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ORIGINAL ARTICLE

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Factors influencing in vivo and in vitro micrografting of sandalwood (*Santalum album* L.): an endangered tree species

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Abstract In vivo micrografting of *Santalum album* was achieved (50%) by grafting 4- to 5-cm-long scions, collected from a candidate plus tree (CPT) of 50–60 years of age, onto 90-day-old nursery-grown rootstock. Scion size, rootstock age, and scion collection season were found to influence graft success. Grafted plants were incubated under greenhouse conditions for 6–8 weeks during the graft union process. In vitro micrografts were achieved by placing 1- to 2-cm-long scions derived from nodal shoot segments (collected from CPT) onto the hypocotyl of 45-day-old in vitro rootstocks. Use of in vitro grown shoots as a source of scion gave better graft success (60%) than scions collected directly from field-grown trees. In vitro grafting was also influenced by scion size and rootstock age. Under favorable conditions, scions and hypocotyls unite to form complete plants that produced two to four leaves after 6–8 weeks. This is the first report on in vivo and in vitro micrografting of *S. album* having potential for production of disease-free clonal plants for conservation and improvement targets.

Key words Graft success · Mature tree · Micrografting · Scion · Rootstock

Introduction

Santalum album, L. family Santalaceae, is commonly known as Indian sandalwood or Chandan. It is a small, evergreen hemiroot parasite tree, which attains a height of 12–13 m and a girth of 1.0–2.4 m. Sandalwood is highly valued for its fragrant heartwood and sandal oil used in world-class perfumes, cosmetics, and medicine (Ananthapadmanabha 2000). Sandalwood is also highly prized next to ivory for carving (Srinivasan et al. 1992).

Natural regeneration of sandalwood occurs mainly by seeds and also through root suckers. However, seedlings are extremely heterozygous due to outbreeding (Srimathi et al. 1995). Vegetative propagation through root suckers or cuttings has been reported by Uniyal et al. (1985). In vitro regeneration has been reported through somatic embryogenesis using explants from seedlings as well as from field-grown trees (Lakshmi Sita 1986; Rao and Bapat 1995; Rai and McComb 2002). However, *S. album* is strongly recalcitrant to in vivo and in vitro propagation for which only limited success has been achieved to date (Uniyal et al. 1985; Rao and Bapat 1992). In vitro rooting limits widespread application of micropropagation techniques for tree improvement of sandal. Vegetative propagation of plus trees for the establishment of clonal seed orchards is achieved by cleft grafting on 1- to 2-year-old seedlings (Srinivasan et al. 1992). This method, however, is limited by low graft success and plagiotropic growth of developing scions. To date and as far as we are aware, vegetative propagation from branch cuttings and in vitro clonal propagation of superior genotypes have not been reported. Overexploitation, sandal spike disease (caused by a mycoplasma-like organism), failure of regeneration efforts, and illicit felling have narrowed the gene pool of this heritage species that is now more and more threatened in its natural habitat. There is therefore a need to develop alternative clonal techniques to produce disease-resistant and high oil-yielding clones for urgent conservation and local uses.

The process and application of micrografting for overcoming incompatibility problems was demonstrated by Jonard (1986). This technique has been also used for rejuvenation and production of clonal plants in *Sequoia sempervirens* (Huang et al. 1992), *Acacia mangium* (Monteuuis 1995), *Morus alba* (Fengtong et al. 1996), and *Theobroma cacao* (Mallika et al. 1997). The objective of the

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present study was to assess the influence of different factors, i.e., scion size, rootstock age, and season to produce selected sandalwood clones by the use of in vivo and in vitro micrografting techniques.

Materials and methods

Preparation of rootstocks

Fresh seeds were collected from Nalla clonal seed orchard in Bangalore, India. Decoated seeds were treated with gibberellic acid (GA_3) (0.05% w/v) solution overnight and sown to produce seedlings that were raised in 250-ml or 450-ml single-cell root trainers filled with potting medium (compost 50%, sand 40%, and garden soil 10%) and maintained in a greenhouse (25° ± 5°C). Twenty replicates for each different age group of rootstocks (40- to 110-day-old-seedlings) were used for in vivo micrografting.

MS basal medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 0.6% agar was used for micrografting. The pH of the medium was adjusted to 6.0 with 0.1 N NaOH solution before autoclaving. The medium (25 ml) was dispensed into 25 culture tubes (150 mm; Borosil, India). The tubes with media were autoclaved at 15 psi and 121°C for 20 min. Seeds treated overnight with GA_3 were surface-sterilized with 0.075% (w/v) mercuric chloride for 6–7 min followed by washing with sterile distilled water (6–8 times) under aseptic conditions before inoculation (one per tube) onto the autoclaved medium. Cultures were incubated at 28° ± 1°C temperature and under a 12-h photopheriod of 60 μmol m^{-2}s^{-1} light intensity provided by cool-white fluorescent tubes (Philips, India). Twenty replicates for each different age group (20–60 days old) were used as rootstocks for in vitro grafting.

Preparation of scions

Actively growing shoots with apices were collected from a high oil-yielding (4%–5%) 50- to 60-year-old candidate plus tree (CPT) growing on the campus of the Institute of Wood Science and Technology, Bangalore, India, and were dipped in 0.1% (w/v) fungicide (Bavestin) for 3–4 min and then washed with distilled water. These shoots were used to determine suitable scion size for in vivo micrografting.

Nodal shoot segments (2.5–3.5 cm in length) with dormant axillary buds were collected from the same CPT, surface sterilized with 70% (v/v) ethanol for 30–40 s, followed by 0.075% (w/v) mercuric chloride (Hi-media, India) for 5–6 min. Surface-sterilized explants were washed (6–8 times) with sterile distilled water and inoculated onto MS medium supplemented with α-naphthalene acetic acid (NAA) (0.53 μM) and benzylamino purine (BA) (11.09 μM) for multiple shoot induction (MSI). All the cultures were incubated at 28° ± 1°C under a 12-h photoperiod with a light intensity of 60 μmol m^{-2}s^{-1} provided by cool white fluorescent lamps. Subculturing was carried out at intervals of 4 weeks onto fresh MSI medium for production of shoots. In vitro shoots derived from third subculture of 0.5 to 2.0 cm in length (scion) were used for the various in vitro micrografting experiments (20 replicates each). In another set of experiments, 20 shoot tips of 1.0–2.0 cm in length that were collected directly from a CPT and were dipped in 0.1% (w/v) fungicide (Bavestin) for 3–4 min followed by 0.075% (w/v) mercuric chloride (Hi-media) for 5–6 min. Surface-sterilized explants were washed (6–8 times) with sterile distilled water and were also used as scion for in vitro micrografting.

In vivo micrografting

An incision of 0.5–1.0 cm was made on the decapitated greenhouse-grown seedling (40–110 days old) using a surgical blade. Shoot apices derived from a plus tree were inserted into the incision, which was then held together with the help of an elastic strip. Scions (shoots) collected from the same CPT during winter (November–January) and the monsoon period (May–July) were used to study seasonal effects on in vivo micrografting.

In vitro micrografting

Under aseptic conditions, a vertical split measuring 0.25–1.0 cm in length was made on the top of the decapitated rootstock (20–60 days old) using a surgical blade. In vitro grown scions derived from nodal shoot segments (0.5–2.0 cm long) and shoot apices (collected directly from a CPT) were inserted into the rootstock incision. After grafting, plants were transferred to culture tubes containing liquid half-strength MS medium with 2% sucrose. The radicle of each grafted plant was pushed through the hole in a filter paper bridge to support the young plant.

Hardening and transplanting

In vivo grafted plants were covered with polythene bags to minimize moisture loss and were maintained at 25° ± 5°C under greenhouse conditions. The percentage of survival and growth of scions were determined after 8 weeks. In vitro micrografted plants were covered with polythene bags, incubated at 25° ± 1°C with continuous light (60 μmol m^{-2}s^{-1}) provided by cool white fluorescent tubes, and observed periodically to evaluate scion and rootstock survival. Well-established in vivo grafted plants with expanded leaves were transferred to a shade house and subsequently moved to a nursery. After 8 weeks, in vitro grafted plants with two to four expanded leaves from the scion were transplanted into 450-ml single-cell root trainers containing sterilized soilrite (Chougule, Bangalore), maintained at 25° ± 1°C under continuous light (60 μmol m^{-2}s^{-1}) provided by cool white fluorescent tubes. Established plants were transferred into the greenhouse, and then 2 weeks later were moved to the nursery. Overall procedures of micrografting are shown in Fig. 1.

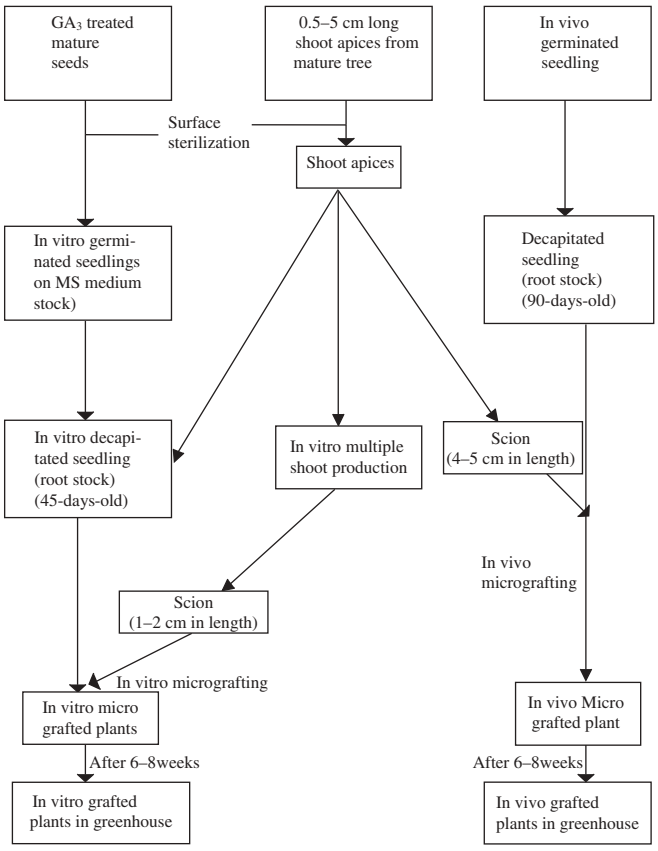

Fig. 1. A schematic representation of in vivo and in vitro micrografting in *Santalum album.* GA_3, gibberellic acid

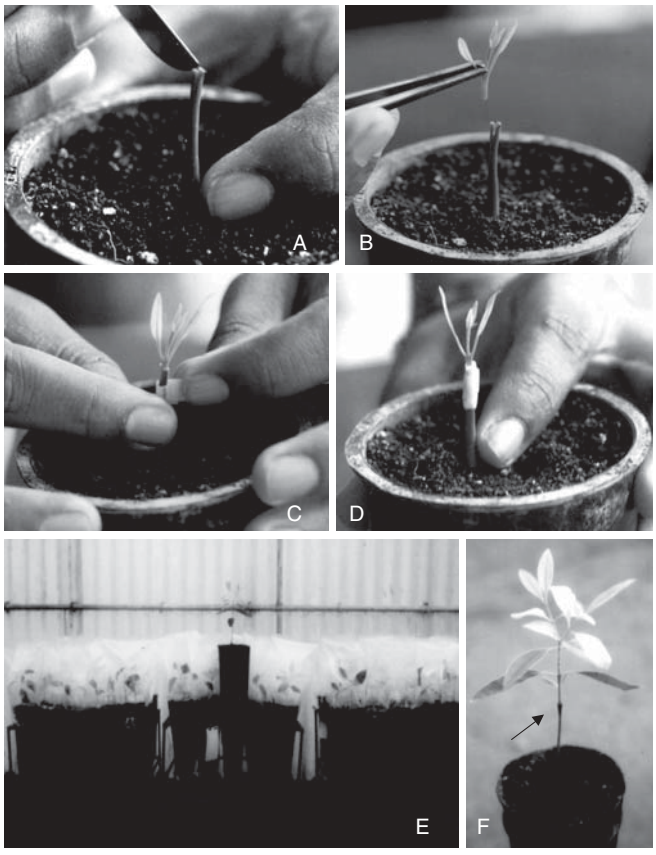

Fig. 2A–F. In vivo micrografting of *S. album.* **A** Incision of root stock. **B** Positioning of the scion. **C**, **D** Applying elastic strip to protect the grafted zone. **E** Large number of grafted plants in greenhouse. **F** Completely established grafted plant (*arrow* indicates grafted zone)

Data analysis

Observations were recorded for 20 grafted plants in each treatment. The survival percentage was determined by counting the number of grafted plants surviving out of the total number of grafted plants at the end of the eighth week.

Results

In vivo micrografting

The graft success of in vivo micrografting varied from 20% to 50%. As depicted in Table 1, the length of the grafted scion had a significant effect on graft success. Graft success was higher (50%) when the scion size was large (4.0–5.0 cm) and low when the size was small (0.5–3.0 cm). The effect of different age group rootstocks was examined (40- to 110-day-old seedlings); higher graft success (50%) was observed when 90-day-old rootstocks were used for grafting (Fig. 2). The percentage of graft success was almost uniform (40%) when 70-, 80-, 100-, and 110-day-old rootstocks were used (Table 2). On comparing success of grafts carried out during different seasons using 4.0- to 5.0-cm-long scions and 90-day-old rootstocks, the grafts made during winter established relatively faster and with high success rate. Low graft

Table 1. Effect of scion size on the success of in vivo micrografting in *Santalum album*

Shoot/scion length (cm)	Number of grafted plants recovered	Graft success (%)
0.5–1.0	0	0
1.0–2.0	4	20
2.0–3.0	4	20
3.0–4.0	8	40
4.0–5.0	10	50

Rootstock age 90 days. Number of rootstocks grafted: 20. Data recorded at the end of eighth week

Table 2. Effect of age of rootstock on the success of in vivo micrografting in *S. album*

Root stock age (days)	Number of grafted plants recovered	Graft success (%)
40	4	20
45	4	20
50	6	30
60	6	30
70	8	40
80	8	40
90	10	50
100	8	40
110	8	40

Scion size 4–5 cm with 4–6 leaves. Number of rootstocks grafted: 20. Data recorded at the end of eighth week

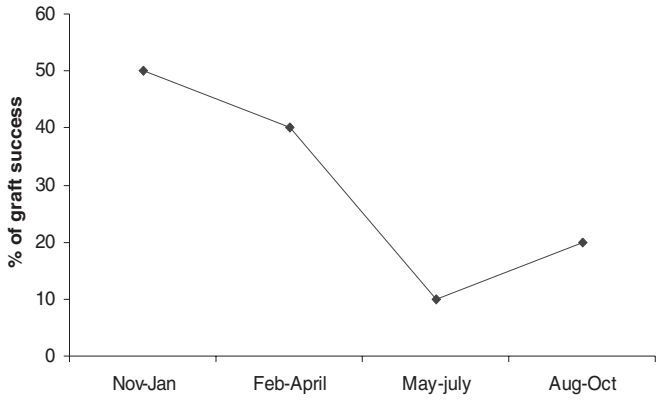

Fig. 3. Effect of season on in vivo micrografting in *S. album.* Scions of 4.0–5.0 cm in length were grafted on 90-day-old rootstocks. Data recorded at the end of the eighth week with 20 replicates for each treatment

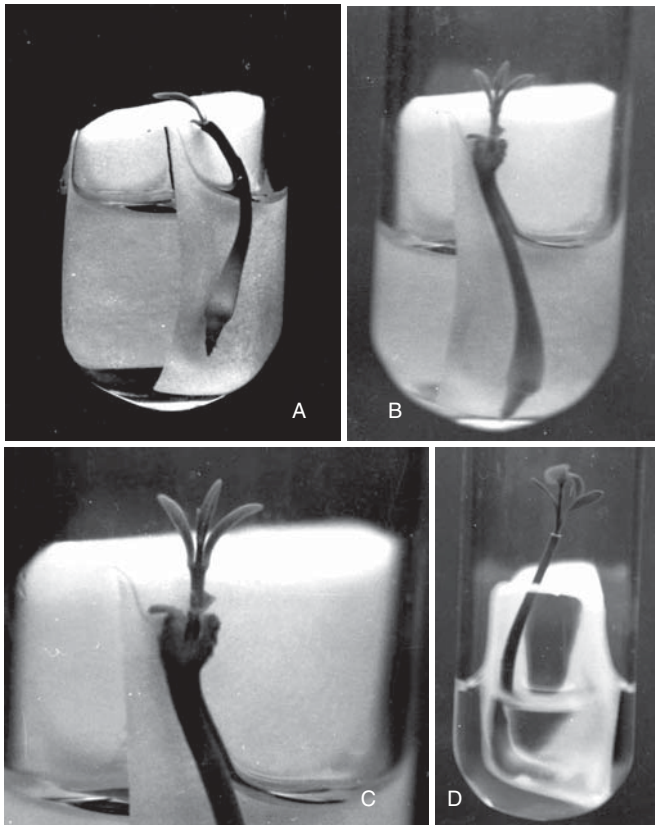

Fig. 4A–D. In vitro micrografting of *S. album*. **A, B** Development of scion on root stock. **C** Close view of point of attachment. **D** Completely established grafted plant

success was observed when the scions were grafted during the monsoon season (Fig. 3).

In vitro micrografting

Table 3 shows the effect of scion size on in vitro micrografting; higher graft success (60%) was observed for scions of 1.5–2.0 cm in length grafted on 45-day-old rootstocks (Fig. 4). Low graft success was observed when

Table 3. Effect of scion size on success of in vitro micrografting in *S. album*

Shoot/scion length (cm)	Number of grafted plants recovered	Graft success (%)
0.4–0.5	2	10
0.5–1.0	8	40
1.0–1.5	9	45
1.5–2.0	12	60

Rootstock age 45 days. Number of rootstocks grafted: 20. Data recorded at the end of eighth week

Table 4. Effect of rootstock age on the success of in vitro micrografting in *S. album*

Root stock age (days)	Number of grafted plants recovered	Graft success (%)
20	4	20
30	4	20
35	10	50
40	10	50
45	12	60
50	10	50
60	10	50

Scion size 1.5–2.0 cm long. Number of rootstocks grafted: 20. Data recorded at the end of eighth week

the scion length was 0.4–0.5 cm, and 1 week after grafting most of the scions had turned brown. The age of rootstock also influenced graft success; 60% graft success was recorded when the 45-day-old rootstock was grafted with scions of 1.5–2.0 cm in length (Table 4). No difference in graft success (50%) was observed when 35-, 40-, 50-, and 60-day-old rootstocks were used.

Discussion

Up to a certain stage, maturation of trees is usually accompanied by a decline of vegetative vigor. Adult trees require rejuvenation treatments for recovering physiological status more favorable to rooting and true-to-type propagation. Rejuvenation can be achieved by micrografting shoot apices (microscions) from selected adult trees onto very young rooted juvenile rootstock in vitro or in vivo (Emmarold and Mantell 2001). Higher graft success and growth was observed for in vitro grafted plants when compared with in vivo grafted plants, which might be due to juvenility of in vitro derived scions. Low success of in vivo micrografting may be due to rootstock and scion age, injury to scion during grafting, and water loss, especially in the smallest scions (0.5–1.0 cm long). Graft success rate was comparatively increased with scion length and no graft union was observed with the smallest scion (0.5–1.0 cm). Similar observations were reported for *Gossypium hirsutum* (Jinhua and Gould 1999).

The success of in vivo grafting declined with increased age of rootstocks (90 days old and onward). This may be due to active cytodifferentiation and lignification in the

hypocotyl region and also due to less wound-healing effect. This indicates that sandal is quite weak in scion capacity to produce callus, which helps ultimately in graft success. In *Camellia sinensis* (Prakash et al. 1999), maximum graft success was achieved in 4-month-old rootstock and low graft success was recorded when 12-month-old rootstocks were used. The season also affected the success of in vivo grafting, which was evident by our observations. Winter and early summer (spring) favored graft union with fewer occurrences of scion rotting. This method provides a cost-effective technique for clonal propagation and could be used in graft-based phase change and long-distance signaling studies in sandalwood and other tree species.

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