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Research Note

In vitro Regeneration of Sandal (*Santalum album* L.) from Leaves

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Abstract: The first successful induction of adventitious shoot buds on *Santalum album* L. leaves is reported. *De novo* shoots were induced directly on leaves without any callusing stage. A leaf length of 0.5-1.5 cm only showed bud inducing potential. Bud formation occurred on both MS and WPM basal media, although, liquid media were more responsive. Among the plant growth regulators, BAP at low concentrations (0.44 and 2.22 µM) was effective in this organogenetic process but exogenous auxin application failed to illicit a similar morphogenetic response. Epiphyllous shoot formation was more pronounced on leaf lamina in which the dorsal and ventral leaf surfaces were equally highly regenerative; the response, however, varied in different parts of the leaf.

Key Words: Woody plant medium, BAP, Shoot bud, *Santalum album*

Introduction

Santalum album L. is an important tree species cultivated in a wide range of areas because of its many applications. In vitro and particularly somatic embryogenesis, technology has been used for quite some time in sandal for the regeneration of plants (Lakshmi Sita et al., 1980; Bapat et al., 1985; Bapat et al., 1990; Mujib et al., 1997; Surajit et al., 1998). There are no published reports on shoot bud formation directly from in vitro cultured leaves for sandal. In woody species relatively little information is available on shoots formed directly on leaves without a callus stage (Oka & Ohyma, 1981; Simola, 1985; Swartz et al., 1990; Preece et al., 1993; Economou & Maloupa, 1995; Martens et al., 1996). Leaves obtained from in vitro grown plants provide a useful source of explants which eliminate the risk of contamination. Furthermore, simultaneous direct shoot bud formation on such leaves will reduce the possibilities of genetic variation common in plants regenerated from cultured cells or tissues. The first incidence of shoot bud production using leaf explants in sandal is reported here. The effect of various growth regulators on leaf and various in vitro responses were also studied.

Materials and methods

Young and immature leaves obtained from aseptically germinated seeds (3-4 weeks old) were used as primary inocula. For germination, the seeds were surface sterilised in 0.1% $HgCl_2$ for 4 min, and rinsed 3 times in sterile distilled water. All the cultures were grown on MS (Murashige & Skoog, 1962) and WPM (Lloyd & McCown, 1980) basal media. The basal media contained 3% (w/v) sucrose and 0.8% (w/v) agar (Sigma Chemical Company). Cytokinins, BAP (0.44, 2.22, 4.44, and 8.87 µM), kinetin (0.46, 2.32, 4.65 and 9.29 µM) and 2,i-p (0.49, 2.46, 4.92 and 9.84 µM), were used individually at the above concentrations. Two auxins, 2,4-D (2.26 and 4.52 µM) and CPA (2.68 and 5.36 µM), were also separately tested. The pH of the media were pre-adjusted to 5.8 and 5.2 (MS and WPM, respectively) before sterilisation (at 121 °C for 20 min). The cultures (explants with 25 ml of medium) were kept in a growth room at a temperature of 25 ± 2 °C and were provided with cool, white fluorescent light with a 16-h photoperiod. Leaves used as explants were categorised into different groups according to their sizes. Excised intact leaves or half leaves (sections cut perpendicular to the mid-vein) with the adaxial (i.e. the rough dorsal side) and abaxial (i.e. the smooth ventral side) surfaces of the leaves were separately tested by placing them in contact with the media. In another set of experiments, explants were placed on solid media either horizontally or vertically. Similarly, the distal and proximal ends of the leaves were tested for their organogenetic responses. Statistical analyses were performed using analysis of variance and means in which each of the experiments was conducted at least twice with 3 replicates per treatment.

Histology

For histological analysis, leaves were fixed in formalin:acetic acid:alcohol (FAA, 1:1:18). Then they were sequentially dehydrated in ethyl alcohol and tertiary butyl alcohol (TBA) series. Following adequate paraffin infiltrations, paraffin blocks were made and sections were cut with a rotary microtome (Reichert Ultracut) at 8-10 µm, stained with a 1% (w/v) aqueous solution of crystal violet and examined under light microscope for shoot bud development.

Results

In the media supplemented with kinetin, the leaf segments remained green for long periods (3-4 months) with no shoot bud formation. With the addition of 2,i-p a similar response was observed. Shoot bud production was exclusively obtained in BAP supplemented media (Figure 1a). Of the levels evaluated 0.44-2.22 µM BAP was effective for shoot bud formation. Frequency of shoot bud formation was, however, relatively low, i.e at 2.22 µM, 13.95% of the cultured leaf explants showed direct shoot bud formation in solid medium. In addition to the formation of buds, BAP also occasionally induced small, slow growing creamy white calli. Kinetin and 2,i-p also produced little callus from the leaf surfaces. However, callusing potential was poor (2.32-17.14%), in all the tested cytokinins callus was only induced in solid media at lower concentrations. Liquid media were ineffective in the callus induction process. Similarly, among the auxins, 2.26 and 4.52 µM 2,4-D and 2.68 and 5.36 µM CPA produced a low creamy white callus from the leaf surfaces. More commonly, at these plant growth regulator levels, curling and subsequent necrosis of leaves were observed.

Shoot bud formation

The first incidence of bud formation was noted after 20-25 days in the form of small protuberances, and in many cases the developing buds fused together. The number and size of shoot buds increased over the course of time (Figure 1b). Shoot bud formation was less evident in the petiole and was mostly restricted to the leaf lamina. It is clear from Table 1 that both the dorsal and ventral surfaces showed shoot forming ability. However, at the ventral surface the level of shoot bud production was high compared to dorsal surface. The frequency was again better at the ventral surfaces.

Leaf length had a strong influence in determining shoot bud formation. Table 2 shows the different categories and their responses. Adventitious shoot buds were mainly formed on small leaves (0.5-1.5 cm in length); 13-20 adventitious shoot buds were formed from such leaves. In some rare instances as many as 60 shoot buds were counted under the microscope (Olympus OIC 68768). Leaves 1.1-1.5 cm long were more responsive in this regard; about 22% of cultured leaf explants showed buds of varying sizes. Leaf groups longer than 1.5 cm lost their bud inducing property completely.

Induced shoot buds regularly developed on the laminar region. The distal end was found to be more responsive than the proximal end. Histological analysis indicated that shoots originated from the leaf epidermal regions (Figure 1c and 1d). These developing buds always had the usual dome-shaped meristems surrounded by 2 leafy primordia which are connected with the vascular system.

Table 1 shows that multiple shoots were produced both on solid and liquid WPM media. In liquid media, the number of shoot buds was significantly high compared with solid basal media. In medium supplemented with 2.22 µM BAP, 15 ± 1.41 shoot buds were formed in liquid media, whereas 4.2 ± 1.09 shoot buds were produced in solid media. Similarly, the frequency of adventitious bud formation was high in liquid media. Adventitious buds grew at a lower rate in solid media compared with that in liquid media. In liquid media, shoot buds were induced in submerged leaves only, whereas fully-floated or immersed leaves never produced any shoot primordia under the tested conditions. Leaf explants when placed horizontally produced epiphyllous shoots, but leaves placed vertically were unresponsive. Shoots induced from these buds grew slowly and only a few developed into individual shoots (Figure 1e). They were transferred to pots after in vitro root formation (WPM + 5.71 µM IAA).

Discussion

In the present study, adventitious shoot bud development was noted from leaf explants. Young green leaves, measuring 0.5-1.5 cm in length, usually showed

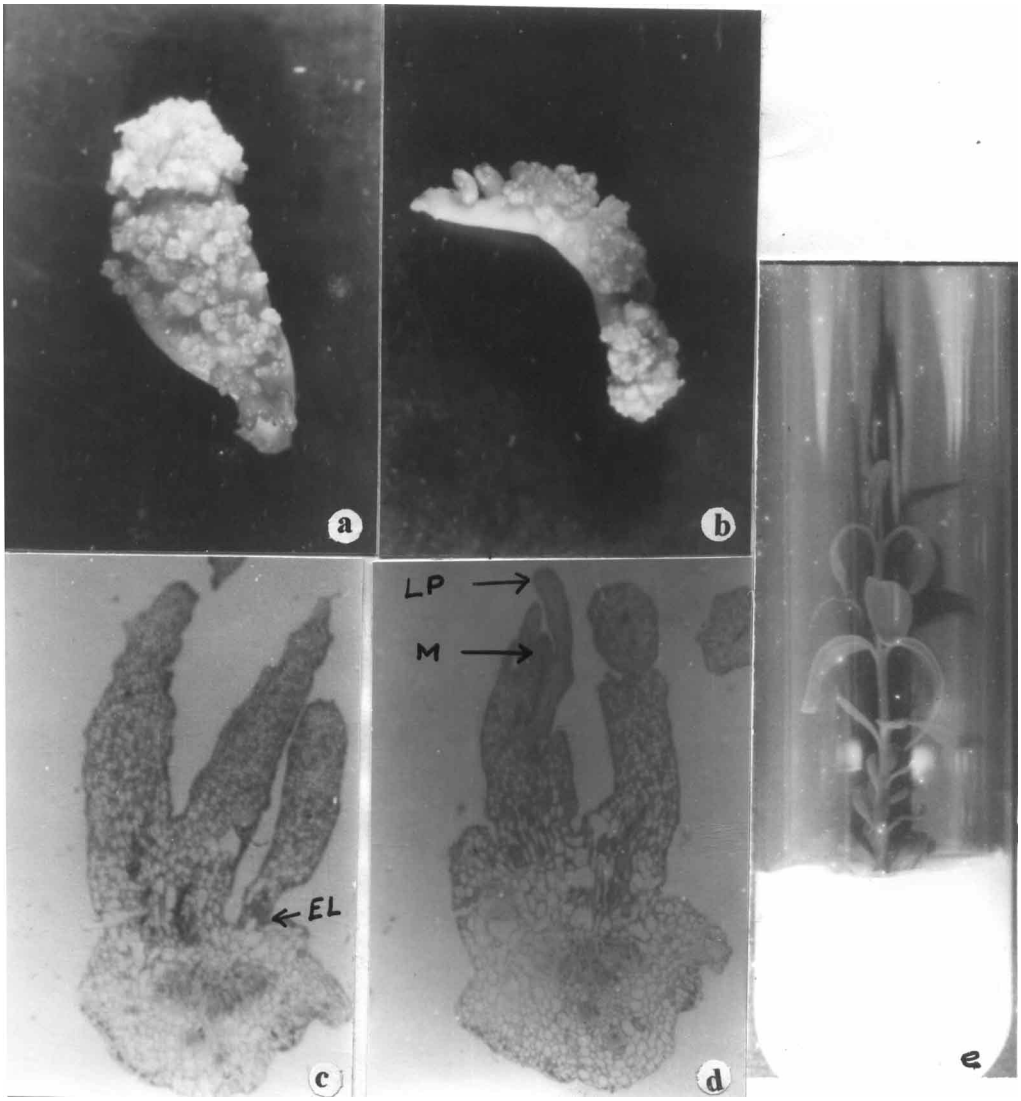

Figure 1. Direct shoot bud formation on sandal leaves
a,b: Two different stages of shoot formation from leaves (x 1.85).
c,d: Longitudinal section showing direct bud formation on leaves (x 100),
(EL: epidermal layer, M: meristem, LP: leafy primordium).
e: Rooted individual shoot before transplantation.

higher in vitro organogenetic responses. Longer leaves showed a total loss of morphogenetic potential. Bud inducing properties were largely seen at the leaf laminae and compared with the dorsal (adaxial), ventral (abaxial) surfaces were highly responsive. This study further demonstrated that explants obtained from or near the apical meristematic part produced a better response compared with explants from the rest of the leaf lamina. Orientation and positioning of the leaf explant on nutrient media had a significant impact on shoot bud development. Leaf sections placed vertically did not show any response, while a high frequency shoot bud formation was noted when the leaf lamina were kept horizontal. Therefore, leaf segments, positioning and surfaces play a significant role in inducing shoot buds. Although the reason is still unclear, differential physiological gradients, of which the endogeneous levels of growth regulators were the most important, existing in different parts of the leaf are

Table 1. Various cytokinins and shoot bud formation from leaves on WPM after 45 days of incubation.

PGR (µM)	No. of explants cultured	% of callusing (degree)	% explant producing shoot buds		No. of shoot buds/explant		Shoot height (mm)
			Solid	Liquid	Solid	Liquid	
Control	135	0	0	0	0	0	0
Kinetin							
0.46		0					
2.32	135	17.14(+)					
4.65		14.28(+)	0	0	0	0	0
9.29		2.85(+)					
BAP							
0.44		0	5.71	8.57	3.4 ± 0.54	12 ± 1.87	4.4 ± 1.14*
2.22	143	11.62(++)	13.95	18.60	4.2 ± 1.09	15 ± 1.41	3.8 ± 0.83*
4.44		9.30(+)	0	0	0	0	0
8.87		2.32(+)	0	0	0	0	0
2,i-p							
0.49		0					
2.46	135	11.42(+)	0	0	0	0	0
4.92		11.42(+)					
9.84		0					

All the values are expressed as mean± standard error.
*Data scored from liquid media
+,++ represent small and moderate callus, respectively

Table 2. Direct shoot bud induction from leaves in WPM medium supplemented with BAP (2.22 µM) after 45 days of culture.

Leaf length (cm)	No. of explants cultured	% explant producing shoot bud		No. of shoot buds / explant	
		Dorsal	Ventral	Dorsal	Ventral
0.0-0.5	136	2.71	2.77	11.8 ± 2.16	20.6 ± 2.07
0.6-1.0	124	5.71	11.42	10.8 ± 1.92	15.8 ± 3.70
1.1-1.5	135	12.5	21.87	11.4 ± 3.36	13.0 ± 2.34
1.6-2.0	132	0	0	0	0

probably involved in such differential morphogenetic responses (Wernicke & Milkovits, 1986; Mujib et al., 1996). Similarly, the optimal age, different physiological gradient in explants and genotypes are among those involved in differential behaviour, as has been known for quite some time in other systems (Tibok et al., 1995; Bandyopadhyay et al., 1999).

Buds were formed on both MS and WPM basal media, and preferably in liquid media. Higher nutrient uptake by plants/cultures may be the reason for the better response in liquid medium. Shoot bud formation from different sources and unique somatic embryogenesis had previously been established (Rao & Bapat, 1992). This and earlier studies indicate that genetic constitution may

be the determining factor in this high morphogenetic ability. BAP was found to be very active plant growth regulator, and at relatively lower levels it favoured shoot bud formation, while other cytokinins (kinetin and 2,i-p) were ineffective. Similarly, in earlier reports, adventitious shoots from leaves were obtained in BAP supplemented media (Welander, 1988; Ostry et al., 1990). BAP thus proved to be a very effective plant growth regulator in sandal, too. Shoot buds developed on leaves showed slow growth, and normal development up to the individual shoot was not that high, although better optimisation of in vitro conditions may improve this epiphyllous shoot development process.

References

Bandyopadhyay S, Cane K, Rasmussen G & Hamill JD (1999). Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species - *Eucalyptus nitens and E. globulus. Plant Science* 140: 189-198.

Bapat VA, Fulzele DP, Heble MR & Rao PS (1990). Production of sandalwood somatic embryos in bioreactors. *Current Science* 59: 746-748.

Bapat VA, Gill R & Rao PS (1985). Regeneration of somatic embryos and plantlets from stem callus protoplasts of the sandalwood tree (*Santalum album* L.). *Current Science* 54: 978-982.

Economou AS & Maloupa EM (1995). Regeneration of *Elaeagnus angustifolia* from leaf segments of in vitro derived shoots. *Plant Cell Tissue and Organ Culture* 40: 285-288.

Lakshmi Sita G, Raghava Ram NV & Vaidyanathan CS (1980). Differentiation of embryoids and plantlets from shoot callus of sandalwood. *Plant Science Letters* 15: 265-270.

Lloyd G & McCown B (1980). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. (Combined) *Proceedings of the International Plant Propagation Society* 30: 421-427.

Martens M, Werbrouck S, Samyn G, Botelhodos Santos Moreira da Silva H & Debergh P (1996). In vitro regeneration of evergreen azalea from leaves. *Plant Cell Tissue and Organ Culture* 45: 231-236.

Mujib A, Bandyopadhyay S, Jana BK & Ghosh PD (1996). Growth regulator involvement and somatic embryogenesis in *Crinum asiaticum. Indian Journal of Plant Physiology* 1: 84-86.

Mujib A, Das S & Dey S (1997). Free cell culture, organization and plant regeneration in *Santalum album* L. *Plant Tissue Culture* 7: 63-69.

Murashige T & Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

Oka S & Ohyama K (1981). In vitro initiation of adventitious buds and its modification of high concentration of benzyladenine in leaf tissues of mulberry (*Morus alba*). *Canadian Journal of Botany* 59: 68-74.

Ostry ME, Skilling DD, Lee Stadelmann OY & Hackett WP (1990). Recovery of somatic variation in resistance of popular to *Septoria musiva*. In: Rodriguez R, Tames RS & Durzan DJ (eds.). *Plant aging: Basic and applied approach*. pp. 113-116, New York: Plenary Press.

Preece JE, Imel MR & Shevade A (1993). Regeneration of *Rhododendron* PJM group plants from leaf explants. *Journal of the American Rhododendron Society* 47: 68-71.

Rao PS & Bapat VA (1992). Micropropagation of sandalwood (*Santalum album* L). In: Bajaj YPS (eds.). *Biotechnology in Agriculture and Forestry, Vol 18, High Tech and Micropropagation II*, pp.193-210, Berlin: Springer-Verlag.

Simola LK (1985). Propagation of plantlets from leaf callus of *Betula pendula* f. *purpurea. Scientia Horticulturae* 26: 77-85.

Surajit D, Susobhan D, Mujib A, Pal S & Dey S (1998). Influence of carbon source and pH on rapid mass propagation of *Santalum album* by somatic embryogenesis: the application of biotechnology in agroforestry. In: Radomiljac AM, Ananthapadmanabho HS, Welbourn RM & Satyanarayana Rao K. (eds.). *Sandal and its product.* ACIAR Proceedings No. 84, Canberra, Australia, pp. 66-68.

Swartz HJ, Bors R, Mohammed F & Naes SK (1990). The effect of in vitro pretreatments on subsequent shoot organogenesis from excised *Rubus* and *Malus* leaves. *Plant Cell Tissue and Organ Culture* 21: 179-184.

Tibok A, Blackhall NW, Power JB & Davey MR (1995). Optimized plant regeneration from callus derived from seedling hypocotyl of *Eucalyptus urophylla. Plant Science* 110: 139-145

Welander M (1988). Plant regeneration from leaf and stem segments of shoot raised in vitro from mature apple trees. *J Plant Physiol* 132: 738-744.

Wernicke W & Milkovits L (1986). Development gradient in wheat leaves. Responses of leaf segments in different genotypes cultured in vitro. *Journal of Plant Physiology* 115: 49-58.