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Interspecific Hybridisation between *Santalum album* and *S. spicatum*

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Abstract

The possibility of producing inter-specific and intra-specific hybrids of *Santalum album* and *S. spicatum* was investigated. A study of the reproductive biology of the species showed that both *S. album* and *S. spicatum* are obligate out-crossing species, with pre-and post-fertilisation barriers preventing self-pollination and inter-specific pollination. Initial fruit-set was low with 70–100% fruit abscission. Reasons for failure of fruit-set included lack of fertilisation, lack of an embryo sac, and failure of endosperm development. An in-vitro culture technique was developed which could induce embryogenesis and thus 'rescue' embryos of intra-specific crosses at 3.5–6 months old, but not from putative inter-specific hybrid fruit, which abscised at 1–3 months old. Putrescine spray increased fruit-set and delayed fruit abscission for both intra-specific and inter-specific crosses. However only a few putative hybrid fruits were retained until four months after pollination; they were not harvested for ovary culture, but allowed to develop to maturity. These mature putative hybrid seeds did not germinate, but their endosperms were confirmed to be hybrid by random amplified polymorphic DNA (RAPD) analysis.

Key words: *Santalum album, Santalum spicatum*, inter-specific hybridisation

WEST AUSTRALIAN SANDAL, *Santalum spicatum*, is indigenous to Australia. Although it initially grew in more temperate areas, it is now restricted to arid and semi-arid areas in South Western Australia and South Australia (Applegate and McKinnell 1991).

It typically has an oil content of two per cent, and in its natural habitat is a slow-growing species. Some 2000 t of wood are harvested each year and utilised for incense. The better known Indian sandal, *S. album* L. has a higher oil content (6–7%) and more valuable oil, and grows in a wide range of temperatures and soil types in tropical and subtropical areas of India, Sri Lanka and Indonesia (Anon. 1990). We investigated the possibility of producing inter-specific hybrids between *S. album* and *S. spicatum* with the objective of combining the drought and stress tolerance of *S. spicatum* with the high oil content and faster growth of *S. album.*

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The breeding biology of *Santalum* species has not been extensively studied, and published information on *S. album* is contradictory. Bhaskar (1992) and Jyothi et al. (1991) found it to be an obligate outcrossing species, while Sindhuveerendra and Sujatha (1989) reported partial inbreeding. Less is known about *S. spicatum*, although the phenology of flowering has been described; in common with other *Santalum* species, it has a very low fruit-to-flower ratio (Barrett 1987).

The peculiarities of the *Santalum album* placenta, ovule and embryo sac structure and development of the endosperm and embryo were recognised early (Griffith 1843; Iyengar 1937; Rao 1942), and Rugkhla (1997) has described the *S. spicatum* ovary. In *Santalum* the unilocular ovary has 2–3 fused carpels, and is almost filled with an enlarged placenta (mamelon) which bears 2–3 'ovules'. The embryo sacs protrude from these ovules, but initially remain embedded in the placenta. As the flower matures, the embryo sacs emerge from the placenta. The 'micropylar' end bears the egg apparatus and is at first curved to point towards the base of the ovary, but

later turns and grows up to the base of the style. After fertilisation the endosperm from one embryo crushes the other embryo sacs and the mamelon, so at maturity there is a mass of endosperm directly inside the fruit wall. There is no seed coat. Inside the endosperm is embedded a small dicotyledonous embryo.

Intra-specific and inter-specific hybridisation has not been widely attempted in sandal, and to understand the problems of production of inter-specific hybrids it was first necessary to study intra-specific crosses in some detail.

Materials and Methods

S. spicatum (seed origin, Kalgoorlie and Bullock Holes, Western Australia), and *S. album* (seed origin, Bangalore and Marayoor, India) 7–12 years old, growing at Curtin University W.A., were utilised. Pollinations were carried out between February and August. One to two days before anthesis, spiral wire frames were placed over inflorescences and they were then covered with perforated polyethylene bags. Where necessary, flowers were emasculated before anther dehiscence. Pollen from freshly dehisced anthers was used. Preliminary analyses of pollen-tube growth in the style, and observations of stigma receptivity, showed the optimum time for pollination to be 2–3 days after anthesis. Success of pollination was scored as:

- failure—when flowers abscised before ovary enlargement;
- initial fruit-set—when ovaries enlarged to 1.5mm; and
- mature fruit-set.

Anatomical studies involved fixation of material in 3% gluteraldehyde (*pH* 7, 0.025M phosphate buffer), dehydration in an alcohol series, and embedding in Spurr's resin. Longitudinal sections were cut at 1-3μ and stained with 'toluidine blue O' (Feder and O'Brien 1968). Assessment of pollen-tube growth and developmental stages of embryo sacs were also observed after fixing material in Carnoy's solution, dehydration in an alcohol series, then softening in 0.8N NaOH at 60°C for up to two hours followed by staining in 0.1% aniline blue in phosphate buffer for 10 minutes squashing in 80% glycerol and observation under fluorescence microscopy (Martin 1959).

In some experiments aimed to extend the time of fruit retention, all flowers on an inflorescence were hand-pollinated; putrescine or arginine (in 0.1M 2-N-morpholino-ethan sulfonic acid (MES) and 0.01% *Tween-80*, pH 7) was sprayed onto inflorescences when 25 per cent of the flowers reached anthesis.

Embryos extracted from endosperm—or cut out with a piece of endosperm and pericarp attached to the basal end of the embryo—were cultured from fruits of different ages. The media were:

- K—Kao and Michayluk (1974) medium with 10 μM GA_3, 0.5 μM BAP, 0.5 μM kinetin, 1 μM zeatin and 1 μM IAA;
- 0—Murashige and Skoog (1962) medium with 5 μM 2,4-D and 1.8 μM kinetin; and
- T—Murashige and Skoog (1962) medium with 2–4 μM thidiazuron.

DNA from fully expanded leaves or endosperm of *S. spicatum* × *S. album* putative hybrids was extracted using the method of Doyle and Doyle (1987). After extraction it was found necessary to include a treatment with RNase A at 10μg mL and to incubate at 37°C for 30 minutes. PCR amplification was conducted using the methods of Williams et al. (1990) and eight different primers were used of which OPA-08 (Operon Technologies) was the best to discriminate between the species. The parameters of the reaction were optimised for each species. For *S. album* 1.9 mM $MgCl_2$ and 0.18 units of Taq polymerase was optimal, while for *S. spicatum* it was 2.4 mM Mg Cl_2 and 0.22 units Taq polymerase.

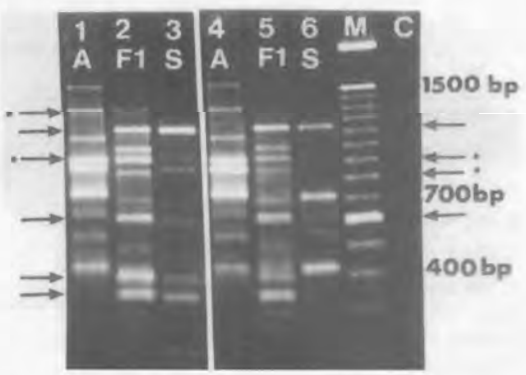

Figure 1. RAPD bands of *S. album* (lanes 1 and 4), *S. spicatum* (lanes 3 and 6), and endosperm of two putative hybrid seeds of *S. spicatum* × *S. album* (lanes 2 and 5)
Notes: Arrows with an asterisk indicate unique bands from the *S. album* parent (pollen parent) that are present in the hybrid. Arrows without an asterisk are unique bands from *S. spicatum* (female parent) present in the hybrid. Lane M is molecular marker 100 bp ladder, lane C the negative control without DNA. Lanes 1–3 were run under conditions optimised for *S. spicatum*, and lanes 4-6 conditions optimised for *S. album*.

Results

The number of pollen tubes observed in styles and ovaries was highest after intra-specific cross-pollination and lowest after self-pollination or inter-specific crossing (Table 1). Histological examination of ovaries showed that the frequency of fertilisation, based on the division of the endosperm nucleus, was higher in the *S. spicatum* × *S. album* cross than in the reciprocal cross. Variation in fruit-set between genotypes was observed, with one *S. album* tree setting no fruit despite using four other *S. album* pollen trees (Table 2). The average final fruit-set was low for both species (Table 3). The rare mature fruit formed after self-pollination of each species was seedless. Although there was an initial low level of fruit-set after inter-specific crossing, none reached maturity. Fruits of *S. album* reached maturity after 7–8 months, while those of *S. spicatum* required 9–11 months.

The female gametophytes were immature at anthesis, and did not reach full differentiation until three days after anthesis. The egg apparatus showed aniline blue induced fluorescence which became intense 2–4 days after anthesis and faded after fertilisation. In *S. album* up to 18 per cent of flowers lacked embryo sacs, and in the *S. album* tree which failed to set fruit, the embryo sacs were very slow to mature.

Histological studies showed that a coenocytic endosperm developed and began to become cellular after 1–2 months. We did not observe development of two compartments in the endosperm with the chalazal nucleus resting as reported by Iyengar (1937) and Rao (1942). The zygote remained quiescent for one month. In inter-specific crosses the endosperm formed only 6–10 nuclei, aggregated on one side of the embryo sac, then degenerated. The hybrid zygote did not divide.

Embryos from intra-specific crosses extracted from fruits 2–3 months old and cultured in vitro, produced only callus. Embryos of *S. spicatum* from fruits 3.5–6 months old, when cultured with a portion of endocarp at the basal end of the embryo, produced adventitious embryos on Kao and Michayluk (1974) medium with a combination of five growth regulators, or on a more simple medium (Murashige and Skoog (1962) with 5 μM 2,4-D and 1.8 μM Kinetin) (Table 4). Zygotic embryos of *S. spicatum* at six months or older did not require the attached endosperm and peri-

Table 1. Pollen tubes observed in the style, ovary and embryosac two days after hand-pollination (intra-specific) crosses, and three days after pollination (inter-specific) crosses

Cross	Pollen tubes (mean no.)		Ovaries with pollen tubes (%)	Fertilised ovules (%)
	Style	Ovary		
S. spicatum selfed	2.0 (0.2)	0.3 (0.3)	20	0
S. spicatum cross	23.5 (4.8)	1.3 (0.1)	50	40
S. album selfed	6.8 (5.0)	0	0	0
S. spicatum cross	14.0 (2.9)	6.3 (2.2)	65	10
S. album × *S. spicatum*	13.6 (5.1)	1.0 (0.6)	20	0
S. spicatum × *S. album*	3.0 (2.0)	0.5 (0.3)	20	0

Notes
- Data are means of 5–20 replicates with SE in parenthesis.
- Unpollinated control flowers showed no pollen tubes.

Table 2. Frequency of fertilisation as determined by the division of the endosperm nucleus (% ovaries)

Cross	Unfertilised	Fertilised	Unidentified
S. spicatum × *S. spicatum*	30	64	6
S. album × *S. album*	38	54	8
S. album × *S. spicatum*	70	5	10
S. spicatum × *S. album*	57	40	3

Note
- 20–24 ovaries were examined for each cross.

Table 3. Fruit development and seed germination after intra-specific or inter-specific pollination

Cross	Fowers pollinated (No.)	Initial fruit-set (%)	Mature fruit-set (%)	Germination (%)
S. spicatum × *S. spicatum*	2500	4.2	1.3	70.4
S. album × *S. album*	865	19.3	7.2	52.0
S. spicatum selfed	370	0.2	0.2[a]	0
S. album selfed	400	0.5	0.3[a]	0
S. album × *S. spicatum*	680	0.4	0	–
S. spicatum × *S. album*	1250	0.3	0	–
Bagged, unpollinated	150	0	0	–

[a] Seedless fruit

Note

• Data from crosses using 22 trees of *S. spicatum* and 7 trees of *S. album* are pooled.

carp, and could develop adventitious embryos on medium with thidiazuron. *S. album* immature embryos were less responsive than those of *S. spicatum* in culture.

Best results for *S. album* were obtained with embryos attached to a portion of endosperm and cultured on media with thidiazuron (Table 4).

When inflorescences were sprayed with putrescine or arginine, there was an increase in initial and final fruit-set in most treatments (Table 5). Putrescine spraying of unpollinated flowers did not induce ovary enlargement. Some inter-specific fruit-set was obtained after spraying; histological examination of such fruit showed that, by Day 60, the endosperm had grown to fill half the embryo sac and the embryo had developed to the octant stage. Those few *S. spicatum* fruit from inter-specific crossing which were retained to maturity did not contain an embryo.

Table 4. Effect of growth regulators and the presence of endocarp and endosperm on in-vitro development from immature zygotic embryos 3.5–6 months after pollination (% of explants)

Species	Medium	Attached tissues	Callus	Somatic embryos	Radicle elongation	Shoot development
S. spicatum	K	endocarp and endosperm	20	30	40	30
		none	100	0	0	0
	O	endocarp and endosperm	40	60	0	60
		none	100	0	0	0
	T	endosperm	0	0	0	0
		none	0	60	0	5
S. album	K	endocarp and endosperm	80	0	0	0
		none	100	0	0	0
	O	endocarp and endosperm	100	0	0	0
		none	100	0	0	0
	T	endosperm	0	40	60	40
		none	0	0	0	0

Notes

• There were 10–20 samples in each treatment.

• Media K, O and T are explained in the text.

The RAPD bands generated using different primers showed that 3–15 per cent were common to both species. DNA from the endosperm of two putative *S. spicatum* × *S. album* hybrid seeds showed most marker bands of both parents when tested under the different sets of PCR conditions optimal for each parent (Fig. 1). A few paternal bands were missing or weakly expressed in the putative hybrid, and there were also some non-parental bands present.

Discussion

From observations of pollen-tube growth and seed set, *S. spicatum* and *S. album* were shown to be outcrossing species in which pre-and post-fertilisation incompatibilities prevent inbreeding. The rare fruits which matured after selfing were seedless. For *S. album* we thus confirmed the evidence of Bhaskar (1992) and Jyothi et al. (1991) and contradicted the results of Sindhuveerenda and Sujatha (1989). For most genotypes, stigma receptivity and female gametophyte maturity were well coordinated, but in one *S. album* tree, embryo sacs were not receptive at the same time as the stigma. The placenta had no ovules in 18 per cent of *S. album* flowers. *S. acuminatum* has also been reported to develop ovaries without ovules in some flowers (Sedgley 1982).

Attempts to produce inter-specific hybrids between *S. album* × *S. spicatum* were unsuccessful. Pollen-tubes were able to grow down the styles in the reciprocal crosses but fertilisation rarely occurred, particularly when *S. album* was used as the maternal parent (Tables 1, 2). When ovules of the *S. spicatum* × *S. album* cross were successfully fertilised, abnormal endosperm development precluded zygote development. As in the fruit species apple and pear (Costa et al. 1986; Crisosto et al. 1988), putrescine spray improved fruit-set after hand pollination of *S. album* and *S. spicatum*. Increased fruit-set (0.2%), was also obtained after inter-specific crossing of the *Santalum* species. After putrescine spray the embryos in the hybrid seeds developed to at least the octant stage, but fruits at maturity did not contain an embryo. Specific RAPD markers for each species were used to confirm the hybrid nature of the endosperm of such seeds. Only 3–15 per cent of the RAPD bands were common between the species indicating that the relationship between *S. spicatum* and *S. album* is distant.

A successful method of rescuing immature embryos from intra-specific crosses was developed. As with the other species (Liedl and Anderson 1992), culturing very small embryos was either unsuccessful or resulted in production of callus only. Embryos from *S. spicatum* fruits 4–6 months old could be induced to produce somatic embryos in vitro. For *S. spicatum* it was found to be important to retain the attachment of the basal end of the embryo to the endosperm and pericarp. This suggests that the suspensor and the vascular connection between the ovule and the endocarp (Rao 1942) play an important role in embryo nutrition.

If hybrid embryos are to be raised successfully, different genotype combinations or a more effective putrescine treatment will need to be developed to allow embryos to grow to sufficient size for in-vitro rescue. In addition it may be possible to raise triploid embryos from hybrid endosperm as has been done for *S. album* (Lakshmi Sita et al. 1980). An alternative pathway for production of hybrids between *S. album* and *S. spicatum* may be to raise somatic hybrids through protoplast fusion. This has been shown to be possible, and fusion products have grown to the stage of small calli (Rughkla 1997).

Table 5. Effect of putrescine and arginine sprays on fruit-set of hand-pollinated flowers

Cross	Initial fruit-set				Mature fruit-set			
	Putrescine			Arginine	Putrescine			Arginine
	0	10 mM	100 mM	100 mM	0	10 mM	100 mM	100 mM
S. spicatum × *S. spicatum*	6.5	5.4	6.5	7.2	0.3	2.9	3.8	3.2
S. album × *S. album*	4.2	11.6	6.0	9.1	1.2	4.3	2.2	3.5
S. spicatum × *S. album*	0.1	2.0	2.1	0.4	0	0	0.2	0.1
S. album × *S. spicatum*	0	0	0	0	0	0	0	0

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