodal segments ± S.E.[a]	Multiplication co-efficient
1 st	69.4 ± 2.8	4.6 ± 0.1	3.1 ± 0.1	2.3 ± 0.1	1.5
2 nd	72.1 ± 5.5	5.3 ± 0.1	3.5 ± 0.1	2.7 ± 0.1	1.9
3 rd	72.2 ± 2.8	6.5 ± 0.1	3.6 ± 0.1	2.8 ± 0.1	2.0
4 th	72.2 ±2.8	8.0 ± 0.4	3.9 ± 0.1	3.2 ± 0.1	2.3
5 th	72.2 ± 2.8	8.1 ± 0.3	4.1 ± 0.1	3.3 ± 0.1	2.3

[a]) = Mean ± S.E. for three replications (12 cultures for each replication)

Figure 1. – Clump of proliferating shoots obtained in the 5th subculture on modified MS with 4.0 mg/l BA and 0.5 mg/l NAA.

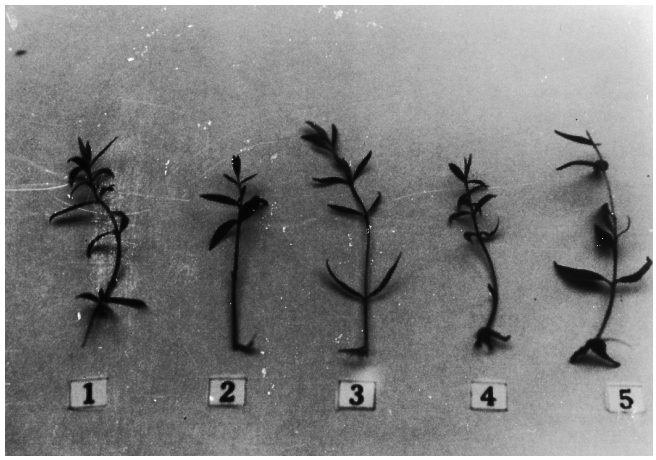

Figure 2. – Root formation in shoots harvested from the 1st to the 5th subcultures of shoot multiplication on ½ MS with 1.0 mg/l IBA after 6 weeks.

Table 5. – Effect of the subculture on rooting of *in vitro* formed shoots of *S. alternifolium* (after 3 weeks of transfer).

Subculture	Rooting (%) ± S.E. [a]	Mean number of roots per shoot ± S.E.[a]	Mean root length (cm) ± S.E.[a]
0.0[b]	0.0	0.0	0.0
1 st	13.6 ± 2.6	1.6 ± 0.3	0.8 ± 0.1
2 nd	16.6 ± 4.8	1.6 ± 0.3	1.1 ± 0.1
3 rd	44.4 ± 2.8	3.6 ± 0.1	1.9 ± 0.1
4 th	66.6 ± 4.8	3.6 ± 0.1	1.9 ± 0.1
5 th	69.4 ± 2.8	3.9 ± 0.1	2.0 ± 0.1

[a]) = Mean ± S.E. for three replications (12 cultures for each replication)
[b]) = culture of shoot initiation

Nodal segments cultured during November to January period showed the best response of shoot initiation as compared to other periods of explants harvest *(Table 3)*. The greater shooting response of nodal segments during this period could be attributed to the active vegetative growth phase of *S. alternifolium* trees in this period, a finding similar to that of *Psidium guajava* (AMIN and JAISWAL, 1987). Another advantage of initiating cultures during November to January months are the availability of a large number of nodal segments.

Shoot multiplication

Repeated subculturing of 5 passages of nodal segments from shoot cultures helped to achieve continuous production of healthy shoots *(Table 4)*. A similar phenomenon was also observed in *Aegle marmelos* (AJITKUMAR and SEENI, 1998). At the end of the 5th passage of shoot multiplication, 72% to 73% of nodal buds produced 8 to 9 healthy, green shoots, each having an average of 4 cm length and 3 to 4 nodes on modified MS medium supplemented with 4.0 mg/l BA and 0.5 mg/l NAA *(Fig. 1)*. Multiplication co-efficient was also increased from the 1st subculture (1.5) to the 5th subculture (2.3). Shoot cultures have been maintained in our laboratory for the past 2 years without loss of vigor or change in the multiplication-coefficient. Stabilization of shoot cultures has taken a period of years in *Eucalyptus* spp (MCCOMB and BENNETT, 1986), while it was achieved after a period of 6 months in *S. alternifolium*.

Rooting

Shoots taken from the cultures of shoot initiation failed to form roots on half-strength MS medium containing 1.0 mg/l IBA. In contrast, shoots collected from the 1st to the 5th subcultures of shoot multiplication rooted following a first treatment with 1.0 mg/l IBA for root initiation (RI), and a second one without auxin for root elongation (RE). A two step method for rooting also demonstrated in other members of Myrtaceae (AMIN and JAISWAL, 1987; YADAV *et al.*, 1990; BLOOMSTEAD *et al.,* 1991). However, the rooting response varied with the subculture of shoot multiplication *(Table 5)*. The percentage of shoots rooted, number of roots per shoot and root length gradually increased form the 1st passage to the 5th passage of shoot multiplication. About 70% of shoots harvested from the 5th subculture of shoot multiplication produced 3 to 4 white thick roots, each having 2 cm length within 3 weeks of transfer *(Fig. 2)*. Similar results were also reported in *Psidium guajava* (AMIN and JAISWAL, 1988) and *Artocarpus heterophyllus* (AMIN and JAISWAL, 1993). Repeated subculturing may change the physiological state and gradually rejuvenate the shoot, which in turn promotes better rooting (ECNOMOU and READ, 1986).

Hardening

Direct transfer of plantlets from culture tubes to pots showed a high rate of mortality. Plantlets transferred from culture tubes to 10 cm plastic pots with vermiculite covered

Figure 3. – Three months old micropropagated plant established in mixture (1:1) of soil and sand.

with polyethylene bags benefited by high humidity and high PAR (100 µmol m^{-2} sec^{-1}) during 3 weeks hardening and showed about 70% survival (97 out of 140). Hardened plants produced new leaves and an adequate root system for further establishment in soil. High PAR hastened the hardening process by accelerating the development of new leaves and the photosynthetic ability of rooted shoots (GROUT and MILLAM, 1985). All the hardened plants, so far, transplanted into 20 cm pots containing a mixture (1:1) of soil and sand were successfully established and showed apparently uniform growth and true-to-type morphology *(Fig. 3)*.

To the best of our knowledge, no data on micropropagation of *S. alternifolium* using mature nodal segments were published, the results presented in this paper represent the first report on clonal multiplication procedure for this rare Indian medicinal tree. The *in vitro* technique has the advantage of establishing long-term proliferating shoot cultures from the 10-year-old experimental tree with a high multiplication co-efficient (2.3 at 6 week interval). Therefore, it offers a high potential for mass propagation and conservation of this species. It seems likely that this protocol, possibly with modifications, can be used for clonal multiplication of other species of the genus *Syzygium* using mature nodal segments.

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Short Note: Identification of Natural Hybrids *Juglans x intermedia* CARR. Using Isoenzyme Gene Markers

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Summary

Isoenzyme polymorphism in bud tissues from *Juglans nigra* L., *Juglans regia* L., and their natural hybrids *Juglans x intermedia* CARR. was analysed. The results reveal that isoenzyme variants of the enzyme systems Aspartat aminotransferase (AAT) and Phosphoglucomutase (PGM) are suitable to identify hybrids of known as well as unknown origin genetically. The use of this method is suggested for forest nursery practice to distinguish hybrids at an early ontogenetic stage.

Key words: Juglans regia, Juglans nigra, Juglans x intermedia, isoenzymes, hybrids, varietal test.

FDC: 165.71; 165.3; 176.1 *Juglans nigra;* 176.1 *Juglans regia;* 176.1 *Juglans intermedia.*

Introduction

Juglans x intermedia CARR. is a natural hybrid between *Juglans nigra* L. and *Juglans regia* L.. When compared to the parent species most of hybrids show an increased vegetative vigour, distinct disease resistance characteristics and good wood quality (SCHEEDER, 1990; SAUTER *et al.,* 1994). Especially in southwest Germany, there is a great demand in plants of *J. x intermedia* for forest utilisation. However, because of temporal separation between flower maturity of both parent species depending mostly on weather conditions and apparent incompatibility mechanism between some individuals of each species (SARTORIUS, 1990), controlled crosses remained yet unsuccessfully (SCHEEDER, 1990). Thus, the production of