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EDITOR'S NOTE

This issue of the SRN has two interesting scientific papers related to *Santalum album*. Ma and Bunn present a detailed examination of the embryology and reproductive biology of *Santalum album* grown in China, which provides useful information for plant breeding and improvement activities. This study provides an in-depth examination of the cellular development of the reproductive tissues in the sandalwood flower and phenological flower development that may assist our understanding of potential gene flow in natural populations and plantations.

Balachandran and Kichenamourthy describe the structure of natural sandalwood stands in Pondicherry India, with particular reference to adaptation to different soil types and the assemblages of associated species. This study reveals that sandalwood is both preferential and opportunistic in its parasitism of different plant species, which includes a range of cultivated and weedy species.

Tony Page

Embryology and pollination trials support dichogamy in *Santalum album* L.

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Abstract

Embryo development and pollination trials were studied in *Santalum album* L. The formation of the male (microspore) and female (megaspore) tissues in the same flower were synchronized during the early stages of flower-bud development. However, at anthesis when pollen was mature, the megaspore had developed only to the stage of a 1-2 nucleate embryo sac. The development from 2-nucleate embryo sac to matured 8-nucleate embryo sac lasted up to 10 days. These results indicate that the flower of *S. album* is dichogamous where the pollen matures before the embryo sac. Following fertilisation of the ovule the endosperm developed prior to division of the zygotic embryo, and 1-3 embryos and endosperms were formed in the same fruit. Seed-set resulting from open pollination was less than 3%. No seed set was observed when inflorescences were covered with a bag; however artificial pollination increased fruit set to 14%. Mature seed usually germinated to produce one seedling, but two- and three-seedlings from one seed were also observed at low frequency.

Introduction

Sandalwood (*Santalum album* L., Santalaceae) is one of the most valuable oil-bearing species in the world, which was introduced to Mainland China in the 1960's and 1980's where it has been widely cultivated in most tropical and subtropical areas. Improving breeding programs and crop regeneration requires greater understanding of the reproductive biology in *S. album*. Previous studies have shown that natural pollination and fruit set in *S. album* are most often low and characterised by high genetic variation and abnormality in successive generations (Bagchi and Kulkarni 1987; Brand 1994; Fox *et al.* 1995; Suma and Balasundaran 2003).

Various reports have dealt with floral morphology and embryology in *S. album* (Griffith 1836, Henfrey, 1856;

Iyengar 1937; Srinivasa 1937; Rao 1942; Paliwal 1956; Bhatnagar 1959; Sindhuveerendra and Sujatha 1989) and indicate that flowers in the *Santalum album* has three ovules (four ovules not reported) and three mature embryo sacs in an 'N' or 'S' shape in the ovary, and that 'selfing' is possible therefore supporting claims of partial inbreeding (Sindhuveerendra 1989). However, Veerendra and Padmanabha (1996) indicated that *S. album* was predominantly out-breeding and self-incompatible. The mechanism of out-crossing in *S. album* is not clear, which prompted the present study. Observations on flowering, pollination and fertilization in *S. album* are presented here in addition to results of studies on embryological development of pollen, embryo sac and embryo.

Materials and Methods

The observations were made on *S. album* L. trees in the Sandalwood Garden of South China Botanical Garden (Figure 1). Flowers were tagged and their developmental process (flowering and fruit setting) was investigated as follows: different sized flowers or fruits were collected and fixed (for 2 days at room temperature) in FAA solution (70% ethanol- glacial acetic acid- 37% formaldehyde, 18: 1: 1) and then transferred to 70% ethanol and stored at 4 °C until use. The flowers and fruits were then stained with haematoxylin, dehydrated in ethanol series, embedded in paraffin and 6-12 µm TS and LS sections cut. Transverse sections were used to estimate the number of ovules in the ovary; LS and TS samples to observe pollen, embryo sac and embryos development.

inside...

Embryology and pollination in *Santalum album*.
Santalum album in the Pondicherry region of India

Pages 1-4
Pages 4-9

Pollination

For pollination experiments three primary treatments were applied (1) no pollination (autogamous selfing) and artificial (2) self- and (3) cross-pollination. Fruit set for each of these treatments were recorded. About 5-10 flowers (of ~ equal size) selected from every inflorescence were bagged 2-days before anthesis (with thin paper bags) to avoid open pollination. In artificial pollination treatments anthers were severed by forceps (sterilized in 75% v/v ethanol) and pollen collected for pollination. Anthers were left intact for autogamous selfing treatments, and then paper bags removed following fertilization.

Results

Micro- and mega-sporogenesis

In early flower development (flowers-1 mm), archesporial cells in anthers are just beginning to develop and one archesporial cell starts to divide to form one parental cell and one sporogenous cell (Table 1). The sporogenous cell divides to form a mass of pollen mother cells. The numerous pollen mother cells undergo meiosis and mature pollen is present by the time the flower is about 3 mm in size (Table 1).



Figure 1. Author with a three year old *S. album* in Leizhou, Guangdong.

During earlier floral development, the hypodermal mother cell enlarges, cytoplasmic density increases and the nucleus becomes larger, gradually embedding deeper into the nucellar tissue mass. The meiotic phase is followed by linear tetrad cell formation;

Flower (fruit) size (mm)	Development of microspore and pollen	Development of megaspore, embryo sac and fertilization
1	Archesporium	Archesporium
1	Sporogenous cell	Sporogenous cell
1.5	Pollen mother cell	Megaspore mother cell
2	Microspore	Tetrad and functional megaspore
2.5	Mature pollen	1-2 nucleate Embryo sac
3	(Flower opens) Pollination	2-4 nucleate embryo sac
3.5	Pollination	4-8 nucleate embryo sac
4	Falling of petal and anther	Nucellar cell Programmed Cell Death to form inactivation in the ovary and embryo sac matured
4		Double fertilization
4-7		Development of endosperm and ovary wall
7-8		Development of zygote
9-10		Fruit matured

Table 1. Developmental stages of megaspore and gametophyte in the same flower in *Santalum album* as indicated by flower or fruit diameter

with the micropylar cell becoming the functional spore while the remaining 3 cells degenerate. Rapid micropylar elongation then ensues and a one-nucleate embryo sac is present by the time flowers reach 3 mm in size.

Comparison of microsporogenesis and megasporogenesis shows that the early development phase is synchronous, however at the time that the microspore is developing into mature pollen, the corresponding megaspore has only just developed to the stage of a uni- or bi-nucleate embryo sac.

Embryo sac development

Development from single to mature 8-nucleus embryo sac takes 2 weeks, during which flower size and embryo sac volume increases slowly. The uninucleate embryo sac develops firstly into a 2-nucleate embryo sac then a 4-nucleate embryo sac by vertical nuclear division. The nuclei in the 4-nucleus embryo sac migrate to the two poles of the embryo sac, and then develop into an immature 8-nucleus embryo sac by the third nuclear division. As the 8-nucleus embryo sac forms, it distinctly elongates at the two poles. Concurrently, some ovular mamelon cells undergo programmed cell death (apoptosis), which provides

nutrition and space for the maturing embryo to become the familiar 'S' or 'N' shape.

The chalaza extends to the top of ovary while the micropyle extends down out of the ovule. There is one egg cell and two synergids at the micropylar end and two polar nuclei in the middle of the embryo sac. The three nuclei develop into antipodal cells. The antipodal cells are able to survive until fertilization. Three embryo sacs in one ovary can usually develop to maturity. The period from the immature 8-nucleus embryo sac to a mature embryo sac lasts approximately 7-10 days. We also observed a change in the number of ovules (from 3 to 4) in different seasons. Usually ovaries consisting of 3-ovules occurred at higher frequencies in Mar-Apr and those with 4-ovules occurred at higher frequencies in Nov-Dec. During the later periods, microspores and megaspores showed far higher rates of abnormal development.

The later developmental stage of the megaspore is relatively slower than the microspore, especially the developmental stage of the 8-nucleus embryo sac (usually requiring 7-10 days for maturity). Hence developmental synchronicity of gametophytes in the

same flower is not realized. When the embryo sac is fully matured, the pollen of the same flower had already shed and dispersed. This dichogamy or lack of overlap limits self-pollination within the same flower.

Development of endosperm and zygotic embryo

Flowers undergoing double fertilization continue growth and development while unfertilised flowers spontaneously drop. Endosperm development precedes zygote development and typically begins at the micropyle site, with a mass of primary endosperm nuclei that gradually forms the endosperm. Concurrently the zygote remains at the single cell stage and zygotic embryo development and differentiation only occurs after endosperm development. As there are several embryo sacs present in the same ovary, fertilization time may

chronous. In some plant species megaspores develop earlier than microspores (e.g. *Magnolia delavayi*) (Gong *et al.* 1998); while the reverse is true for other species such as *S. album*. Dichogamy in some plants with larger flowers having easily visible stamens and pistils (phanerogamous) may be empirically distinguished based on floral developmental characteristics. With *S. album* flowers are very small so it is not easy to determine developmental differences between male and female organs. Our embryological observations show that megasporogenesis and microsporogenesis is concurrent during early floral development, but the microspore develops more rapidly and pollen is mature (*i.e.* pollen is mature), approximately 7-10 days before the embryo sac is mature. Given this, a reduced fertilisation rate and seed

(2000) proposed that pollen sterility, pollen-pistil incompatibility or pistil dysfunction may account for very little or no fruit production in clonal remnant populations of *S. lanceolatum*.

'Self' pollen have been shown to germinate on the stigma and pollen-tubes are able to enter the embryo sac in *S. acuminatum* (Sedgley 1982) and *S. album* (Rugkhla *et al.* 1997) indicating a lack of self-incompatibility mechanism in the stigma and style of these species. Therefore partial self-compatibility in *S. album* may be true to some extent (Sindhveerendra and Sujatha 1989; Veerendra and Padmanabha 1996), but this study found the difference between the onset of mature pollen and mature embryo sac in the same flower is approximately 10 days. The time taken from pollen germination to pollen-tube entry into the embryo sac is approximately 2-4 days (Bhaskar 1992; Jyothi *et al.* 1991; Rugkhla *et al.* 1997), so natural self-pollination that results in self-fertilization within a flower is unlikely.

In this study we have shown that the development of the embryo sac and pollen is not coincident, and even if the pollen could germinate on the stigma and enter into the ovary, the chances of self-fertilization within a single flower are remote. These findings support previous studies that seeds of *S. album* are generally heterozygous through cross-pollination and cross-fertilization (Veerendra and Padmanabha 1996). In addition, our studies offer an explanation of previously conflicting conclusions. *S. album* appears to have a degree of self-compatibility (Sindhveerendra and Sujatha 1989), but self fertilization may only be achieved if the development of pollen and embryo sac is made coincidental (*i.e.* through intervention via artificial pollination) or pollination results from pollen of a different flower same on the same plant (geitonogamous pollination).

Treatment	No. Flowers Tested	Percent fruit set
No pollination (with cover bag)	50	0
Artificial self-pollination	50	12
Artificial cross -pollination	100	14
Open pollination	100	3

Table 2: Percentage fruit set following different pollination treatments

vary between embryo sacs. Embryo sacs undergoing early fertilization may occupy the micropylar site, develop earlier and mature normally. Embryo sacs squeezed to alternative positions usually exhibit delayed or abnormal development.

Pollination trials

No seed sets when inflorescences are bagged, however, 3% of open pollinated flowers set seed. Increased levels of seeds developed following artificial pollination with both 'self' pollen (24%) and 'cross' pollen from another tree (14%) (Table 2). Therefore seed set was limited by pollen transfer under the conditions in the South China Botanic Garden.

Discussion

In the course of evolution, many plant species have developed mechanisms for avoiding 'selfing' and the development of inbred progeny. Dichogamy is one such mechanism where maturity of microspores (*i.e.* pollen) and megaspores (ova) are not syn-

set would be expected when artificial pollination is carried out during pollen shed (anthesis). While no data was recorded, application of pollen to the stigma approximately 7-10 days after anthesis may help maximise potential seed set.

The developmental sequences of microspore and megaspore are asynchronous during the most favourable period for self-pollination within a flower. Even if pollen can germinate partially on the stigma, they are prevented from completing fertilization because the embryo sac is immature. So the flower must accept pollen from other flowers which can include 'cross' pollen from other genotypes or 'self' pollen from flowers of different maturity on the same plant (geitonogamous pollination). The natural cross-pollination rate depends on climatic factors that influence wind dynamics and insect activity, which can contribute to an unpredictable fruit set in *S. album*. Warburton *et al.*

Acknowledgements

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Profile of natural stands of *Santalum album* L. in the Pondicherry region, India

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Abstract

The enumeration of sandal and its habitats, soil type, girth, height, status of reproduction and associated plants were studied within the Pondicherry region in India. A total of 463 mature sandal trees (girth at breast height (gbh) ≥ 10 cm) and 360 sapling trees (<10 cm gbh) were recorded from 10 different locations, 5 habitats and 4 soil types. The analyses of growth (girth and height), reproduction and associates were compared with different soil types. Generally clay with limestone and sandy soils had substantially greater numbers of sandal trees than clay and red earth soils. *Azadirachta*, *Glycosmis*, *Morinda* and *Phoenix* were the most predominant associated species across all soil types.

Introduction

Santalum album is a small evergreen tree that naturally occurs in India in areas from sea level to 1800m altitude in areas with a rainfall of between 600 – 1600mm with a distinct dry season. It grows in a range of soil types but generally performs in soils that are slightly acidic to neutral, well drained and moderately fertile. Sandalwood in India attains a height of 12 -13 m and a stem girth up to 2.5m with slender drooping as well as erect branching habit. The sandal tree can attain sexual maturity from 2-3 years after germination. It is a partial root parasite, which can host over 300 species of

plants from grasses to other sandal, but shows different growth patterns with different host species (Nagaveni and Vijayalakshmi, 2003).

In India *Santalum album* is distributed in the dry scrub forest of Salem, Mysore, Coorg, Coimbatore, Nilgiris and Thiruvannamalai. It is also found in Andhra Pradesh, Bihar, Gujarat, Karnataka, Madhyapradesh, Maharashtra and Tamilnadu. Kadamban and Balachandran (2005) reported the presence of natural sandal in the Coromandal coastal plains of Pondicherry and Tamilnadu and the aim of this study

was to undertake a broad inventory of sandalwood and its associated species in this area.

Materials and Methods

A survey of the sandalwood resources in the Pondicherry region was undertaken over a period of one year during April 2005 to March 2006. The region is 292 km² in area, and is bounded by Cuddalore and Villupuram districts of Tamilnadu (north, west & south) and Bay of Bengal to the east. The maximum daily temperature ranges between 28 and 32° C during the sunny days and the average relative humidity is 80 %. It receives around 800 mm rain mainly from the north-east monsoon during September to December and has a distinct dry season typically from May to August.. The region consists of the broad soil types of pure sandy, alluvial, loam, black cotton, red earth-laterites, clay and clay with lime stone. The survey was conducted in all the villages of Pondicherry region but the presence of Sandal trees were recorded in only 10 of the 264 villages, which were Abishegapakkam, Edayarpalayam, Jipmer, Madhikrishnapuram, Moolakulam, Pillyarkuppam, Poornankuppam, Pondicherry town, Sedrapet and Thavalakuppam.