Vegetative focused propagation of *Santalum austrocaledonicum Vieillard* (sandalwood) and the reproductive biology of *S. lanceolatum*, *S. album* and *S. austrocaledonicum* for the domestication of sandalwood

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Statement of the Contribution of Others

Nature of Assistance	Contribution	Names, Titles and Affiliations of Co-Contributors
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Abstract

Sandalwood *(Santalum spp.)* is a commercially important forest product that has been traded for many centuries. The trade has been based on the exploitation of wild stands of the wood, but as supplies have dwindled interest in its cultivation has been increasing within agroforestry systems, both as an industrial crop and a small scale product. More recently, interest has increased in the domestication of the more commercially valuable species. Recent studies in Vanuatu into *Santalum austrocaledonicum* have identified individuals with high oil yield and quality that can form the basis of a breeding programme. A lack of appropriate knowledge of the reproductive biology within the genus and the capacity for clonal propagation has hindered progress towards genetic improvement. This situation has led to this study, which has the following objectives: (i) to determine the amenability of cuttings for propagation for *S. austrocaledonicum*; and (ii) elucidate key features of the reproductive biology of *S. austrocaledonicum* and other *Santalum* species that can inform their breeding and domestication.

Method

To develop an effective method for the vegetative propagation of clonal sandalwood (*S. austrocaledonicum*), a series of four experiments were conducted. In these experiments I evaluated the effects of cutting (genotype, type of cutting, and leaf area,) and environmental (exogenous auxin, rooting media, and light intensity) treatments on adventitious root initiation and development (mean root number and length) in leafy stem cuttings using non-mist propagators. The results demonstrated that *S. austrocaledonicum* seedlings can be successfully propagated by cuttings.

Genotype: Cuttings taken from genotypes from the island of Erromango outperformed those from Tanna for all three measures of rooting across all experiments. In experiment 2, for instance, the level of adventitious root induction in Erromango genotypes ranged from 63 to 92% and those from Tanna 0 to 6%. Significant variation was also found among genotypes from Erromango, with genotype 'j-erro' demonstrating greater root induction compared with others across three of the four experiments.

Cutting Type: Cuttings taken from the apical and medial stem positions on the stockplant outperformed those taken from basal stems across the two clones examined. In a complementary experiment (4^{th}), apical cuttings were found to have significantly greater rooting percentage than medial cuttings across the three clones.

Leaf area: The effect of leaf area (400 vs. 800mm^2) was not significant for adventitious root induction, root number or root length. The proportion of leaves retained by the cutting during propagation positively influenced the percentage of cuttings that form adventitious roots. Significantly greater rooting percentage was found in cuttings with no leaf abscission (85%), followed by quarter (71%), half (48%), three quarters (35%) and all (21%) leaves abscised. This result indicates that the retention of some of the leaves is important in propagation by cuttings of this species.

IBA: The application of the exogenous auxin (3000, 4000 & 8000 ppm indole-3-butyric acid) did not have a significant effect on adventitious root induction, mean root number and length when compared with the control (0 IBA).

Rooting Media: I examined the effect of three rooting media 1. Gravel-5mm (29% Air Filled Porosity AFP), 2. Vermiculite and Perlite at 1:1v/v (46% AFP) and 3. Vermiculite, Perlite and Peat 2:2:1v/v/v (42% AFP). No significant differences in percent adventitious root induction or mean root number were found between these three media. The mean length of the roots was, however, significantly shorter in the gravel medium compared with the other two media.

Light Intensity in the propagator: The effect of four different mean daily light levels (116, 86, 56 and 48 μmol m^{-2} s^{-1}) in the propagator on cutting performance (mean root number and mean root length) was examined. The level of light had a significant effect on the percentage of cuttings with adventitious root induction. The influence of light on root induction was not consistent among clones, with a significant interaction found between light and clone. The greatest percentage of root induction was found for a mean daily light level of 86 μmol m^{-2} s^{-1} across all clones, which was achieved in a propagator positioned under 50% high-set shade with an additional 25% shade cloth over the top of the propagator. No significant effect of propagator light level was found for the mean number of roots or mean root length.

Reproductive Biology: To understand the breeding system in sandalwood (*Santalum species*), two experiments were undertaken. In these experiments I evaluated floral phenology in five species (*S. lanceolatum, S. austrocaledonicum, S. album, S. macgregorii* and *S. yasi*), and controlled hybridisation between three species (*S. lanceolatum, S. austrocaledonicum* and *S. album*) of sandalwood.

Floral Phenology: Systematic investigation of *S. lanceolatum, S. austrocaledonicum, S. album, S. macgregorii* and *S. yasi* has found that flowers in all species open rapidly (over 3-4 hrs), primarily in the morning. Flower life of an un-

pollinated flower varies significantly among the species, with mean days of flower life ranging from 24 hours (*S. macgregorii*) to 8.7 days (*S. album*). Full closing of flowers after opening was displayed among some of the species. *S. lanceolatum* (12-24 hrs), *S. austrocaledonicum (12-36 hrs)* and *S. macgregorii* (up to 12 hrs) closed after opening, while *S. album* and *S yasi* remained open. A change in colour of the tepals was observed in *S. album S. yasi* and *S. magregorii.* The tepals changed from white to pink after opening in *S. album* (19-24 hrs) and *S. yasi* (4-12hrs), and then changed progressively to dark red/purple. In *S. macgregorii*, the flower bud turned pink just before opening, and changed colour to red/purple after flower opening.

Breeding system of *Santalum lanceolatum*: Self- and intraspecific cross-compatibility was examined in 13 genotypes of *S. lanceolatum,* and interspecific cross-compatibility between *S. lanceolatum* with each of *S. austrocaledonicum* and *S. album*. A total of 20% of genotypes formed seed after self-pollination. Conversely, a total of 62% of genotypes pollinated with outcross (intraspecific) pollen set seed. *S. lanceolatum* was found to be cross compatible with both *S. austrocaledonicum* or *S. album*. However, although the percentage of seed set was similar between intra- (7.5%) and interspecific (7.6%) crosses, the germination of seeds produced through interspecific pollination was greater (*S. album* = 114% (some seeds producing 2 seedlings), and *S. austrocaledonicum* = 70%) than those produced through intraspecific (41%) pollinations. The implications of this breeding system on both the domestication of sandalwood as well as the genetic conservation issues surrounding introduction of a foreign species into a natural population are discussed.

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Chapter 1. Introduction

1.1 Background

Many rural communities in developing countries continue to depend on natural forests and their products to varying degrees for food, fibre, timber, medicine and a source of income (Costanza *et al.*, 1997; Sunderlin *et al.*, 2005; Syampungani *et al.*, 2009). This reliance, combined with population growth, has placed increasing pressure on these forests, and in many cases led to their degradation (Burley *et al.*, 2011). One strategy to limit further degradation involves developing key species for production, and thus reducing community reliance on natural sources. Cultivation of previously wild trees has the potential to provide an alternative source for these products and is an attractive proposition for many rural communities (Meyfroidt & Lambin, 2011). However, the long term success of this activity will require the development of effective methods of cultivation, as well as the development of improved domesticated tree forms (Simons & Leakey, 2004).

Domestication is the process by which plants and animals become genetically distinct from their wild relatives through the process of human-imposed selection (Harlan, 1992; Purugganan & Fuller, 2009; Gross & Olsen, 2010). A domesticated plant invariably possesses more desirable characteristics, which increase the benefits of its cultivation relative to wild harvest (Diamond, 2002). With their short generations annual crops, have been the first crops to be cultivated and domesticated (Harlan, 1992), although domestication of tree crops followed closely (Zohary & Spiegelroy, 1975), as people began to live a more sedentary life (Zohary & Hopf, 2000).

Trees have been important to the cultural and economic development of many civilizations (Dafni 2006). Olives (Olea spp.), dates (*Phoenix dactylifera*), figs, (*Ficus carica*) and pomegranates (*Punica granatum*) were among the earliest trees to be domesticated in the near East. These domestications dated between 3200-2700 BC (Zohary & Spiegelroy, 1975). In Mesoamerica, avocado (*Persea americana*) trees were possibly planted as early as 6500 BC, with domestication evident by 900 BC (Smith, 1966). Evidence of the frequent use of *Spondias* fruit dates back to 7000 BC, with variants high in sugar proving important to the production of alcoholic beverages (Zizumbo-Villarreal & Colunga-GarciaMarin, 2010). In the Pacific, the nut-producing *Canarium* genus has been cultivated in Melanesia for thousands of years (Matthews & Gosden, 1997; Thomson & Evans, 2006), with multiple domesticated lineages of desirable fruit characteristics being incorporated from both cultivated and wild-harvested species (Weeks, 2009).

Tree domestication is often brought about through vegetative propagation of desirable forms (McKey *et al.*, 2010). Many tree crops have outcrossing/self-incompatible breeding systems, where the fixation of desirable alleles is not possible through inbreeding (Khan *et al.*, 2014). Vegetative propagation offers the benefit of fixing agronomically important characteristics (Zohary & Spiegelroy, 1975; Zohary & Hopf, 2000; McKey *et al.*, 2010), particularly those that have non-additive heritability (Dickmann *et al.*, 1994). Vegetative propagation also has the benefit of reducing the lengthy juvenile-phase of tree crops after propagation from seed (Martin-Trillo & Martinez-Zapater, 2002; Miller & Gross, 2011). Clonal propagation continues to remain a feature of tree crop improvement (Leakey *et al.*, 1982; Mesen *et al.*, 1997b) and the domestication of wild trees (Amri *et al.*, 2010).

Forest trees provide communities with many valuable products, but the demand for wood and agricultural land have contributed greatly to forest degradation (Lambin *et al.*, 2003; Gibbs *et al.*, 2010; Meyfroidt & Lambin, 2011). Wood has been valued since prehistoric times for its unique physical and structural properties, and has long been harvested from natural sources (Fenning & Gershenzon, 2002). As the need for planted forests increased with the increasing density of human population, there was a need to develop its cultivation from exploitative extraction, to natural forest management and plantation forestry (Evans, 1999; Victor & Ausubel, 2000; Meyfroidt & Lambin, 2011). It is within this developmental context that the domestication of forest trees has become important (Boerjan, 2005), and it is only in recent times that forest trees, other than fruit trees, have undergone the initial stages of domestication (Taylor, 2002). Forest trees such as various eucalypts (Turnbull, 1999), conifers (Hermann & Lavender, 1999; Schultz, 1999; Sutton, 1999), and poplars (Heilman, 1999) are now widely planted and have undergone various stages of domestication and improvement.

It is only in more recent times that tropical trees have become the focus of domestication (Evans, 1999; Simons & Leakey, 2004; Leakey *et al.*, 2012). This development possibly reflects the more recent depletion of tropical forest resources in comparison with subtropical and temperate forests. As global food supplies are dominated by only a few highly domesticated species, it is possible that planted forests will also be comprised of a limited number of species from genera such as *Pinus*, *Eucalyptus*, *Populus*, *Acacia* and some species of the Verbenaceae (Jagels, 2006). Despite the importance of these species to global timber supply, some so-called minor forest products have also been severely depleted through selective harvesting. These minor forest products thus require attention. High-value non-timber forest products

(NTFPs) can be of particular importance to the development of small-scale planted forests (Belcher *et al.*, 2005). This development is particularly relevant to rural smallholders in developing countries, such as Vanuatu.

Vanuatu is a small island nation in the South Pacific Ocean with a very small population (~260,000). A significant proportion of Vanuatu's export earnings come from tourism, agriculture and the exploitation of its timber resources, with future growth in the forestry sector to come largely from plantation establishment (Australia. Department of Foreign Affairs and Trade, 2006). The development of forestry in Vanuatu will depend on an expansion of the planted resources of species that meet the demand for specialty products and which are unlikely to be supplied by other countries (Australia. Department of Foreign Affairs and Trade, 2006). Sandalwood (*Santalum austrocaledonicum*) is one such tree species, which has a significant potential for development. Sandalwood trees produce scented heartwood oils that are highly valued in the manufacture of incense products, aromatic oils and scent, carvings, soaps and pharmaceutical products (Sen-Sarma, 1977; Thomson, 2006). The popularity and high price of sandalwood has resulted in intense harvesting of its natural stands, and has resulted in a reduction in product supply (Gillieson *et al.*, 2008).

Santalum is a hemi-parasitic genus with plants that form parasitic root connections with other plants through specialized organs known as haustoria (Tennakoon & Cameron, 2006). They obtain a part of their water and nutrient requirements through these haustorial connections, while the remaining component is derived directly from the soil (Loveys & Tyerman, 2001). It is this feature of the genus' biology that has presented challenges for its production in plantations, since sandalwood needs to be produced in combination with other plants, and will not

perform under monoculture (Fox & Barrett, 1994). Sandalwood requires access to suitable hosts over the entire production rotation, including during its time in the nursery (Radomiljac, McComb*, et al.*, 1998) (Radomiljac, 1998; Annapurna *et al.*, 2004), during establishment (Fox *et al.*, 1996; Radomiljac *et al.*, 1999) and during its maturation phases (Brand, 2009; Page, Tate, Tungon*, et al.*, 2012; Lu *et al.*, 2014). Sandalwood can potentially make haustorial connections with many different species, but can have a preference for some plants. In a review of hosts reported in the scientific literature, da Silva *et al.* (2016) listed 118 suitable and 49 non-suitable sandalwood hosts across three species (*S. album*, *S. acuminatum* and *S. spicatum*) grown in Australia, China and India. Half of the suitable host species were from nitrogen fixing families with Fabaceae (22 species), Mimosaceae (22) and Casuarinaceae (10) having the most species represented. While it is evident that sandalwood has a preference for nitrogen fixing hosts, species from each of these families (6, 3, & 1 respectively) were also recorded as non-suitable.

The development of a viable planted sandalwood industry in Vanuatu will be assisted through domestication and improvement of sandalwood for productive and high quality forms (Page, Potrawiak*, et al.*, 2010). Early work on the species has demonstrated that significant variation exists within the species for important oil production and quality traits, which can form the basis of improvement (Page *et al.*, 2007). The process of sandalwood domestication will, in turn, depend upon scientific understanding of its reproductive biology and vegetative propagation characteristics. These areas of research were considered important for the development of new genetic variation through sexual reproduction and the capturing of genetically unique, commercially valuable individuals through clonal reproduction. Thus, they form the basis of this research.

In this thesis, I examine the clonal propagation of *Santalum austrocaledonicum* using leafy stem cuttings in order to determine the optimal environmental and morphological conditions for root initiation and development. I also examine the fundamental aspects of sandalwood reproductive biology with respect to floral and fruiting phenology. I also examine the level of self and intra-specific compatibility within *S. lanceolatum* and inter-specific compatibility with two other species of sandalwood (*S. album* and *S. austrocaledonicum*).

1.2 Description of the thesis

1.2.1 Research aim

The aim of the research was to develop a greater understanding of the breeding of *Santalum lanceolatum, S. album* and *S. austrocaledonicum* through controlled pollination, and the vegetative propagation of *Santalum austrocaledonicum* through leafy stem cuttings. The specific objectives of the research were to (a) develop robust protocols for the cutting propagation of *Santalum austrocaledonicum* for the development of clonal cultivars, (b) improve the understanding of variation in floral phenology among five commercial species of sandalwood and (c) determine the predominant breeding system in *Santalum austrocaledonicum* in order to assist in the design of appropriate conservation and domestication strategies.

1.2.2 Research questions

1) What are the genetic, cuttings and environmental factors that influence the initiation and development of adventitious roots in leafy stem cuttings of *Santalum austrocaledonicum,* including:

   (a) Genotype origin (Erromango vs. Tanna) and clone,

(b) Relative cutting position (apical, medial and basal) from the stem of the stock plant,
(c) Cutting leaf area and stem dimensions,
(d) Propagation media,
(e) Use and concentration of indole-3-butyric acid (IBA), and
(f) Level of irradiance in the propagation environment.

2) What are the main phenological stages of flowering in five species of sandalwood (*Santalum album, S. austrocaledonicum, S. lanceolatum S. magregorii* and *S. yasi*), and what is the variation in expression and duration of each stage among the species?

3) What is the relative level of self- and cross- compatibility in *S. lanceolatum*, and the inter-specific compatibility between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum*?

1.2.3 The study

The research aim of this thesis was carried out to advance our understanding of the practical propagation and breeding of sandalwood. This knowledge was considered to be essential to the domestication of sandalwood, by informing the practices of:

1) Cutting propagation particularly for the bulking up of selected individuals for production, and

2) Controlled pollination, which is essential for generating new genetic diversity in the breeding population by crossing selected individuals and generating hybrid individuals. Understanding the nature of inter-specific hybridisation was also of importance to determining the impact of

introducing exotic sandalwood species within the natural range of other commercially important sandalwood species.

1.2.4 Thesis structure

1.2.4.1 Chapter 2

This chapter comprises a review of literature relevant to the studies of the experimental chapters. The subject of this review is the theory of and methodologies for vegetative propagation and reproductive biology in perennial woody species. The review compares procedures and methodologies used to successfully domesticate a range of tropical timber and ornamental tree species and their relevance to breeding of *Santalum*. The literature review is divided into two parts, the first part of the review covering vegetative propagation and the second part related to reproductive biology.

1.2.4.2 Chapter 3

Clonal propagation through cuttings is a desirable option for developing improved germplasm as well as routine propagation within the sandalwood plantation sector. Clonal propagation is considered to have advantages over propagation by seeds in terms of fixing desirable characters in selected individuals and improving uniformity in growth, oil yield and quality within the planted population. Chapter 3 comprises four experiments on vegetative propagation to examine the different environmental and morphological conditions required to achieve maximum rooting. The scope of some of the experiments has been limited by the availability of suitable material.

1.2.4.3 Chapter 4

This chapter covers two key aspects in the reproductive biology of *Santalum* species. Firstly I conducted a study on the floral phenology of five species of

sandalwood (*S. album*, *S. austrocaledonicum S. lanceolatum*, S *macgregorii* and *S. yasi*), in which I defined the characteristics and duration of particular floral stages within each species and quantified variation in expresssion and duration between the species. In the second part of the study I conducted controlled pollination experiments to determine the level of self- and intra specific compatibility within *S. lanceolatum* as well its interspecific cross-compatibility with *S. album* and *S. austrocaledonicum*.

1.2.4.4 Chapter 5

This chapter covers general discussions and observations in relation to chapters three and four.

1.3 Introduction to the genus *Santalum*

Santalum is a genus from the family Santalaceae (Merlin & Van Ravenswaay, 1990; Harbaugh, 2005) and comprises 16 species (Butaud *et al.*, 2006) with a natural distribution from India to the Juan Fernandez islands (Applegate *et al.*, 1990). *Santalum* species occur in seasonal tropical and semi-arid temperate environments in the Indo-Pacific region including in Australia, Cook Islands, Timor Leste, Fiji, French Polynesia, Hawaii, India, Indonesia, New Caledonia, Papua New Guinea, Sri Lanka, Tonga and Vanuatu (Applegate *et al.*, 1990).

Sandalwood is highly sought after and valued for the scented aromatic oils that accumulate within its hardwood (Bule & Daruhi, 1990). Sandalwood has significant cultural importance in many parts of Asia. Many Hindus, Buddhists, Chinese and Muslims have used the oil and burnt the wood during ceremonies over many centuries (Yusuf, 1999). The main consumer uses of sandalwood in eastern markets include incense, handicrafts and carving, toiletries, mouth freshener and medicinal uses. In

addition it is used as a non-alcoholic fragrance and flavouring agent (Sen-Sarma, 1977; Thomson, 2008). Sandalwood is also valued in western societies. There it is used as incense and its oil is used as a key component in perfumes, toiletries, cosmetics and aromatherapy (Australian Agribusiness Group, 2006). In the Pacific, the heartwood is also traditional used for medicinal purposes as well as in combination with coconut oil as a scented body lotion (Brennan & Merlin, 1993).

Santalum species generate significant income for many rural communities. For instance, in Vanuatu sandalwood owners were paid a total of $US55 million in between 2007 and 2014 for trading sandalwood. Because of this high commercial value, sandalwood species have been over exploited in almost all areas where they grow naturally. In the Pacific region, sandalwood has been exploited since the early 1800s, at which time it was traded for tea in China by foreign merchants (Shineberg, 1967). As demand has increased, excessive sandalwood harvesting has led to the degradation of current wild stocks leading to supply shortages. This has resulted in the resource being able to only supply 10% of the potential world demand for sandalwood oil (Sita & Bhattacharya, 1998).

Growers typically propagate *Santalum* species through seeds collected from the wild. In Vanuatu the seeds normally come from populations with high tree-to-tree variation found within and between populations (Page, Southwell*, et al.*, 2010). In *Santalum album* (Indian sandalwood) the breeding system is mixed with outcrossing, accounting for 53% to 75% of seed production (Veerendra & Padmanabha, 1996). This mixed breeding system derived from wild collections results in great variability in important heartwood oil and productivity characters for the seedlings. For all sandalwood species there is a need to understand the predominant breeding system in

order to determine the potential variability generated from wild collected seed. In addition, the potential for cross hybridisation between species needs to be determined.

Given the predominantly outcrossing nature of many woody tree species (Griffin *et al.*, 1987; Hall *et al.*, 1994; Heliyanto *et al.*, 2005; Wallwork & Sedgley, 2005; Ward *et al.*, 2005; Nayak & Davidar, 2010; Pei *et al.*, 2011), including *Santalum album* (Veerendra & Padmanabha, 1996; Rugkhla *et al.*, 1997; Kulkarni & Muniyamma, 1998; McComb & Jones, 1998; Ma *et al.*, 2006; Muir *et al.*, 2007), it is probable that a degree of outbreeding exists in other species of *Santalum*. Therefore, to develop a line of good quality trees for cultivation, clonal propagation could be considered in the development of an improvement strategy. Therefore, it is important to determine the feasibility for routine cutting propagation as a potential avenue for multiplying and propagating clonal cultivars.

Chapter 2. Literature Review

Reproduction and multiplication of flowering plants can be achieved through

1) Sexual reproduction, which involves the fusion of 'male' and 'female' haploid gametes to form a diploid zygote (Hale *et al.*, 1995),

2) Asexual reproduction, in which new plants are produced through replication of vegetative structures (Hartmann *et al.*, 1997), and

3) Apomixis, defined as the formation of seeds without sexual reproduction typically forming an embryo through mitosis within the tissues of the ovule.

The domestication of woody perennial species often exploits the relative benefits of both sexual reproduction in order to generate new genetic variation in which to make new selections and clonal propagation to capture and disseminate these selections. In this chapter, the literature on vegetative propagation and reproductive biology is reviewed and discussed.

2.1 Vegetative propagation

Vegetative propagation is the asexual means for plant reproduction through vegetative plant parts, also known as clonal propagation (Hartmann *et al.*, 2002). Clonally propagated plants are exact genetic copies of the parental/donor plant. Common vegetative propagation techniques include cuttings, tissue culture, grafting, budding and layering. In the practical breeding of plants, vegetative propagation is a tool used to capture and disseminate individuals with combinations of desirable genes

(Eldridge *et al.*, 1993; Libby & Ahuja, 1993). The benefit of establishing plantings with clonally propagated plants is the high uniformity between individuals.

Vegetative propagation has been successfully achieved with many tree species that were previously considered 'difficult-to-root', and the methodologies applied to promote higher survival rate have been well documented. This information is particularly relevant for the vegetative propagation of many *Santalum* species which have been reported to be difficult to propagate clonally (Rao & Srimathi, 1976; Balasundaran, 1998).

Clonal forestry through vegetative propagation has been practiced for several centuries. Species such as *Cryptomeria japonica* in Japan (Toda, 1974), *Cunninghamia lanceolata* in China (Minghe & Ritchie, 1999), *Salix* and *Populus* spp. in Europe (Evans & Turnbull, 2004) have long been propagated by clonal means. In the 1960s and 70s, these techniques were applied through national programmes to conifers and tree species within *Eucalyptus*, such as Aracruz in Brazil (Eldridge *et al*. 1993), and later through private sector programmes such as Shell Forestry in Chile (Zobel, 1992). In the 1980s and 90s, vegetative propagation techniques were extended to include fruit, nut and medicinal tree species for tropical agroforestry. Some of these species include *Irvingia gabonensis* (Shiembo *et al.*, 1996), *Dacryodes edulis* (Tchoundjeu, Avana*, et al.*, 2002), *Ricinodendron heudelotii* (Anigbogu *et al.*, 1996), *Gnetum africanum* (Shiembo *et al.*, 1996; Fondoun & Manga, 2000), *Prunus africana* (Tchoundjeu, Kengue*, et al.*, 2002) and *Pausinystalia johimbe* (Tchoundjeu *et al.*, 2004).

The primary vegetative propagation techniques employed are cuttings, grafting, air-layering and tissue culture (Leakey, 1985; Leakey, 2004a). Suckering is a natural vegetative propagation method (Leakey *et al.*, 1982), by which the shoot regenerates

from either a stump or an injured root. Development of tissue culture techniques, which involves culturing of organs, is capital intensive, highly technical and requires skilled labour. In the tissue culture of *S. album* Ananthapadmanabh, Rai (1998) reported problems with adventitious root development for tissue cultured propagules, although Bele *et al.* (2012) indicated better success using this method. Other methods involving stem cuttings under fog or intermittent mist have been extended to a range of plant species (Hartmann *et al.*, 1997). The non-mist propagator (Leakey *et al.*, 1990) has been used successfully in rooting several 'difficult-to-root' tropical species (Taylor & Dimsey, 1993). The non-mist propagator is cost effective and low technology (not requiring running water or electricity), and may well suit the situation and needs of remote and local communities.

2.1.1 Benefits of vegetative propagation

Plants developed through vegetative propagation (ramets) are genetically identical to the 'parent' plant (ortet) and therefore very little variation should be observed between their phenotypes (Mesen *et al.*, 1997b; Hartmann *et al.*, 2002). When the ortet has been specifically selected for particular desirable characteristics, these characteristics are also expressed in its ramets, provided no somatic mutation occurs. Therefore, vegetative propagation has been a practical and successful method for plant improvement (Zobel, 1992; Hartmann *et al.*, 2002).

Clonal propagation is particularly important for improvement of tree species because its processes (usually grafting) can shorten the juvenile period (relevant for tree fruit crops). Traditional breeding through recurrent selection is time consuming for trees, due to the length of time it takes for them to attain sexual maturity. This long juvenile period means that one generation of breeding may take many years. This time

lapse substantially reduces the rate of genetic gain when compared with annual crops that have one or more generations per year (Acquaah, 2007).

Additionally, many tree species have an obligate outbreeding system, which means that it is not possible to fix characters in the homozygous form for reliable replication in subsequent seed generated cultivars. The use of clonal propagation circumvents this limitation through mass production of desirable forms that reliably express the selected heterozygous characters (Acquaah, 2007). The combination of clonal propagation and sexual reproduction through the vegetative propagation of selected progeny within the F1 generation is a very successful method for reproducing the desirable characteristics of both parents in a small number of progeny, and for securing the potential benefits of hybrid vigour (Acquaah, 2007).

The advantages of vegetative propagation are particularly evident among trees and plants that have poor fruit and seed production capabilities, seeds with low viability and which are difficult to collect and/or store, seeds that are very short lived (recalcitrant), seeds that are difficult to mass propagate, and for those setting hybrid seeds that are sterile (Leakey & Simons, 2000). Vegetative propagation offers an alternative form of reproduction that does not require successful seed production.

Cutting propagation is most suited to operational propagation of forestry trees, since there is a requirement for a greater number of individuals, each of which have a lower market value compared with horticultural trees. Therefore, the focus of this review is on cutting propagation, since it most suitable for routine mass clonal propagation of sandalwood. Particular prominence is accorded to stockplant management, the influence of pre- and post-severance factors and environmental conditions that influence cutting success.

2.1.2 Vegetative propagation by cuttings

Vegetative propagation through cuttings for clonal forestry has been reported to be successful for the establishment of clonal plantations of *Eucalyptus* spp. (Eldridge *et al.* 1993), *Acacia* spp. and *Gmelina arborea* (Evans & Turnbull, 2004). Many 'difficult-to-root' tropical trees have been successfully grown through considered management of stockplants (Leakey, 1983), cutting stem length (Leakey & Mohammed, 1985), leaf area (Leakey & Courtts, 1989; Mesen *et al.*, 1997a) and propagation environment (Mesen *et al.*, 1997a). Factors affecting root initiation and development such as rooting hormones, temperature, light and humidity and their effect on rooting have also been well documented (Hartmann *et al.*, 1997). Collectively, this information could assist with development of experimental work for cuttings propagation of *Santalum austrocaledonicum*.

2.1.3 Biological factors affecting cutting performance

2.1.3.1 Stockplant management (pre-severance)

Stockplants are the source plants from which cuttings are collected. These may be raised either as seedlings, clones, or mature coppiced plants. The survival and performance of cuttings is influenced by stockplant physiology, which can be manipulated through its management (Leakey, 2004b). Therefore, to promote suitable shoot material from the stockplant it is essential to understand and, where appropriate, modify the environmental conditions of the plant (Hoad & Leakey, 1996).

While cuttings may be collected from wild grown plants, successful mass propagation of a majority of tree species usually requires stockplants to be managed as pruned hedges or coppice stools. Stockplant hedges may be established in the ground (nursery beds) or in pots, and aspects such as soil water availability, nutrition, light

environment, and juvenile vigour are usually managed intensively. The aim of stockplant management is to maintain the health and vigour of the shoots, in order to maximise the number of cuttings produced and and to optimise their rooting capacity.

2.1.3.2 Stockplant nutrition

The positive effect of stockplant nutrition on adventitious rooting of cuttings is generally known, but the specific role of various nutrients in stimulating adventitious rooting has yet to be fully understood for many species (Hartmann *et al.*, 1997). A vital part of nutrition management is to ensure that stockplant health and vigour is maintained. The application of fertilisers to stockplants may have both positive and negative effects on adventitious rooting (Becker *et al.*, 1991). It is therefore thought that the effect of nutrient application may not be a stand-alone factor, but may interact with other factors including light, water and the physiological condition of the stockplant.

In *Triplochiton scleroxylon*, stockplant fertilisation had a positive effect on the formation of adventitious roots in cuttings (Leakey, 1983). The cuttings from the basal nodes increased rooting when the stockplant was given fertiliser (N 23%, P 19.5%, K 16%) 16 weeks prior to collection of cuttings; the number of roots doubled for cuttings taken from stock plants with higher fertiliser concentrations (4.0%) than stockplants with low fertiliser concentrations (0.4%). McDick *et al.* (2004) noted that increasing fertiliser (NPK) to *T. scleroxylon* stockplants increased growth rate of stockplants as well as rooting in cuttings. They also found that rooting percentages increased from 27% from stockplants with lower rates of nutrients to 64% from stockplants treated with high nutrient rates. They also reported that cuttings taken from fertilised *T.*

scleroxylon stockplants achieved 20% rooting in 20 days, while cuttings from unfertilised stockplants took longer, achieving 20% rooting in 40 days.

The effect of nutrients can be modified by other environmental factors such as water availability, light quality, photoperiod and temperature to impact on the physiological and morphological condition of the stockplant and root formation in its cuttings. For example, low irradiance combined with low nutrients has been observed to result in longer internodes with high rooting ability in *Albizia guachapele* (Mesen *et al.*, 2001). The authors reported a negative relationship between rooting percentage with each level of irradiance (200 -500 μmol m^{-2} s^{-1}) and increase in nutrients (20%N 20%P 20%K) (0.25-1.25%). They found that increasing irradiance and nutrients (NKP) decreased rooting percentage from 53.8% to 11.2%.

In the above examples, the addition of fertiliser as a supplement for plant nutrition had either positive or negative influences on adventitious rooting, a result that varied among species. Nutrients alone may not affect rooting in cuttings, and any assessment should consider the broader context and the effects of other factors which form the environment of the stockplant. High success of rooting may be achieved by manipulation of nutrients and irradiance simultaneously, as shown in *Albizia guachapele* (Mesen *et al.*, 2001). Finally, species effects play a significant role in rooting, meaning that each species may respond differently to different NPK applications, and that such responses must be treated separately for each species.

2.1.3.3 Stockplant irrigation

Water is crucial for plant growth, acting as a solvent and transport medium for mineral solutes within the plant. Together with carbon dioxide, it is essential in photosynthesis. The movement of mineral nutrients from the root to the leaves, and

that of carbohydrates from the leaves to other parts depends on the movement of water (Loach, 1977). The role of water in maintaining cell turgidity, particularly in juvenile meristematic tissues, as well as cell division and growth, highlights its importance in the functioning of a plant (Hartmann *et al.*, 2002). Therefore, maintenance of adequate soil moisture through irrigation is particularly crucial in maintaining stockplant health, vigour and longevity.

The rooting capacity of cuttings is intimately related to the water balance between transpiration and absorption (Loach, 1977). Rooting capacity is reduced when the plant is affected by water stress. In stockplants, cell turgidity needs to be retained when preparing stockplants for collection of cuttings. Loach (1977) noted that cuttings taken from water stressed stockplants may produce gas bubbles in the xylem tissues, which may block water vessels from the transportation of water, carbohydrates and mineral nutrients.

To ensure that cuttings maintain cell turgidity, the time of collection is important. Cuttings are best collected during the earlier part of the day, when the plant material still retains much water (Hartmann *et al.*, 2002). The best collection periods would then be either in the morning or late afternoon on sunny days, or on cool rainy and cloudy days, when hedges are less likely to be stressed. According to Loach (1977), propagules collected from actively growing stems performed better than those collected from 'dormant' stems. Cuttings collected during periods of high cell turgidity should have a higher chance of survival. Water stress may result in cell water stress, which may result in suppression and shrinkage of the cell wall, and lead to a delay in root initiation (Loach, 1977).

2.1.3.4 'Age' of stockplant

The age of a plant can be characterised in three ways:

1) Chronological –gradient occurs with time;

2) Ontogenetic – gradient towards reproductive maturity, and

3) Physiological – occurs on individual shoots as they mature and increase in secondary metabolites (Leakey, 2004b).

The ontogenetic effects on reproduction and on the growth and development of woody plants have two distinct stages of development:

1) The juvenile stage in which plant growth and development is restricted to only vegetative growth; and

2) The mature stage in which the plant has the capacity to initiate development of sexually reproductive tissues (Hackett, 1986; Wendling *et al.*, 2014; Rasmussen *et al.*, 2015).

The maturity of the plant brings with it cellular and physiological changes, which include lignification of shoots resulting in difficulties in vegetative propagation.

The adventitious development of roots in many tree species is often more difficult in more mature plant tissues. Rooting success in *Robinia pseudoacacia* was lower in cuttings taken from mature plant tissues (66%) compared with juvenile tissues (83%) (Swamy *et al.*, 2002). Poor root initiation in cuttings collected from mature plant parts has also been reported for other species, including *Pinus taeda* (Hartmann *et al.*, 1997), *Prunus avium* (McDick & Leakey, 2006), and *Eucalyptus grandis* (Paton *et al.*, 1970). McDick and Leakey (2006) demonstrated that mature cuttings have very

different physiology and so the difficulty of rooting mature cuttings may not necessarily be due to ontogenetic aging, but rather to the attributes of physiological age. For instance, they found that *Prunus avium* had a higher rooting percentage (71%) of physiologically juvenile compared with mature (5%) cuttings, despite both being collected from ontogenetically mature parts of the canopy.

Two possible causes may be attributed to the negative influence of a mature physiology on cutting success. Firstly, various types of endogenous rooting inhibitors have been found in higher concentrations in mature compared with juvenile stems of *Eucalyptus* (Paton *et al.*, 1970; Fogaca & Fett-Neto, 2005), *Backhousia* (Kibbler *et al.*, 2002) and *Picea* (Bollmark & Eliasson, 1990). Secondly the increasing complexity of a tree as it matures leads to lignification of mature tissues, with the reduction in endogenous auxins and a high concentration of rooting inhibitors (Swamy *et al.*, 2002) and propensity of wound-induced compounds (De Klerk *et al.*, 1999; da Costa *et al.*, 2013).

Although the 'rooting' of cuttings from old and sexually mature trees can be difficult to achieve, a state of ontogenetically juvenile growth can be retained in order to ensure that rooting is achieved over long periods. Despite such difficulties, rooting from mature plants is possible and can be achieved through rejuvenation (Leakey, 2004b), by techniques such as coppicing (McComb & Wroth, 1986), root sprouts (Leakey, 1985; Balasundaran, 1998), serial grafting (Huang *et al.*, 1992; Hartmann *et al.*, 1997), or air layering (Evans & Turnbull, 2004). Juvenility can be maintained through regular pruning of seedling hedges. In species such as *Pinus taeda* (Hamann, 1998) and *Eucalyptus* spp. (Hartney, 1980), the material from juvenile maintained hedges has a similar 'rooting' capacity to seedlings. Thus, maintaining juvenility

through well-managed hedges enables longer-term clonal propagation of elite family lines through cuttings.

2.1.3.5 Genetic variation

The capacity for cuttings to initiate adventitious roots may vary within a provenance between species, provenances and even genotypes. For instance, (McDick *et al.*, 1996) observed variable rooting percentages between genotypes from the same provenance in leafy cuttings of *Calliandra calothyrsus.* However, the authors indicated that variation of the morphological and physiological factors affecting stem growth within the ortet and the cutting itself was the contributing cause, rather than genetic variation in the rooting process that conferred the apparent variation.

Variation in rooting capacity between clones has been described for several tree species. Shepherd *et al*. (2005) found such variation to exist between cuttings from hybrid clones of *Pinus elliottii* × *P. caribaea,* in which F1 genotypes rooted better (73% ± 0.9) than F2 genotypes (65% ± 0.9). Husen (2013) found similar variation in rooting of two clones of *Tectona grandis* (clone FG 1 = 45.89% and clone FG 11 = 26.82%). Strong genotype variation has also been found for adventitious root induction in cuttings of *Backhousia citriodora* (Kibbler *et al.*, 2004a) and mango (Reuveni & Castoriano, 1993).

2.1.3.5.1 Light quality

The qualities of the light spectrum to which stockplants have been exposed can have an effect on their internal physiology and influence success rates in cutting propagation, although specific effects vary between species (Newton *et al.*, 1996; Hartmann *et al.*, 2002; Nissim-Levi *et al.*, 2014). In *Eucalyptus grandis*, the rate of

photosynthesis per unit leaf area and concentration of chlorophyll increased when the R:FR (red: far red) ratios were increased (Hoad & Leakey, 1996). In *Artocarpus heterophyllus* the highest rooting percentage (100%), maximum number of roots (6.3) and root dry weight (62 mg) per cutting was found in cuttings sourced from stockplants that were exposed for 45 days of low light (3% of full sun) with a low R:FR ratio (0.4;) followed by 15 days exposure to full sun before cutting collection (Hossain & Kamaluddin, 2011). Different responses to stockplant physiology were found between *Terminalia spinosa* and *Triplochiton scleroxylon* exposure to different ratios R:FR, which also affected adventitious root development in their cuttings. Success in the former species was promoted by a higher R:FR ratio (3.1), and in the latter species by the lower R:FR ratio (0.5) (Newton *et al.*, 1996).

2.1.3.6 Post-severance cutting factors

Both morphological and physiological factors of a cutting can affect its survival, and rooting during propagation (Hoad & Leakey, 1996). Internal carbohydrate concentration in a cutting is an important characteristic that can influence its capacity for successful root initiation (da Costa *et al.*, 2013). This initial carbohydrate can be drawn upon for metabolic functions following its severance from the ortet for hardwood cuttings, while softwood leafy stem cuttings will depend more on carbohydrates produced from photosynthesis in the propagator (Leakey, 2004b). The level of carbohydrate is influenced by the characteristics of the cutting such as stem volume and leaf area.

2.1.3.7 Leaf area

The retained leaves in a cutting strongly influence the carbohydrate dynamics following its severance from the stockplant (Leakey & Courtts, 1989; Tchoundjeu, Avana*, et al.*, 2002). In particular, they have two roles:

1) To produce essential sugars through photosynthesis, and

2) To affect water loss through transpiration (Loach, 1977).

Leaves have been found to have a significant effect on successful rooting of many tropical hardwood species (Leakey, 1985; Leakey & Courtts, 1989; Mesen *et al.*, 2001; Tchoundjeu, Kengue*, et al.*, 2002). The optimal leaf area for a cutting will maintain a balance between photosynthesis and transpiration. A small leaf area may provide inadequate assimilates to meet the energy requirements of the metabolic process that maintain the cutting and support adventitious root initiation and development. A large leaf area may result in higher transpiration rates causing water stress and consequent cutting death or stomatal closure that limits further photosynthesis, resulting in low rooting percentages (Leakey & Courtts, 1989).

Mesen *et al*. (1997a) reported that, immediately after severance, cuttings undergo physiological stress, which may result in gradual depletion of carbohydrates in each cutting. This decrease in stem carbohydrates may take a few days, and only begins to increase once roots are established (Veierskov, 1986). The contribution of carbohydrates from current photosynthesis varies according to a range of factors including light intensity (environmental) (Mesen *et al.*, 1997a), stomata aperture (physiological) (Leakey & Courtts, 1989; Chaves, 1991; Flexas *et al.*, 2002) and leaf

area (morphological) (Mesen *et al.*, 1997a). This variable carbohydrate assimilation influences the rates of carbohydrate depletion.

The influence of cutting leaf area on rooting percentage and root number has been demonstrated for cuttings of woody plants such as *Prunus africana* (Tchoundjeu, Avana*, et al.*, 2002), as well as non woody ornamentals such as *Begonia spp*. (Ottosson & Welander, 1997). The former study found the percentage of cuttings that developed roots and the number of roots on each cutting were significantly greater in cuttings with larger leaf areas (20 and 25 cm^2 - 79% and 14 roots per cutting respectively) than those with smaller leaf areas (5 and 10 cm^2 - 40% and 5 roots per cutting respectively). Optimal cutting leaf area will vary between genotypes and species (Leakey, 2004b).

The effect of leaf area on increased dry-weight has been observed in *T. scleroxylon* cuttings (Leakey & Courtts, 1989). In this case, the authors reported a successive increase in total dry weight of cuttings during six weeks in the propagator, indicating the important function that leaves play in supporting growth during propagation. They also reported that rooting in cuttings with 10 cm^2 and 50cm^2 leaf area exceeded 80% compared to cuttings with 100cm^2, which achieved only 65% rooting, indicating that large leaf areas are subject to greater transpiration rates than photosynthesis.

2.1.3.7.1 Leaf area and light effects on rooting

In a study on *Cordia alliodora*, Mesen *et al.* (1997a) found that, under high irradiance, the number of roots in stem cuttings with leaf areas of 20 cm^2 and 30 cm^2 were lower than cuttings with leaf areas of 5 cm^2. In *Alnus incana*, cuttings exposed to photon flux densities of 40 *u*mol m^{-2} s^{-1} and 190 *u*mol m^{-2} s^{-1} achieved good rooting.

However, when light reached the base of cuttings in both light treatments, it reduced the number of roots as well as length of roots (Hussdanell *et al.*, 1980).

2.1.3.8 Cutting stem length

Cutting stem length can have a considerable effect on the cutting's capacity for adventitious root development. Leaky and Mohammed (1985) reported a positive correlation between rooting percentage and cutting length in *T. scleroxylon* resulting from the contribution of accumulated carbohydrates towards adventitious root initiation and growth. In hardwood cuttings of *Azadirachta indica*, Palanisamy (1997) found that adventitious root formation was greater in cuttings with a stem length of 25 cm (100%) than those of 12 cm (47%). They further noted that at a constant length of 25 cm, rooting percentages declined with increased diameter. For instance, rooting percentage for the diameter of 0.5 cm was 100%, 77% for the diameter of 1.0 cm, and 70% for the diameter of 2.0 cm.

Apart from limited carbohydrate reserves, shorter cuttings may also have physical barriers to successful propagation. Mesen *et al.* (2001) found that cuttings with short-length basal nodes are more susceptible to rot, because their leaves are more likely to touch the propagating media. In considering the importance of stem length of juvenile cuttings to propagation success, stockplants may be managed (by growing under low light conditions) to produce longer shoot internodes, from which longer stemmed cuttings can be collected.

2.1.3.9 Auxins

Auxins are plant growth regulating substances that influence cell division and elongation (Hale *et al.*, 1995). Endogenous auxins are known to stimulate plant growth, particularly the leaf primordia and young leaves (Hartmann *et al.*, 1997), and roots

(Blakesley & Chaldecolt, 1993). Concentrations of endogenous auxins are higher in the shoots and young leaves where it is produced, but they are translocated basipetally (McDavid *et al.*, 1972; Aloni *et al.*, 2003), where it stimulates root growth (McDavid *et al.*, 1972; Ford *et al.*, 2001; Sorin *et al.*, 2005). Auxins have also been found to be important for the initiation and development of adventitious roots in plant cuttings (Day & Loveys, 1998; Hartmann *et al.*, 2002). Tchoundjeu (2002), reported that the application of auxins to the base of a cutting influenced the transfer of carbohydrates from the apical part of the cutting to its base. The translocated carbohydrates provided the energy for the processes leading to adventitious root formation.

Basipetal movement of endogenous auxins (particularly IAA) occurs naturally, and is principally responsible for root polarity (McDavid *et al.*, 1972; Ford *et al.*, 2001). Ford (2001) found that an auxin translocation inhibitor (TIBA - 2,3,5-triobenzoic acid) effectively reduced the basipetal translocation of IAA in *Syringia vulgaris* and *Forsythia intermedia,* resulting in the reduction of rooting percentage from 80% in control treatment to 6.7% when treated with TIBA. This simple example demonstrates the importance of exogenous IAA on rooting as well as the complexities that an inhibitor can have on the formation of roots.

The production and rate of translocation of endogenous auxins differs among species. It has been reported that IAA is metabolised more rapidly and translocated more slowly in tissues of 'difficult-to-root' species than in tissues of 'easy-to-root' species (McDavid *et al.*, 1972; Ford *et al.*, 2001). Species and genotypes with low levels of adventitious root development due to low levels of endogenous auxins may benefit from exogenous application of auxins to their cuttings.

2.1.3.9.1 Endogenous auxin application to cuttings

Artificially synthesised auxins have been used extensively in horticulture and forestry, primarily to enhance adventitious rooting in vegetatively propagated plantlets (Leakey *et al.*, 1982; Mesen *et al.*, 1997b; Hartmann *et al.*, 2002). The most widely used are Indole-3-butric acid (IBA) and alpha-naphthaleneactic acid (NAA) (Hartmann *et al.*, 2002). Indole-3-acetic acid (IAA) has also been reported to enhance root induction, but has a lower efficacy than IBA and NAA (Rout, 2006). It is apparent that plants respond differentially to particular auxins. For instance, when IBA, NAA and IAA were applied individually to *Camellia sinensis*, IBA was found to be more effective in promoting rooting (Rout, 2006).

The optimum concentration of a particular auxin required to maximise adventitious root induction also varies between species. For instance the optimum IBA concentration for cutting propagation was 0.4% for *Triplochiton scleroxylon* (Leakey *et al.*, 1982), 1.6% for *Cordia alliodora* (Mesen *et al.*, 1997a), 0.2% for *Milicia excelsa* (Ofori *et al.*, 1996) and 0.2 to 1.0% for *Prunus africana* (Tchoundjeu, Avana*, et al.*, 2002). This means that any species of interest should be tested to determine their optimum auxin concentrations before considering mass clonal propagation by cuttings.

The physiological role of auxins in promoting adventitious rooting can be complex (Hartmann *et al.*, 2002). For instance, the inability of leafless cuttings to root after treatment with auxins indicate that auxins may interact with other compounds produced in the leaves and shoots, particularly carbohydrates, to promote rooting. According to Tchoundjeu (2002), application of auxins to the base of a cutting influences the transfer of carbohydrates from the apical part of the cutting to the base of

the cutting. The translocated carbohydrates provide the energy for processes leading to root formation.

Despite the role of auxins as a root promoter, high concentrations can have significant negative effects on shoot growth (Mesen *et al.*, 1997b), due to the continuous basipetal translocation of carbohydrates. Young shoots and leaves have an important role in the growth and vigour of cuttings after root induction and therefore an optimal concentration of auxin requires consideration of its effect on shoot growth.

2.1.4 Propagation media

Propagation media can have substantial effects on adventitious root induction and development in cuttings. While the materials of a propagating medium can vary considerably they need to fulfill three basic functions: support, water supply, and aeration for root respiration (Loach, 1985; Handreck & Black, 1994). A good rooting medium must comprise a balance between water holding capacity (WHC) and air-filled porosity (AFP). Excessive water will result in reduced stem respiration and stem decay, while too much air can result in water stress (Handreck & Black, 1994). The appropriate balance of these two factors can vary substantially between species and genotypes.

Mesen *et al*. (1997a) investigated the effect of rooting media on cuttings of *C. alliodora* and found that these cuttings rooted better in gravel (89%) and sand (88%) than in sawdust (76%). Sawdust can retain excessive moisture, which limits oxygen diffusion into the rooting medium for root respiration. Ansari (2013) reported that in Pomegranate " *Malas torsh"* cv (*Punica granatum*), mixing sand with vermiculite achieved greater rooting percentage (72.1%) compared to pure sand (40.43%). The author attributed the success to the potential release of nutrients by vermiculite. The

authors didn't quantify air-filled porosity or water holding capacity between the media, even though these could have been contributing factors to the differences.

2.1.5 Propagator and relative humidity

When cuttings are harvested, each cutting loses its ability to draw water from a well-established root system, and has to rely on moisture intake through the cutting base (Loach, 1988a). Failure to maintain sufficient moisture in the cutting will lead to water stress (Loach, 1977). Severe water stress will reduce the cutting's ability to effectively photosynthesise, as well as its ability to produce roots. Water relations in cuttings are affected by irradiance, humidity, temperature and foliage wetting (Loach, 1985). Mudge (1995) observed that water stress contributed enormously to poor rooting in bougainvillea, hibiscus and kei apple. Maintaining a high relative humidity in the propagation environment is important for minimising moisture loss through open stomata, allowing photosynthesis to aid cutting metabolism (Flexas *et al.*, 2002). Propagation systems are designed to maintain an atmosphere with high relative humidity to facilitate cutting development.

A range of propagation systems exist, and vary primarily in terms of the method employed to maintain atmospheric humidity. The most widely used systems are those using intermittent mist or fog through irrigation heads to maintain relative humidity (Hartmann *et al.*, 2002). The interval between and length of misting cycles will influence the relative humidity in the propagator. Non-mist propagators maintain a high relative humidity by moving water from a water table below the medium to the atmosphere of the propagator by capillary action through the propagation medium (Leakey *et al.*, 1990). These types of low-cost propagators have sometimes proven to be effective for cutting propagation in a range of species (Leakey *et al.*, 1990).

According to Loach (1977), the non-mist propagator would be preferred over the intermittent mist propagator, in terms of humidity control and management, because the enclosure of a non-mist propagator keeps atmospheric humidity at a constant level.

2.1.5.1.1 Media moisture

The level of available water in the propagation medium affects the water balance in the cutting (Loach, 1985). It is therefore critical to ensure that moisture in the rooting medium is maintained at a level that maximises rooting potential. Other effects can include a reduction in photosynthesis as well as effective translocation of sugars. The movement of auxins could also be affected (Davis, 1986; Davis & Zhang, 1991; Flexas *et al.*, 2002). For instance, Darbyshire (1971) reported that, in pea plants, increased water stress (-2 to -14.5 bars) increases IAA oxidation, thereby reducing the endogenous auxin and its activity, and resulting in decreased root initiation and development.

Optimum moisture levels in the propagation medium can vary between species. Those from arid regions require a lower moisture content than those from wet tropical regions (Mesen *et al.*, 1997a) to achieve better rooting percentages. Specific moisture requirements can be easily managed by using appropriate blends of media components based on their water holding capacity, such as milled pine bark, forest soil and sand (Akwatulira *et al.*, 2011) or sawdust (Mesen *et al.*, 1997b).

2.1.5.2 Temperature

Temperature plays a significant role in modulating plant growth and development (Salisbury & Ross, 1992). Ambient temperature in both the propagator and rooting medium influences the capacity and rate of adventitious root induction in

cuttings (Shibuya *et al.*, 2014; Zhao *et al.*, 2014). Some of the influences that air and soil temperature can have on cuttings are:

1) Increased metabolic processes (Leakey & Mohammed, 1985; Webster *et al.*, 1990; Reuveni & Castoriano, 1993),

2) Increased transpiration and respiration rates (Loach, 1988a), and

3) Increased water stress as a result of increased water loss through transpiration (Loach, 1988b).

Increased temperature in the propagator is positively correlated to increases in light intensity (Loach, 1988a), and therefore can be controlled by managing the amount of direct light that enters the propagator (Dykeman, 1976). Optimum cutting performance will occur within a specific temperature range, which is species dependent (Reuveni & Castoriano, 1993). Palanisamy *et al.* (1997) noted that rooting is lower in propagators exposed to high irradiance/temperature (66%) than shaded/low temperature (77%). Therefore, to ensure maximum cutting performance, it is crucial that the air and rooting medium temperatures in the propagator are maintained to the level that maximises root initiation and development. In the non-mist propagator, air temperature is usually maintained at around 20^{o}C but can reach up to 34^{o}C (Leakey *et al.*, 1990). Whenever the propagator is opened, the air temperature drifts to ambient temperature, then slowly stabilises again after the propagator is closed.

The temperature of the rooting medium is known to affect root formation in leafy stem cuttings. Hartmann *et al.* (1997) noted different optimum temperatures for temperate climate species, ranging from 18 to 23^{o}C, while tropical species have a higher optimum range: from 25 to 32^{o}C, which again may differ from species to species.

Applying heat to the base of the rooting medium has been found to promote rooting in species such as *Chrysanthemum* and *Forsythia* (Dykeman, 1976), *Mangifera indica* (Reuveni & Castoriano, 1993) and *Camellia* spp. (Goodwin & Cowell, 1998)

2.1.5.3 Light

Plants require radiant energy as the main energy source for photosynthesis (Salisbury & Ross, 1992; Hartmann *et al.*, 2002). Photosynthesis in cuttings is important in the provision of energy for metabolic processes involved in root initiation and development (Davis, 1986). The level of irradiation and photoperiod are important for variables that influence cutting success (Hartmann *et al.*, 2002).

2.1.5.3.1 Irradiance

Cuttings can differ in their response to light (Hartmann *et al.*, 2002), even though, generally, the effect of different levels of irradiance on rooting may be insignificant if water stress is reduced or eliminated (Leakey, 1985). Generally, cuttings perform well under low-light conditions, as this reduces temperature and associated water loss from the retained leaves, but supports an adequate level of photosynthesis in the cutting (Dieleman & Meinen, 2007). In the cuttings propagation of *Backhousia citriodoria* light-levels in the propagator were reduced to 22% of ambient sunlight (Kibbler *et al.*, 2004b). In cultivars of *Grevillea* sp. light intensity in the propagators was maintained at around 440 μmol m^{-2} s^{-1} (Krisantini *et al.*, 2006), which equates to around 25% of the irradiance level under full-sun conditions.

2.1.5.3.2 Photoperiod

Exposing stock plants or cuttings to extended day lengths have influenced rooting percentage in some species (Couvillon, 1988; Loach, 1988a; Cameron *et al.*,

2005). A significantly greater rooting percentage was found for cuttings collected from *Cotinus coggygria* stockplants grown under a 16hr photoperiod (84%), compared to those under an 8-hour photoperiod (50%) (Cameron *et al.*, 2005). The authors also reported a more rapid development of apical shoot tips on cuttings from stockplants grown under long days than short days. In contrast to this, Pellicer *et al*. (1998) found that artificially extending the ambient daily photoperiod reduced the rooting percentage of cuttings in *Larix* x *eurolepis*.

2.1.6 Cutting propagation in the genus *Santalum*

Several species of *Santalum* can be reproduced clonally by cuttings, although success is moderated by ontogenetic effects, such that cuttings are produced more readily using juvenile than mature plants (Havea, 2012) and optimum conditions required for successful cutting propagation vary between species (Collins *et al.*, 2000; da Silva *et al.*, 2016). It has been proposed that *S. yasi* and *S. austrocaledonicum* are more amenable to stem cutting propagation than *S. album*, *S. lanceolatum*, and *S. macgregorii* (Collins *et al.*, 2000; Thompson *et al.*, 2005). Collins (2000) conducted cuttings studies with tropical sandalwood species (*S. album*, *S. austrocaledonicum*, *S. lanceolatum*, *S. macgregorii* and *S. yasi*) and reported substantial variation in the percentage of rooting between experiments, genotypes and species. Differences between experiments were recorded for *S. album* (9.5 and 0% rooting), *S. austrocaledonicum* (63.5 and 20.2%), and *S. yasi* (46.1 and 10.1%). Differences were observed between 15 *S. austrocaledonicum* genotypes with root induction ranging from 25 to 88.9 %. The rooting success of *S. yasi* was examined in six propagation media with percentage rooting as follows 8% (Sand/Peat (1:1)), 15% (Sand/Coir (1:1)), 11% (Sand/Mahogany compost (1:1)), 17% (Sand/Sawdust (1:1)), 13% (mahogany compost) and 6.5% (coir) (Collins *et al.*, 2000).

Srimathi (1995) reported that clonal propagation of *S. album* was problematic with less than 3% rooting success in stem cuttings propagated under intermittent mist. Rooting success was improved to 25% by taking cuttings from root suckers, which may be more likely to be juvenile. Uniyal *et al.* (1985) demonstrated that *S. album* can be propagated by root cuttings with 60 % success when they used Seradix B2Ò at the time of planting during the first week of April under conditions of its natural range in southern India (dry deciduous forests of Deccan Plateau at the edge of the Western Ghat Range (Rai, 1990)). Havea (2012) recommended treating cuttings of *S. album* and *S. yasi* with IBA and NAA (1.0 mg/L each) and striking in a medium comprised of sand: peat moss (30:70, w/v). Batabyal et al (2014) reported that propagation of *S. album* can be achieved using 15-cm-long stem cuttings from 3- to 4-year-old trees and treating with IAA (1.5 mg/L) and GA3 (1.5 mg/L). They reported that this method resulted in the most shoots per branch, but they failed to report rooting percentages between the treatments. The general lack of published systematic studies pertaining to stem cutting propagation in the genus *Santalum*, means that there are opportunities to undertake experiments to improve propagation knowledge.

2.2 Reproductive biology

The breeding systems of flowering plants have a significant influence on the genetic structure in a population and the evolution of individual plant species. A species breeding system incorporates aspects of floral morphology and phenology, pollen biology, stigma receptivity, pollination biology, compatibility and self-incompatibility (Richards, 1997). Breeding systems operate on a continuum from full outcrossing through selfing to apomixis. In many woody perennial plant species out-breeding is important for the maintenance of genetic diversity within the species and

reducing the possible harmful effects of inbreeding depression (Richards, 1997). The discovery and description of floral morphology and pollination vectors by Christian Konrad Sprengel in the 18th Century demonstrated an early recognition of its scientific importance (Vogel, 1996). Knowledge of reproductive biology in plants is of particular importance to plant breeding and domestication. Plant breeders use knowledge of a crop's breeding system to bring about new generations of unique genotypes. With an interest in the development of improved cultivars of sandalwood (*Santalum austrocaledonicum*) for use in agroforestry in Vanuatu, there is a need to develop an improved understanding of the breeding systems of the genus and species. This review of plant breeding systems, with reference to woody perennial species, is considered relevant to the evaluation of the characteristics of the breeding system in *Santalum*.

Reproductive biology can be referred to as the science of reproduction through sexual means, involving fusion of functionally male and female gametes to produce offspring, and asexual means (apomixis and vegetative reproduction). In the sexual reproduction of flowering plants, the successful fusion of parental gametes can only be achieved when viable pollen lands and germinates on a genetically compatible and receptive stigma (Dafni, 1992; Rugkhla *et al.*, 1997; Uyenoyama, 2000; Silva & Goring, 2001). However, even successfully pollinated flowers may not lead to successful fertilisation and such failures can be attributed to:

1) Incompatible reactions, particularly rejection of the male gametes,

2) The stage of maturity of the pollen and receptivity of the stigma, and

3) The viability of the pollen grain when it landed on the receptive compatible stigma (Gonzalez & Coque, 1995; Richards, 1997; Rugkhla *et al.*, 1997; Uyenoyama, 2000; Silva & Goring, 2001).

2.2.1 Floral biology

A flower is a complex sex organ of angiosperms that is central to their reproduction, ensuring that parental genes are carried into the next generation (Percival, 1965; Richards, 1997). Percival (1965) described floral biology to be the study of the life of a flower, largely concerned with pollination; involving a series of stages from flower opening to fertilisation of the embryo. Sprengel (1996) described nectar production and protection and floral structure as a means for facilitating pollen transfer between flowers of the same species. An understanding of these aspects of floral biology by plant breeders is important in order to develop efficient and effective methodologies for controlled pollination techniques and understanding possible mating patterns and levels of diversity. The specific details of both floral morphology and floral phenology are further evaluated for a range of different perennial plant species.

2.2.2 Floral morphology

Floral morphology refers to the form and structural arrangement of the flower's perianth, androecium, and gynoecium, which occur in concentric whorls on the receptacle (Clarke & Lee, 1987). Flowers have typically evolved in association with pollination vectors (biotic and abiotic), and predominant breeding systems (inbreeding and outcrossing) of the plant species. In general, flowers with coloured petals that produce scents, nectar and large pollen grains are better adapted to biotic pollination while dull, unscented flowers that produce abundant small pollen grains are more suited for abiotic pollination agents (Percival, 1965). The influence flower morphology has on the success of reproduction has been reported for several species including *Prunus dulcis* (Yi *et al.*, 2006), *Pedicularis* spp. (Sun *et al.*, 2005), *Alpinia nieuwenhuizii* (Takano *et al.*, 2005) and *Lythrum salicaria* (Waites & Agren, 2006).

2.2.2.1 Perianth

The perianth comprises two outer whorls known as the calyx and corolla. The calyx is the outermost whorl and consists of a minimum of two sepals, which typically surround the flower during its development as a bud (Clarke & Lee, 1987). The calyx acts as a bud-scale, protecting the flower parts during the developing stages, and in some species sepals are coloured and can attract pollinators (Lersten, 2004).

The corolla is comprised of petals that are usually equal in number to the number of sepals. The petals vary greatly in shape, size and colour between species, and are often a prominent visual feature of flowers in species with biotic pollination. Prominent exposure and display of petal colours act as pollinator attractants, while petal colour changes may be used to encourage or discourage further pollination in species such as *Pedicularis monbeigiana* (Sun et al., 2005).

2.2.2.2 Androecium

The androecium consists of one or more stamens, which are positioned in the subsequent whorl(s) within the corolla. The stamens are composed of a filament and anther. Numerous filaments can occupy more than one whorl within the flower. The filament has a specific role in positioning the anther where it is accessible to pollinators. The elongation of the filament , however, is secondary to the subsequent maturity of the anther (Lersten, 2004). The anther harbours pollen grains, which contain the male gametes that are released during anther dehiscence.

2.2.2.3 Gynoecium

The central whorl known as the gynoecium consists of one or more reproductive structures called carpels or pistils. Each carpel contains the female reproductive organs (Lersten, 2004). The carpel contains a smaller structure known as a stigma, which

protrudes from the ovary. The main role of the stigma is to capture pollen and provide favourable conditions for pollen germination. The stigma is positioned at the immediate end of the style. Apart from stigma support, the style's role is to provide a medium for the male gametes to travel towards the ovary. Similar to an animal ovary, the ovary of a flower contains the ovules or female gametes, and each ovule is the site of fertilisation. If fertilisation is successful, the ovary will develop into a fruit.

2.2.3 Floral phenology

Understanding the life cycle of a flower from anthesis, which involves release of pollen, through stigma receptivity, pollination and fertilisation is important for development of effective methods in controlled pollination, understanding biotic interactions and mating systems (Elzinga *et al.*, 2007), and evolutionary biology (Levin, 2006). Floral phenology may be studied at various levels, within a flower, a tree, or a population (Wyatt, 1982). Variation in phenology at all these levels influences gene flow within and between individuals in a given population and also between populations (Augspurger, 1983; Gross & Werner, 1983).

2.2.4 Stigma biology

The stigma provides a surface that facilitates contact between the pollen grain and the style (Percival, 1965). The stigma is the first site for interaction between male gamete and female sporophyte, that determines whether they are compatible. Stigma receptivity in flowering plants is the period in which the stigma surface provides conditions suitable for capturing pollen and promoting its germination. The onset of stigma receptivity differs widely among species (Richards, 1997) and the receptive period may vary from a few hours to several days (Dafni, 1992). Boland and Sedgley (1986) reported that in *Eucalyptus uncinatum*, stigma receptivity began three days after

anthesis but effective pollination occurred between 7 to 10 days. In contrast, the same authors found that in *E. regnans*, maximum receptivity occurred 10 to 14 days after anthesis. In *S. album*, it has been observed that the stigma starts to become receptive four to six hours after anthesis (Kulkarni & Muniyamma, 1998), but stigma receptivity is at its peak only two to three days after anthesis (Rugkhla *et al.*, 1997).

Stigma morphology varies greatly between species (Clarke & Lee, 1987) but can be broadly defined as being functionally 'wet' or 'dry' (Heslop-Harrison & Shivanna, 1977). According to Richards (1997), when receptive, wet stigmas secrete copious fluid, containing free sugars. A dry stigma produces little to no exudate and direct contact is possible between pollen grain and stigmatic papillae (Heslop-Harrison & Shivanna, 1977; Elleman *et al.*, 1992). Both stigma types produce extracellular proteins (Raghavan, 1997), and esterases (Shivanna & Rangaswamy, 1992), that are important for recognition and germination of compatible pollen grains (Fahn, 1982). In wet stigmas proteins and esterases are present as an extra-cuticular layer (Raghavan, 1997).

The primary purpose of the exudate of the wet stigma is to trap and re-hydrate viable pollen grains that land on the stigma. Desiccated pollen grains regain turgidity through osmosis within the stigmatic fluid, enabling pollen germination (Lersten, 2004). Dry stigmas provide a "less hospitable surface" for pollen, and pollen traction to the stigma involves the following three conditions; "(i) an electrostatic force, (ii) surface tension, and (iii) traction through chemical bonds" (Lersten, 2004). Logically, pollen hydration differs between wet and dry stigma types. In the former, pollen hydration occurs externally on the papilla surface, whereas in the latter, pollen hydration occurs internally (Richards, 1997). This internal hydration occurs when the soluble coating of

the pollen degrades and flows out to form a point of contact between pollen and stigma. Through this contact, the pollen then becomes hydrated through osmotic pressure (Elleman *et al.*, 1992).

2.2.5 Pollen biology

Pollen are microspores that contain the male gametophyte (sperm cells), which fuse with the female gametes during fertilisation (Shivanna & Rangaswamy, 1992) to produce a new offspring. A pollen grain contains both a diploid vegetative and haploid generative nucleus (Richards, 1997). The germination and growth of the pollen-tube is controlled by the vegetative nucleus to create a conduit for the generative cell(s). The generative nucleus divides mitotically to form two haploid sperm cells, and during double-fertilisation one fuses with the central cell to form the endosperm and the other with the egg cell to form the embryo (Lersten, 2004).

In some plants, after pollination, mitotic division of the generative nucleus occurs in the pollen grain (trinucleate), while in others the division occurs in the pollen-tube (binucleate) (Raghavan, 1997). In general, binucleate pollen has greater longevity and can better tolerate lower temperature and humidity environments under storage than trinucleate pollen (Richards, 1997). These features of binucleate pollen have been attributed to a lower respiration rate compared with trinucleate pollen (Fægri *et al.*, 1989). The rate of pollen germination (autotrophic phase) in binucleate pollen has also been found to be slower than that of trinucleate pollen. In contrast, the rate of pollen-tube growth (heterotrophic phase) was found to be more rapid in binucleate compared with trinucleate pollen (Mulcahy & Mulcahy, 1983).

The viability of pollen is important as it influences the rate of germination, pollen-tube growth and, ultimately, fertilisation. The viability of pollen from many

flowering plants degrades over time, and pollen longevity varies substantially between plant species (Table 2.1). In *Butea monosperma*, pollen viability at room temperature reduced from 63% at anther dehiscence to 45% then 30% after 24 and 48 hours respectively (Tandon *et al.*, 2003). In some grass species viability may only last up to 30 minutes after dehiscence (Richards, 1997). Dehydration is one of the main factors contributing to the short life in pollen grains for species with trinucleate pollen, which are desiccation-sensitive. The short lifespan in pollen is not necessarily the effect of starvation, because the pollen grains are packed with starchy and lipid metabolites able to support pollen viability (Lersten, 2004).

Table 2.1
Lifespan of pollen for selected genera/species in their natural environment

Species	Lifespan
Hibiscus trionum	3 days
Trifolium	12 days
Paenia	8 weeks
Narcissus	10 weeks
Apple	3 months

Source: Percival 1965

The longevity of pollen can be generally be artificially extended by storage under low temperature and humidity. Recommended pollen storage conditions for *Melaleuca alternifolia* is 3-5 ^{o}C for shorter periods (months), and -18 ^{o}C to -20 ^{o}C for longer periods (years) (Baskorowati, 2006). Storing eucalypt pollen at 0 ^{o}C has been recorded as successful for long-term storage (Moncur, 1995).

Pollen is produced and contained within the anther of the flower until it is shed at maturity. After being shed, pollen grains are transferred from the anther of one flower to the stigma of another flower by pollen vectors. This transfer is influenced by floral morphology, floral phenology and the characteristics/behaviours of pollen vectors (Richards, 1997). In nature, pollen transfer is facilitated by either abiotic (wind and water) or biotic (animal) vectors (Percival, 1965; Richards, 1997). In crop breeding, pollen transfer between selected individuals can be naturally- (open pollination) or human- (controlled pollination) mediated.

2.2.6 Pollen-pistil interaction and fertilisation

The biochemical interactions between pollen grain and stigma are known as pollen-pistil interactions and have a significant influence on whether a given pollination is compatible or not (Knox *et al.*, 1994; Richards, 1997). In compatible pollinations

pollen germination commences after the pollen comes into contact with a receptive stigma. The germinating pollen produces a pollen-tube that penetrates the stigmatic surface and enters the style. The pollen-tube continues its journey through the style until it reaches the ovary and penetrates an ovule. The two haploid generative nuclei travel within the developing pollen-tube. When the pollen-tube enters the ovule, its tip bursts to allow one generative nucleus to fuse with the egg nucleus, and the other with the polar nucleus, thus affecting double fertilisation (Lersten, 2004). At all stages during this process of pollination and fertilisation there is intimate contact between the pollen and pistil. The termination of pollen germination, pollen-tube growth and ovule penetration can occur at any point during this process, due to specific incompatibility responses mediated by interaction between pollen and pistil (Knox *et al.*, 1994; Richards, 1997). The first point of contact between pollen and pistil is on the stigma and the structure of the stigma will influence this interaction (section 2.2.7).

Pollen-tube growth rates can differ significantly between species and genotypes within a species. In a majority of species, the time from pollen tube germination to ovule penetration takes approximately from one to 48 hours, irrespective of carpel distances (Richards, 1997). Flower age and the state of stigma receptivity can affect the rate of pollen germination and tube growth. The genotype of the pollen can also influence the rate of pollen-tube growth in the style, with evidence in some species for depressed growth in 'self' compared with outcross pollen-tubes (Richards, 1997).

2.2.6.1 Effective pollination period

The effective pollination period (EPP) is specifically determined by the viability and longevity of a flower's reproductive parts. The effective pollination period has been determined as the period in which hand-pollinated flowers are at their highest

capacity to produce fruits (Gonzalez & Coque, 1995), and can be measured by seed set capacity (Mesejo *et al.*, 2007). The length of the EPP is influenced by receptivity of the stigma, pollen tube growth rates, and the longevity of ovule (Sanzol and Herrero 2001). Gonzalez and Coque (1995) found that stigma receptivity in kiwi fruit becomes the limiting factor in EPP. Stigma receptivity lasts for four days, while the ovule degenerates seven days after anthesis.

The EPP is also affected by environmental conditions and flower quality (Orlandi *et al.*, 2005). Sanzol and Herrero (2001) reported that flower quality lengthens the longevity of the stigma and ovule, while temperatures can either accelerate or decelerate pollen-tube growth. The effect of temperature on the EEP is not linear, however, since it not only accelerates pollen tube growth but also the degeneration of the stigma, resulting in shortening of the EPP.

2.2.7 Compatible and incompatible reproduction

Successful fertilisation can only be achieved in pollinations where pollen grain and stigma are genetically compatible (Percival, 1965). Incompatibility mechanisms in flowering plants generally promote outcrossing and facilitate increased genetic diversity within a species (Grindeland, 2008). Three levels of incompatibility are known to occur within the flowering plants. These are self-incompatibility, intra-specific incompatibility, and inter-specific incompatibility.

Outcrossing species can possess physical and developmental mechanisms to limit certain types of pollination. These outbreeding mechanisms including dichogamy (protandry and protogyny) and heterostyly (distyly and tristyly) (Dafni, 1992). Some plants have also developed biochemical systems that operate to limit such pollinations from effecting fertilisation and the formation of a zygote (Richards, 1997).

2.2.7.1 Self-fertile plants

There are two prominent features of floral morphology in self-compatible plants (Percival, 1965). In 'cleistogamous' plants, flowers do not open for pollination, but remain closed, concealing the reproductive parts in order to maximise the chances of self-pollination. In 'chasmogamous' plants, flowers open normally but autogamous self-pollination is promoted through synchronized maturity of male and female reproductive parts (Richards, 1997).

2.2.7.2 Self-incompatibility

Self-incompatibility can be defined as the inability of a plant to produce viable seeds after self-pollination (Lundqvist, 1964). Self-incompatibility in flowering plants is a mechanism that operates to prevent successful breeding within identical and between genetically similar genotypes (Raghavan, 1997; Silva & Goring, 2001). It encourages out breeding and has a significant effect on genotypic diversification of flowering plants (Holmes, 2000). The self-incompatibility mechanism in flowering plants are promoted by two systems, the heteromorphic system, which is controlled by differences in floral morphological arrangements (herkogamy), and in some cases differences in maturation of reproductive parts of the flower (dichogamy), and homomorphic system which is controlled by genetic recognition and rejection of the male gametophyte (Matton *et al.*, 1994; Richards, 1997). The self-incompatibility mechanism can manifest as a failure of pollen adhesion, germination on the stigma, and restriction of pollen tube growth down the style (Richards, 1997) and not due to ovule sterility (Brewbaker, 1957).

2.2.7.2.1 Herkogamy

The level of self-pollination is influenced by the arrangement and structure of flowers. Herkogamy in flowering plants refers to the spatial separation of the anther and stigma within a flower that limits the incidence of self-pollination within that flower (Borojevic, 1990; Dafni, 1992; Richards, 1997). In species with heteromorphic flowers, stamens and stigmas are located at different levels on each flower type in a manner that reduces own pollen landing on the same stigma, but promotes cross-pollination and outcrossing. In dimorphic species there are two forms of floral arrangements, one with the stigma higher than the stamens ('Pin'), and the other with stamens higher than the sigma ('Thrum') (de Nettancourt, 1977). In trimorphic species there are three types of flowers, with the style shorter, equivalent and longer than the anthers or vice versa (Percival, 1965; Dafni, 1992; Raghavan, 1997; Richards, 1997; Lersten, 2004; Waites & Agren, 2006). Heteromorphic flowers are typically structured for biotic pollinators, for which pollen is deposited on different parts of the pollinator and transferred to a reciprocal stigma (i.e. high anthers to high stigma). In addition to the structural differences in the flowers between morphs, heterostylous species often also have genetic controlled SI (Richards, 1997). Research suggests that heterostyly is controlled sporophytically (see section 2.2.7.2.5), with one or two diallelic loci in distyly (*S*,*s*) and tristyly (*S*, *s* and *M*, *m*) respectively (Stone & Goring, 2001).

2.2.7.2.2 Dichogamy

The level of self-pollination may also be influenced by temporal separation of male and female stages of flowering. This is prevalent in dichogamous flowers whereby pollen is shed before the stigma is receptive (protandry) or vice-versa (protogyny) (Weberling, 1989; Dafni, 1992; Richards, 1997; Jersakova & Johnson,

2007). The degree of temporal separation between pollen shed and stigma receptivity varies considerably between protandrous species, ranging from 12 to 24 hours in *Crotalaria* spp. (Shivanna & Rangaswamy, 1992), 1-2 days in *Dryandra sessilis* and *Grevillea wilsonii*, (Collins *et al.*, 2008), 3 days in *Chamelaucium uncinatum* (O'Brien, 1996) and 10 to 14 days in *Eucalyptus regnans* (Boland & Sedgley, 1986).

In protogynous flowers, the period of stigma receptivity is completed before the pollen is shed. Plants displaying this type of breeding system may also vary in the number of days of separation. For instance, in *Parietaria judaica*, the stigma was receptive for approximately 6 days, and anther dehiscence occurred 3 days after this (Franchi *et al.*, 2007). In *Spartina alterniflora,* Fang (2002) found that the stigma becomes receptive 2.5 to 5 days before anther dehiscence, while in *Plantago maritima*, Dinnetz (1997) reported that the female receptivity is one to ten days before anther dehiscence.

2.2.7.2.3 Dicliny

Dicliny refers to a breeding system in which not all individuals of a species are regular hermaphrodites (Barrett, 1984; Holmes, 2000). Dicliny specifies spatial separation of male and female flowers and includes monoecy (male and female flowers on one plant), dioecy (individual genotypes bear either functionally male or female flowers) and polygamy (both hermaphrodite and single sex flowers on the one plant). In dioecious plants outcrossing is complete, since there is no opportunity for self-pollination within a genotype (Radford, 1986). Andromonoecy (hermaphrodite and male flowers on the one plant) might be considered to be common in the genus of *Leptospermum* (Thompson, 1989), it being recorded in many of its species (Burrell, 1965; Primak & Lloyd, 1980; Radford, 1986; Andersen, 1990; O'Brien, 1994).

Gynomonoecy (hermaphrodite and female flowers on the one plant) has been recorded in *Hirshfeldia incana* (Horovitz & Beiles, 1980) and *Eucalyptus leucoxylon* (Ellis & Sedgley, 1993).

2.2.7.2.4 Genetically controlled self-incompatibility (SI) systems

Self-incompatibility (SI) in flowering plants is characterized by recognition and rejection of 'self' pollen, and is known to be controlled genetically (Franklin *et al.*, 1994). The incompatibility process in the pistil involves cellular recognition of the *S*-allele(s) in the male gamete by pistil, and subsequent rejection where these alleles are shared between them (Dafni, 1992; Raghavan, 1997; Richards, 1997). The site of recognition and rejection can occur on the stigma, in the style or ovary (late acting) (Richards, 1997).

Self-incompatibility acts by inhibiting the germination of pollen on stigmas, or the elongation of the pollen tube in the styles. Genetic recognition in SI is categorised into the following forms, sporophytic self-incompatibility (SSI), gametophytic self-incompatibility (GSI) and heteromorphic SI. Late acting SI is also examined. Given the importance of these incompatibility systems as a means to promote genetic diversity, each method is examined briefly below.

2.2.7.2.5 Sporophytic self-incompatibility (SSI)

The sporophytic self-incompatibility mechanism is controlled by a multiallelic *S* locus (Matton *et al.*, 1994). The incompatibility mechanism is controlled by the interaction between the diploid somatic tissues of both the pollen-bearing and pistillate parents (Brewbaker, 1957; Heslop-Harrison & Shivanna, 1977). Incompatibility between them occurs when the pollen grain contains at least one of the *S*-alleles also

present in the pistil. Pollen will only germinate if none of the pollen *S*-alleles is the same as those of the stigma (Figure 2.1). The inhibition occurs at the pollen-stigma interface, resulting in the failed germination of pollen (Nasrallah & Nasrallah, 1993). Sporophytic self-incompatibility is often associated with trinucleate pollen and dry-stigmas (Brewbaker, 1957).

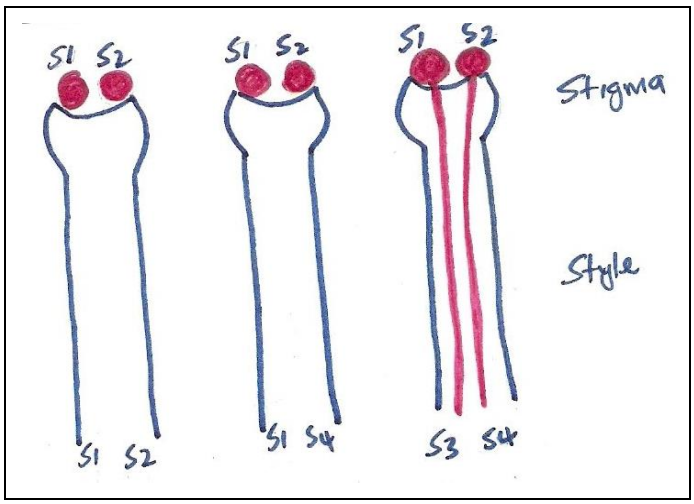

Figure 2.1 Germination behaviour of pollen with the *S*-phenotype of its parent (S_1S_2) on the stigmas with different *S*-alleles in a sporophytic incompatibility system.

2.2.7.2.6 Gametophytic self-incompatibility

Gametophytic self-incompatibility is moderated by the interaction between the S-allele in the haploid pollen grain and its interaction with both *S*-alleles of the diploid pistil parent (Matton *et al.*, 1994; Richards, 1997). Incompatibility is expressed when the *S*-allele of the pollen is the same as either one of the *S*-alleles expressed in the somatic tissues of the style. In a GSI the compatibility of crosses between two different genotypes can be categorised as compatible, semi-compatible or incompatible, depending on the number of *S*-alleles shared between sporophytes (Richman *et al.*, 1996) (Figure 2.2).

The self-incompatibility reaction takes effect within the tissues of the style and manifests in several ways. Symptoms include callose plug formation in the pollen tube,

restricting its growth (Dumas & Knox, 1983; Richards, 1997; Lersten, 2004), pollen tubes bursting, growing in different directions, becoming twisted or coiled, and cessation of pollen tube growth (Lersten, 2004). Gametophytic self-incompatibility is more likely to be associated with binucleate pollen (Brewbaker, 1957; Heslop-Harrison & Shivanna, 1977) and wet stigmas (Heslop-Harrison & Shivanna, 1977), and is considered to be the most widespread incompatibility system in flowering plants (Franklin *et al.*, 1994).

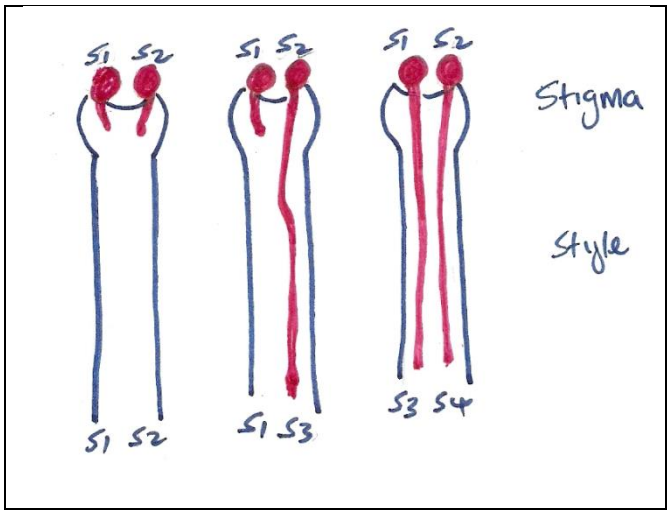

Figure 2.2 Behaviour of haploid pollen from a diploid parent (S_1S_2) with different *S*-alleles, on three stigmas with different combinations of *S*-alleles with gametophytic self-incompatibility

2.2.7.2.7 Late acting self-incompatibility

Late acting self-incompatibility is a mechanism which restricts fertilisation after successful pollen germination and tube growth, and is at times considered as delayed GSI or ovular SI (Seavey & Bawa, 1986; Gibbs & Bianchi, 1999). It occurs in the later stages of pollen tube development, and its actual effect is the prevention of fertilisation (Seavey & Bawa, 1986). There are two possible sites for the incompatibility reaction, just before the pollen tube enters the ovary with the pollen tube failing to penetrate the ovary walls and in the ovary in which the male gametes fail to fertilise the ovules. Late acting SI does not always prevent successful fertilisation. In some instances,

fertilisation occurs successfully, but then the self-fertilised embryos are later aborted (Gibbs & Bianchi, 1999; Gibbs, 2014).

While recognising that this form of self-incompatibility is genetically controlled, Seavey and Bawa (1986) listed the following as possible causes of late acting SI:

1) Failure of pollen-tube to penetrate the ovule,
2) Ovule sterility,
3) Fertilised embryo encounters lethal molecules during development, and
4) Maternal resource allocation

2.2.8 Interspecific incompatibility

Interspecific incompatibility systems operate to restrict gene-flow between species (Boavida *et al.*, 2001). These mechanisms develop during the process of evolutionary divergence between species. Reproductive isolation, barriers to interspecific hybridisation, are broadly categorised as either operating before, or after fertilisation. This section addresses interspecific incompatibility with respect to the mechanisms that prevent pollen germination or pollen-tube development, leading to a failure to develop a hybrid zygote (de Nettancourt, 2001).

Similarities in the rejection sites and pollen-tube morphology between interspecific- and self- incompatibility provide support for a possible relationship between the two types of incompatibility (de Nettancourt, 1984). This author also discussed the possible association between the two mechanisms in which successful hybridisation between self-incompatible (SI) and self-compatible (SC) can only be

achieved when the self-compatible species is used as the female parent. This type of incompatibility has been described as unilateral, in that it occurs in only one direction.

Within subgenera of *Eucalyptus* two major pre-zygotic barriers to hybridisation operate (Potts & Dungey, 2004). The first is unilateral, in which the pollen tubes of species with small flowers are unable to affect fertilisation of species with large flowers. This barrier is due to structural differences between the flowers, in which pollen-tubes of small flowered species are unable to growth the entire length of the style of those species with large flowers. The second barrier manifests as abnormalities in pollen-tube growth ultimately leading to pollen tube arrest in the pistil. This barrier is more prevalent in crosses between taxonomically distant species (Potts & Dungey, 2004).

Incongruity is defined as a pre-zygotic interspecific barrier that is independent of the *S*-locus. Incongruity may be described as a mismatched interface between the pollen and pistil of two species, due to significant genetic differences between them. This incongruence is more passive compared with incompatibility mechanisms, and de Nettancourt (1977) equated it to the analogy of a 'lock and key'. This occurs when the pollen grain requires a certain suite of signals (key) to be recognised by the pistil and supplied with the necessary components for growth to affect fertilisation. This form of incongruity may only be associated with hybridisation between genetically distant species, whereas incompatibility mechanisms are effective barriers between genetically similar species (Knox *et al.*, 1987)

2.2.9 Reproductive biology of *Santalum*

Flowers of *Santalum* spp. are hermaphroditic, containing both functionally male and female reproductive organs, and are pollinated by a variety of insects such as bees, butterflies, moths, beetles, ants, wasps, and flies (Jyothi *et al.*, 1991; Veerendra &

Padmanabha, 1996; Baskorowati, 2011). In *Santalum austrocaledonicum,* there have been anecdotal reports of 'male' and 'female' plants in Vanuatu (Berry, 2005). These plants are not necessary dioecious, but some plants ('female') are noticeably more fecund than others ('male'). The biological mechanisms of low fruit set may be worth investigating to determine possible presence of monoecy in this species, with individuals producing both functionally male and hermaphrodite flowers. Protandry has been observed in *Santalum album,* in which anthers dehisce four- to six-hours before stigma receptivity (Kulkarni & Muniyamma, 1998). In *S. album*, it has been observed that female reproductive parts were still immature at the time of anther dehiscence (McComb & Jones, 1998), which is a mechanism for promoting outbreeding. This outbreeding mechanism is further supported by asynchronous flowering within individuals and some form of gametophytic self-incompatibility (Veerendra & Padmanabha, 1996; Kulkarni & Muniyamma, 1998). Despite displaying self-incompatibility adaptations, these authors also suggest that genetic variation in their expression exists in *S. album,* where some genotypes are capable of producing viable seeds after self-pollination.

Attempted reciprocal hybridisation crosses between *S. album* and *S. spicatum* have failed to result in production of viable hybrid seed (McComb & Jones, 1998). Successful natural hybridisation has been observed between other *Santalum* species. For instance, putative hybrids from natural hybridisation between *S. album* and *S. yasi* have been recorded by the Fiji Forestry Department (Bulai & Nataniela, 2007). However, there is a need for systematic study of *Santalum* reproductive biology, including interspecific hybridisation between commercial species.

The viability of pollen in *Santalum* could be drastically reduced after anthesis. Examination of pollen viability using *in vitro* germination in *Santalum album*, (in 20% sucrose solution) after storage was measured at 81 to 95% directly after anther dehiscence, but was reduced to 52% 12 hours later (Kulkarni & Muniyamma, 1998). The time taken for fertilisation of a successfully pollinated flower has been found to be slightly affected by the age of flowers. For instance, in *S. album* and *S. spicatum,* pollen-tube growth from a 'one day old' pollinated flowers took two days to reach the ovary, while pollen tubes from a '2-3 days old' flowers took only one day to reach the ovary (Rugkhla *et al.*, 1997). The relationship between pollen tube growth and the age of the flower in *Santalum* indicated the importance of selecting the appropriate timing for effective pollination of species in the genus.

Chapter 3. Vegetative Propagation Studies of *Santalum austrocaledonicum*

Significant variation exists within several sandalwood species for commercially important oil traits. The concentration of volatile oils within the heartwood ranges from 0.05 to 8% in *S. austrocaledonicum* (Page *et al.*, 2006) and 0.5 to 5% in *S. album* (Veerendra and Padmanabha 1996) and 0.1-8.2% in *S. lanceolatum* (Page *et al.*, 2007). The variation in the key oil component alpha-santalol ranges from 1 to 47% in *S. austrocaledonicum* (Page *et al.*, 2006), 34.5-40.4% in *S. paniculatum* (Braun *et al.*, 2014) and 2-25.6% in *S. lanceolatum* (Page *et al.*, 2007). To increase the quality and uniformity of heartwood oil from planted sandalwood, further work developing routine methods for clonal propagation of selected individuals is required. The research in this chapter demonstrates the potential for clonal propagation of *S. austrocaledonicum*, but also highlights variation between genotypes in their response to variables examined. The scope of some of the experiments has been limited by the availability of suitable material.

3.1 Study 1: Effects of rooting media and IBA concentrations on adventitious root induction in juvenile *Santalum austrocaledonicum* leafy stem cuttings

3.1.1 Abstract

The effects of IBA (control and 3000ppm) and three different propagation media on adventitious root development were evaluated in leafy stem cuttings of *Santalum austrocaledonicum*. A total of 301 single node cuttings across 10 genotypes were investigated in a non-mist propagator. Adventitious root induction, root number and root lengths were measured at 33, 65 and 113 days after setting the cuttings. No

significant differences in rooting percentage and mean root length were found between 3000ppm IBA and control. There was no statistical difference in rooting percentage between the propagation media used. Rooting was greatest at 65 days (40%), and lower at 33 days (20%) and 113 days (8%). A significant difference in rooting percentage between genotype origins was observed, with genotypes from the island of Erromango rooting better than those from Tanna. It was concluded that the low rooting percentage in 3000ppm IBA indicated that the IBA concentration for optimum rooting of the species may be higher than that used in the experiment, or lie between the control and 3000ppm.

3.1.2 Introduction

In this study, indole-3-butyric acid (IBA), and a range of rooting media were examined for the cutting propagation of *Santalum austrocaledonicum*. Auxin and rooting media can have a strong influence on root development (Day & Loveys, 1998). Externally applied auxins have been known to increase rooting percentages in many 'difficult-to-root' tree species (Hartmann *et al.*, 2002), such as *Cordia alliodora* (Mesen *et al.*, 1997b), *Milicia excelsa* (Ofori *et al.*, 1996), *Triplochyton scleroxylon* (Leakey *et al.*, 1982), *Prunus africana* (Tchoundjeu, Avana*, et al.*, 2002) and *Nauclea diderrichii* (Leakey, 1990). The influence of rooting media on the rooting capacity of several species have also been examined, including *Ficus binnendijkii* 'Amstel Queen' (Shah *et al.*, 2006), *Cordia alliodora* (Mesen *et al.*, 1997b) and *Prunus africana* (Tchoundjeu, Avana*, et al.*, 2002), in which root induction and development has been found to respond to differently to different media types. Therefore, examining their combined effects on the cutting propagation of *S. austrocaldonicum* may provide an insight into the most appropriate combinations to promote adventitious root development.

Cuttings from *Santalum* species are considered difficult to root (Thomson, 2006). Therefore, scientific experiments are required to determine the amenability of species to vegetative propagation by cuttings. The objective of this study was to examine the effect of IBA and propagation media on adventitious root induction and development in leafy stem cuttings of *Santalum austrocaledonicum* set in a non-mist propagator.

3.1.3 Materials and methods

Ten genotypes (ortets) of *Santalum austrocaledonicum* were selected from seedlings raised at the James Cook University nursery in Cairns. All genotypes originated from the Pacific Island nation of Vanuatu, with two and eight genotypes sourced from wild-collected seed originating from the islands of Tanna and Erromango, respectively. The seedlings were approximately 2 years old at the time of cutting collection. The ortets were labelled; b- and j-tanna and d-, e-, f-, g-, h-, i-, j- and k-erro.

Three propagation media treatments were prepared and the percentages of air filled porosity (AFP) measured (

Table 3.1). Indole-3-butyric acid (IBA) at 3000ppm was selected as the single auxin treatment, which was a commercially prepared gel (Clonex). The control cuttings received no rooting hormone or gel solvent.

Table 3.1
Composition of each rooting media, air filled porosity (AFP) and IBA concentrations used for cutting treatment.

Propagation media	Composition	Air-filled Porosity	IBA treatment	Control
Gravel	Sieved gravel (5mm)	29%	3000ppm	0ppm
VP	1 part vermiculite: 1 part perlite	46%	3000ppm	0ppm

VPP	2 part vermiculite: 2 part perlite: 1 part peat	42%	3000ppm	0ppm

The non-mist propagator (Leakey *et al.*, 1990) was divided into three media chambers, each containing one of three propagation media (the first contained 5mm sieved gravel, the second contained vermiculite and perlite (VP 1:1v/v), and the third contained vermiculite, perlite and peat (VPP 2:2:1v/v)). Cuttings were collected on 3rd March 2006 (14 months old), and prepared as single node cuttings with an approximate mean leaf area of 400mm^2 per cutting. Each of the 10 ortets was represented by at least 4 cuttings in each of the three media. Half of the cuttings from each ortet were treated with 3000ppm IBA solution, and the other half was left untreated (control). Due to limited cutting material, each ortet was represented by unequal numbers of cuttings in the media and IBA treatment combinations.

Table 3.2
Number of ramets in each media (5mm sieved gravel, vermiculite and perlite (VP 1:1v/v) and vermiculite, perlite and peat (VPP 2:2:1v/v), IBA and control treatment

Media	% IBA	Control
Gravel	3000ppm (51 Ramets)	0ppm (51 Ramets)
VP	3000ppm (50 Ramets)	0ppm (49 Ramets)
VPP	3000ppm (51 Ramets)	0ppm (49 Ramets)

The cuttings were randomly set into each of the three media types in the propagator. A soil drench (Fongarid 250g/kg Furalaxy) fungicide was applied to the propagation bed after planting was completed. Cuttings were inspected daily and dead leaves removed to discourage fungal growth and disease outbreak in the propagator. Water level at the base of the propagator was also inspected at this time, and water was added when required. Cuttings were manually sprayed with a water mister after each inspection to maintain high humidity within the propagator.

3.1.3.1 Assessments

Cuttings were assessed 5 (33 days), 9 (65 days) and 16 (113 days) weeks after propagation. During each assessment, the number of cuttings with adventitious roots was recorded and for those with roots, the number and length of their roots were measured. Successfully rooted cuttings were transferred from the propagator and 'potted on', with no further measurements included in this experiment. Living cuttings without adventitious roots were returned to the propagator for assessment during the following period. Dead cuttings were removed from the propagator and recorded.

3.1.3.2 Analysis

Pairwise differences in the proportion of cuttings with adventitious roots between ortets and media were evaluated using an equality test of two binomial proportions (Ott & Longnecker, 2001), calculated by:

$$z = \frac{(\hat{p}_1 - \hat{p}_2)}{\sqrt{\frac{\hat{p}_1(1-\hat{p}_1)}{n_1} + \frac{\hat{p}_2(1-\hat{p}_2)}{n_2}}}$$

The two binomial populations are denoted by $\hat{p}_1 = \frac{y_1}{n_1}$ and $\hat{p}_2 = \frac{y_2}{n_2}$, where y_1 rooted cuttings are recorded for the random sample of n_1 cuttings from population 1, and y_2 rooted cuttings are recorded for the random sample of n_2 cuttings from population 2. The null hypothesis was rejected where the absolute value of the statistic z was greater than $z_{0.05} = 1.645$. This statistical approach was used because the low number of cuttings per media/IBA treatment combination did not permit the use of multiple cutting experimental units to generate percentage data that could be evaluated by analysis of variance.

3.1.4 Results

3.1.4.1 Percentage cuttings with adventitious roots

After 113 days of propagation, 68% of cuttings across all clones developed adventitious roots. At this time the remaining cuttings were classified as either alive but without adventitious roots (18%) or dead (14%). The greatest number of cuttings with adventitious roots was recorded between 33 and 65 days in the propagator, with 40.2% of cuttings with roots followed by assessments at 33 and 113 days, with 20.5% and 7.6%, respectively.

No significant difference was found in the percentage of cuttings forming adventitious roots for the three media used in this experiment. Within the VP and VPP media, no significant difference in adventitious rooting was observed between 3000ppm IBA and the control (0ppm IBA). The percentage of cuttings with adventitious roots within the gravel medium, however, was significantly ($P<0.05$) greater in the control compared with 3000ppm IBA (Figure 3.2).

Significant variation was found between ortets with significantly greater rooting percentage for all those sourced from Erromango compared with the two originating from Tanna. No successfully rooted cuttings were produced from the j-tanna ortet. Within the Erromango ortets there was a continuum of variation in rooting percentage from a high of 88% (e-erro) to 63% (d-erro) (Figure 3.1).

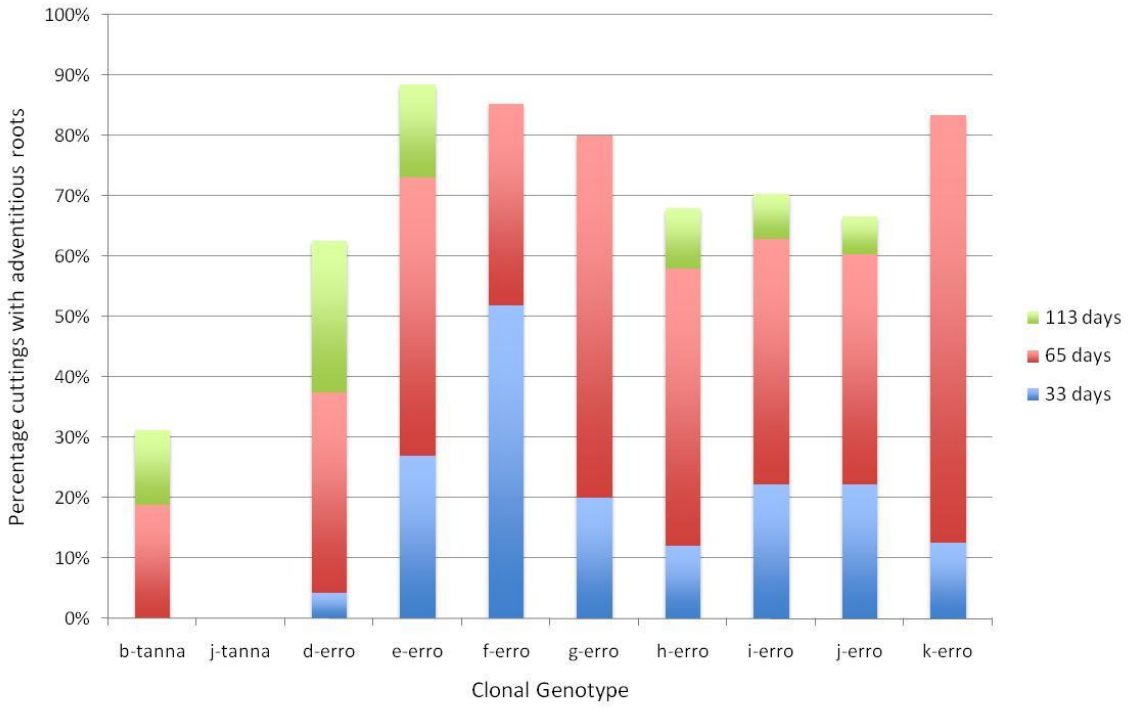

Figure 3.1 Percentage of cuttings with adventitious roots across 10 seedling genotypes for each of the three assessments (33, 65 and 113 days).

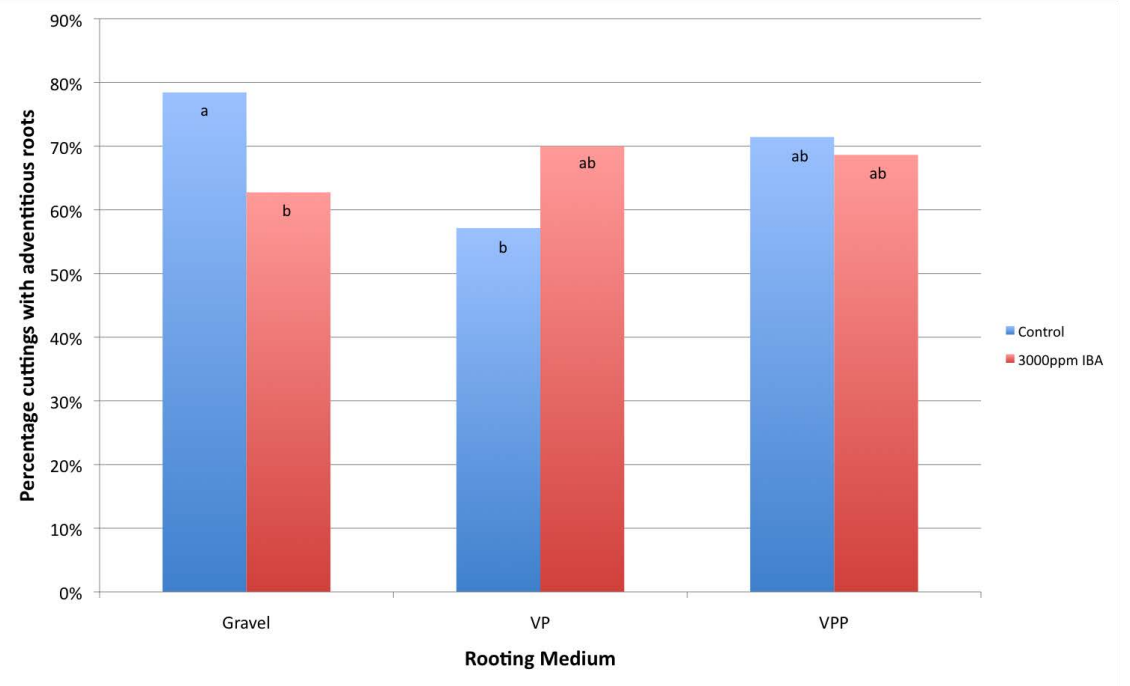

Figure 3.2 Percentage of stem cuttings with adventitious roots for three rooting media (Gravel, Vermiculite/Peat – VP, and Vermiculite, Perlite and Peat – VPP) and two IBA concentrations (0ppm and 3000ppm).

Treatment combinations that share lower case letters within the upper part of the column are not significantly different (P>0.05).

3.1.4.2 Mean root number and length per cutting

Rooting media did not have a significant effect on the mean number of roots per rooted cutting of *S. austrocaledonicum,* with 1.56, 1.43, 1.38 roots recorded for gravel, VP and VPP respectively. Similarly to the rooting media, IBA did not have a significant effect on the mean number of roots per rooted cutting, with 1.46 and 1.47 roots measured for control and 3000ppm respectively.

No significant differences were found for mean root length per week between the 3000ppm IBA and the control within gravel (6.37 and 5.12mm respectively) and VPP (10.45, and 9.13mm respectively) media. In contrast, significantly ($P<0.05$) longer roots were found in cuttings treated with 3000ppm IBA (10.63mm), compared with the control in the VP medium (4.81mm) (Figure 3.3). Cuttings in the VPP medium produced significantly ($P<0.05$) longer roots when compared with gravel, although no significant differences existed between all remaining pairwise comparisons. No significant differences in cutting mean root length were found between any of the ten ortets used in this study.

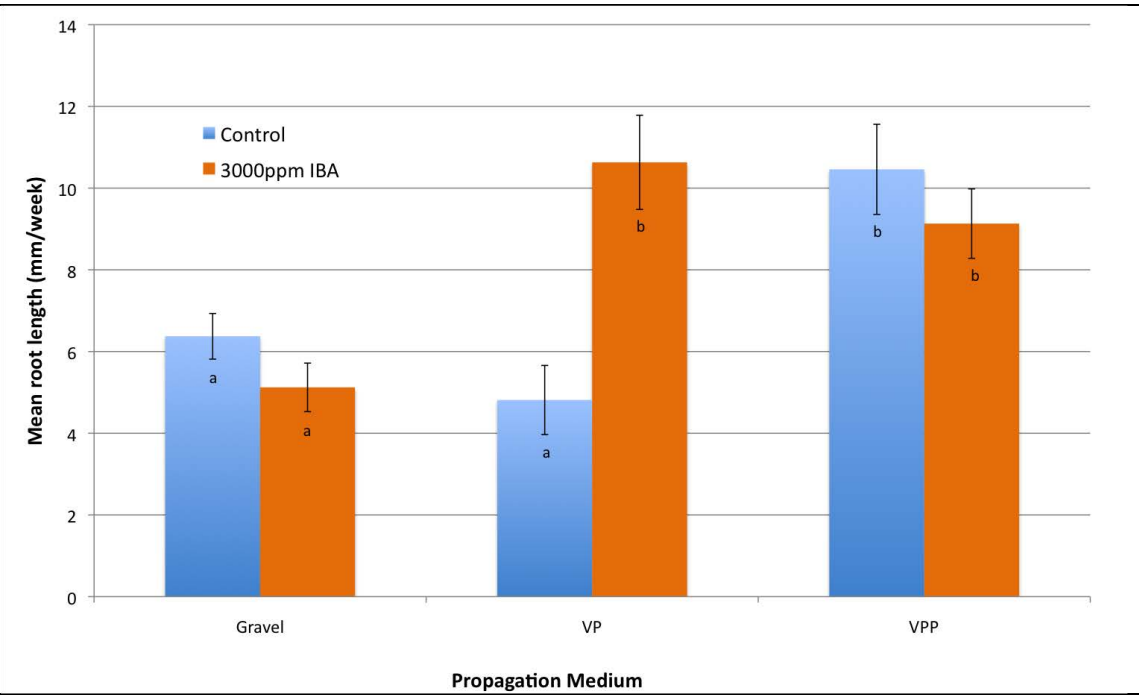

Figure 3.3 Mean root length (mm per week) for cuttings with adventitious roots for three rooting media (Gravel, Vermiculite/Peat – VP, and Vermiculite, Perlite and Peat – VPP), and each IBA concentration (0ppm and 3000ppm).

3.1.5 Discussion

The results from this study show that *Santalum austrocaledonicum* can be successfully propagated through leafy stem cuttings in a non-mist propagator. This study found that 68.3 % of cuttings produced adventitious roots after 133 days, while 18% remained alive but not rooted, and 14% were dead. This rooting success was similar to that reported for *Santalum album* cuttings, in which 70% of cuttings successfully developed adventitious roots under a misting system (Bulai & Nataniela, 2007). Based on the findings, the optimum rooting period for *S. austrocaledonicum* cuttings would be between 33 and 65 days. The greatest percentage (40.2%) of cuttings forming adventitious roots was recorded between 33 and 65 days of propagation. Only 20.5% was recorded on day 33 and 7.6% rooted cuttings were recorded on day 113. The highest rooting percentage at 65 days finding is similar to that reported in another tropical species *Ilex paraguariensis,* in which Tarrago *et al*.(2005) observed the development of root primordia at 21 days and the emergence of roots after 35 days.

For the routine propagation of genotypes originating from Erromango, a propagation period of approximately two months would be required, while the genotypes originating from Tanna may require a longer propagation period.

No appreciable difference in the percentage of cuttings developing adventitious roots was found between the media used in this study. Given that the media differed substantially in terms of air-filled porosity, this result suggests that medium may not be the most important factor in optimising the propagation environment for *S. austrocaledonicum*. It is therefore proposed that subsequent experiments evaluate the effectiveness of other factors such as IBA (a greater range), leaf area and stem volume in a limited number of media. The apparent 'adaptability' of *S. austrocaledonicum* cuttings to media with a broad range AFP means that a propagation medium could be easily prepared using sterilized materials such as coarse sand, pumice, cocopeat (composted coconut husks) and top soil that are available in Vanuatu.

One of the main findings was that of the 68.3% of cuttings that rooted, 34.8% rooted in the control treatment (0ppm IBA), while 33.5% rooted in 3000ppm IBA. Within the VP and VPP propagation media, no significant difference in rooting percentage was found between cuttings treated with IBA compared with the control. This result may indicate that the IBA preparation used was either of insufficient concentration to stimulate of root induction, or that *S. austrocaledonicum* cuttings do not respond to exogenous application of IBA. The apparently negative effect of IBA application on the percentage of root induction within the gravel medium contradicts the result from VP and VPP media. The exact mechanism leading to this contrasting result is not yet clear, but the result does suggest further evaluation of a greater range of concentrations of IBA is necessary. In *Cordia alliodora,* for instance, Mesen *et al.*

(1997b) found that increasing IBA from 0% IBA to 1.6% (16,000ppm) IBA increases rooting percentage from 10% to 70%.

Ortets originating from Erromango produced cuttings with substantially greater root induction (66.5%) than those from Tanna (1.8%). All eight Erromango genotypes successfully rooted with all genotypes exceeding 60% rooting (Figure 3.1). In contrast, only one of the two Tanna clones (b-tanna) rooted with 30% of the cuttings producing adventitious roots, while 70% were still alive but not rooting. In j-tanna, 65% of the cuttings died while 35% remained alive, but none rooted by 113 days. The reasonably high percentage of cuttings that remained alive may mean that clones originating from Tanna may require over 3 months in the propagator to set adventitious roots. Resource limitations in this experiment meant that it was not possible to continue the experiment beyond 3 months. Given the potentially lengthy time spent in the propagator, this method of propagation may only be viable for very important selections, for which a premium could be gained in either their sale or post-planting growth and quality. While the number of genotypes from Tanna (two) is too small to report definitive conclusions, earlier experiments (data not presented) with a greater range of Tanna genotypes had similarly low levels of rooting. Further systematic evaluation of provenance variation in rooting capacity would be required before definitive conclusions could be made with respect to *S. austrocaledonicum.*

IBA had no significant effect on mean root length in either the VPP or gravel propagation media. However, in the VP media adventitious roots were significantly longer in cuttings treated with 3000ppm IBA compared with the control. The mechanism(s) behind the interaction between IBA and media for mean root length is/are unclear. The mean root length of cuttings set in VPP were significantly longer

than those set in gravel. This result is largely due to the damage sustained to the roots when they were manually lifted from the medium during inspection and assessment, which introduced some error into the experiment. Root damage was largely sustained from the very abrasive components of the gravel medium. From this perspective, the gravel media, although suitable for promoting adventitious root induction, was not desirable from an operational perspective. Given that equivalent results for the percentage of root induction were found between the three media, it is recommended that further optimisation of the propagation protocol be focused on the less abrasive media of VP and VPP.

3.1.6 Conclusions

The current study found that 3000ppm IBA does not have any significant effect on rooting of juvenile cuttings of *Santalum austrocaledonicum*, when compared with the control treatment (0ppm) for VP and VPP media. When propagated in a gravel medium, IBA had an apparently negative effect. Since IBA at this rate did not have a significant effect on the rooting of the species, it is recommended that future experiments on vegetative propagation of the species should investigate a wider range of IBA concentrations. This experiment also found that rooting medium does not have a significant effect on adventitious root induction in the species, meaning that medium is not a critical factor in optimising rooting in the species. The difference in rooting percentages between genotypes highlighted the need to include other parameters such as leaf area, cutting length and diameter and light, as these factors have been known to influence adventitious rooting development in some tropical hardwood species. It also indicates that different clones may require different propagation periods to achieve optimum rooting. Under the current conditions, the propagation period for some clones

is approximately two months, while other clones may require a period greater than three months.

3.2 Study 2: Effect of exogenous auxin (indole-3-butyric acid, IBA) and genotype on induction of adventitious roots in stem cuttings of *Santalum austrocaledonicum*

3.2.1 Abstract

The effectiveness of indole-3-butyric acid (IBA) on root induction and development was examined for leafy stem cuttings in nine clones of *Santalum austrocaledonicum,* originating from two islands in Vanuatu (Erromango and Tanna). Two IBA concentrations (4000ppm and 8000ppm) and a control were tested on a total of 324 cuttings in a non-mist propagator. The cuttings were assessed for root induction, number and growth after four, 10 and 13 weeks. No significant difference in the percentage of root induction, or mean root length (mm/week) was found between the control, 4000ppm and 8000ppm IBA, after week 13. Under the propagation conditions in this experiment, the cuttings of genotypes sourced from Erromango outperformed those from Tanna in all three measures. The overall rooting percentage among genotypes was significantly different, with clones sourced from Erromango ranging between 63 and 92% and those from Tanna between 0 and 6%. The variation among Erromango genotypes indicates that further optimisation of the propagation conditions for individual clones may be required.

3.2.2 Introduction

Auxins are plant growth regulators that are specifically involved with the initiation, enhancement and development of adventitious roots in cuttings (Blakesley & Chaldecolt, 1993; Mesen *et al.*, 1997b; Hartmann *et al.*, 2002). Their role in promoting

root initiation and growth in plants is well known. Without the use of exogenous auxins, the cutting propagation of many wood plant species, including tropical timber species, may not be possible (Leakey, 1985).

Endogenous auxins, mainly indole-3-acetic acid (IAA), are produced naturally by plants, mainly in the shoot tips and young leaves (McDavid *et al.*, 1972; Ford *et al.*, 2001; Aloni *et al.*, 2003). The accumulated auxins are then translocated basipetally to the base of the plant, where they influence cell division and elongation (Hale *et al.*, 1995) including root initiation and growth (McDavid *et al.*, 1972; Leakey, 1985; Ford *et al.*, 2001; Sorin *et al.*, 2005). Despite the importance of IAA as a natural product in commercial horticulture and forestry, the most frequently used exogenous auxins for vegetative propagation are synthetic chemicals (Hartmann *et al.*, 2002). The main forms of exogenous auxins are indole-butyric acid (IBA) and naphthalene acetic acid (NAA). When applied to the base of freshly cut stems, these auxins can play a critical role in promoting adventitious root formation.

These trees include *Triplochiton scleroxylon* (Leakey *et al.*, 1982), *Cordia alliodora* (Mesen *et al.*, 1997b), *Irvingia gabonensis* (Shiembo *et al.*, 1996), *Khaya senegalensis* (Tchoundjeu & Leakey, 1995) and *Prunus africana* (Tchoundjeu, Avana*, et al.*, 2002). However, these authors have noted that the type of auxin and the optimal requirements may vary widely between species. Therefore, it is important that the type and concentration of auxin be tested for each species of interest, in order to optimise its use for mass vegetative propagation.

Santalum is an important forestry and agroforestry crop, which is propagated mainly through seeds and seedlings sourced from wild populations. Clonal propagation of the species is regarded as important in order to capture and replicate the superior

selections identified in natural populations. The aim of this experiment was to examine the effectiveness of two auxin (IBA) concentrations (4000ppm and 8000ppm) and nine genotypes of *Santalum austrocaledonicum* cuttings, for promoting adventitious roots in leafy stem cuttings.

3.2.3 Materials and methods

A total of 72 leafy stem cuttings were collected from each of nine clones of potted seedling hedges of *Santalum austrocaledonicum* (648 cuttings across all clones), grown in 300mm planter pots at the James Cook University, Cairns Campus. Cuttings were collected on 4th October 2006 from seedling hedges that were approximately 21 months old. The seedling clones originated from Erromango and Tanna islands in Vanuatu. In this experiment five clones originated from Tanna (f-, n-, o-, u- and v-tanna) and four clones originated from Erromango (f-, h-, j- and k-erro). The hedges were given a slow release fertiliser (Osmocote 15-9-12+2MgO+TE @ 2g/L, applied biennially) and a liquid fertiliser (Peter's Professional 20-20-20 +TE @ 1g/L applied monthly), to supplement their nutrient intake requirements prior to collection of cuttings.

3.2.3.1 Cuttings treatments

The cuttings were prepared with two leaves, with a total combined mean leaf area of 400mm^2 per cutting. The stem length and cutting diameter of each cutting was measured and recorded. Cuttings from each clone were divided into three groups of 24 with each group treated with 0ppm (control), 4000ppm or 8000ppm indole-butyric acid (IBA) dissolved in a 70% ethanol-based aqueous solution. After immersion of the base of cuttings in IBA, the treated bases of the cuttings were held in front of an electric fan to evaporate the ethanol before they were randomly positioned into the non-mist

propagator. The rooting medium used was composed of vermiculite, perlite and peat (VPP 2:2:1v/v), with an air-filled porosity (AFP) of 43%. The medium was then treated with Fongarid (250g/kg Furalaxyl) immediately after the setting of the cuttings to prevent fungal outbreak.

As with the previous cuttings experiment (Section 3.1), cuttings were inspected daily and dead leaves removed to discourage fungal growth and disease outbreak in the propagator. The water level at the base of the propagator was also inspected at this time, and water was added when required. Cuttings were manually sprayed with a water mister after each inspection to maintain high humidity within the propagator.

The experiment was established as a randomised complete block design with 2 blocks (2 non-mist propagators) and three treatments (IBA). For each IBA treatment there were 12 cuttings per block, randomly allocated in groups of four cuttings (forming an experimental unit). A total of two blocks were used, bringing the total number of cuttings for each clone/IBA treatment to 24 (6 experimental units).

3.2.3.2 Assessment and analysis

Adventitious root formation was assessed three times after the cuttings were set, at weeks four, ten and 13. During these assessments, the numbers of rooted cuttings were recorded; a rooted cutting was defined as having at least one root of 5mm or longer. The root number and root lengths were also recorded for each rooted cutting. All rooted cuttings were removed and planted into planter pots with no further assessments. Given this removal from the experiment, root length assessments were divided by the number of weeks since setting (assessment 1) or the previous assessment (assessments 2 & 3), so that mean root length per week were compared between treatments. Shoot growth, callus, dead and rotted cuttings were also recorded during

each assessment. Rooting percentage data were arcsine transformed to ensure normal distribution and equal variance. The main effects and interactions of clone *vs*. IBA were determined by a two-way ANOVA with Tukey's post-hoc test comparisons.

3.2.4 Results

3.2.4.1 Percentage cuttings with adventitious roots

The application of exogenous IBA to the base of the *S. austrocaledonicum* cuttings, at both concentrations (4000ppm and 8000ppm) had no statistically significant effect on adventitious root induction compared with the control (control = 30.6%, 4000ppm = 36.1% and 8000ppm = 34.3%). Furthermore, no significant interaction was found for root induction between IBA and clonal genotype treatments.

All four genotypes derived from seedlings sourced from Erromango contained cuttings with adventitious roots. The rooting percentage among these genotypes ranged from 63 ('k-erro') to 92% ('j-erro'), with an average rooting percentage of 73.5%. In contrast, only one (n-tanna) of five genotypes from Tanna had cuttings with adventitious roots and the rooting percentage of this genotype (6%) was significantly ($P<0.05$) lower than all genotypes from Erromango (Figure 3.4).

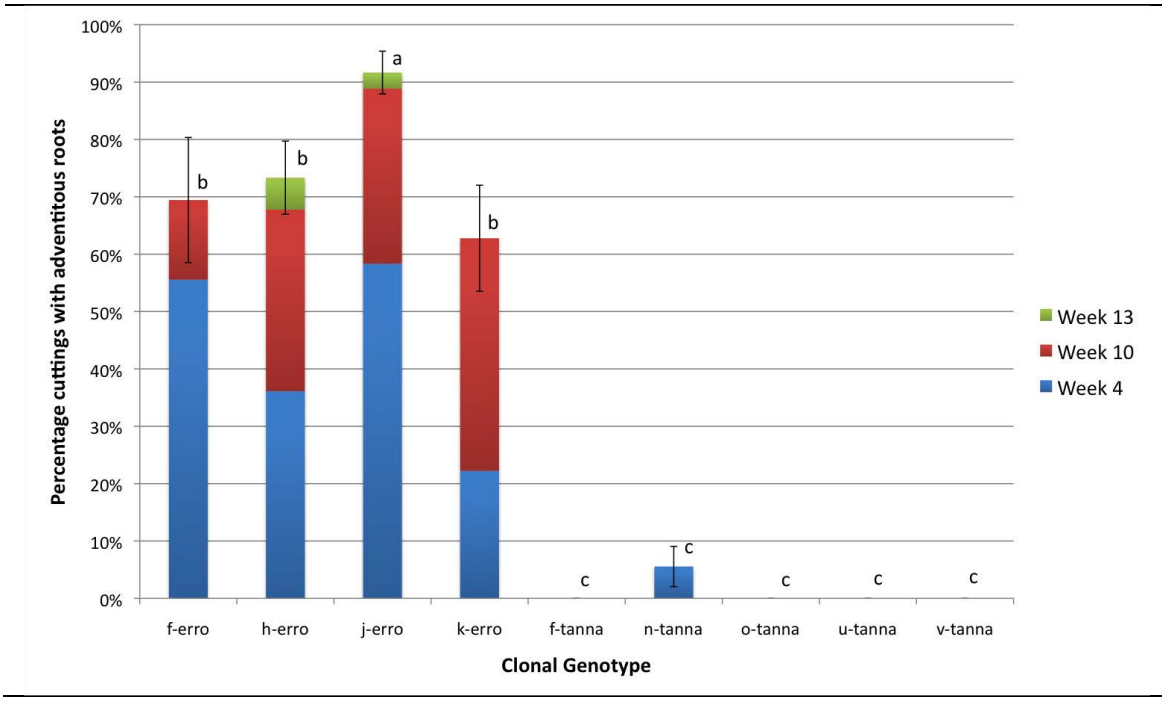

Figure 3.4 Percentage cuttings with adventitious roots across nine seedling genotypes for each of three assessments (Week 4, 10 and 13).

Vertical bars denote standard errors for total rooting percentage across all assessments. Clonal genotypes that share the same lower case letter are not significantly different at the 0.05 level.

3.2.4.2 Mean number of roots in rooted cuttings

IBA and clone did not have any significant effect on the mean number of roots per rooted cutting. The greatest number of roots per cutting was found in 8000ppm IBA (1.57), while 4000ppm IBA has the second highest number of roots (1.42) and 0ppm IBA (1.28), although no statistical differences were found between them. The mean number of roots per cutting in n-tanna was 1.0, but given that this result represented only two cuttings, this genotype was not included in further statistical analysis. Clonewise, f-erro had the highest mean number of roots per cutting (1.65), followed by j-erro (1.48), k-erro (1.32) and h-erro (1.19) (Figure 3.5). Statistical differences in root number were found only between j- and h-erro, with no differences between all remaining genotypes.

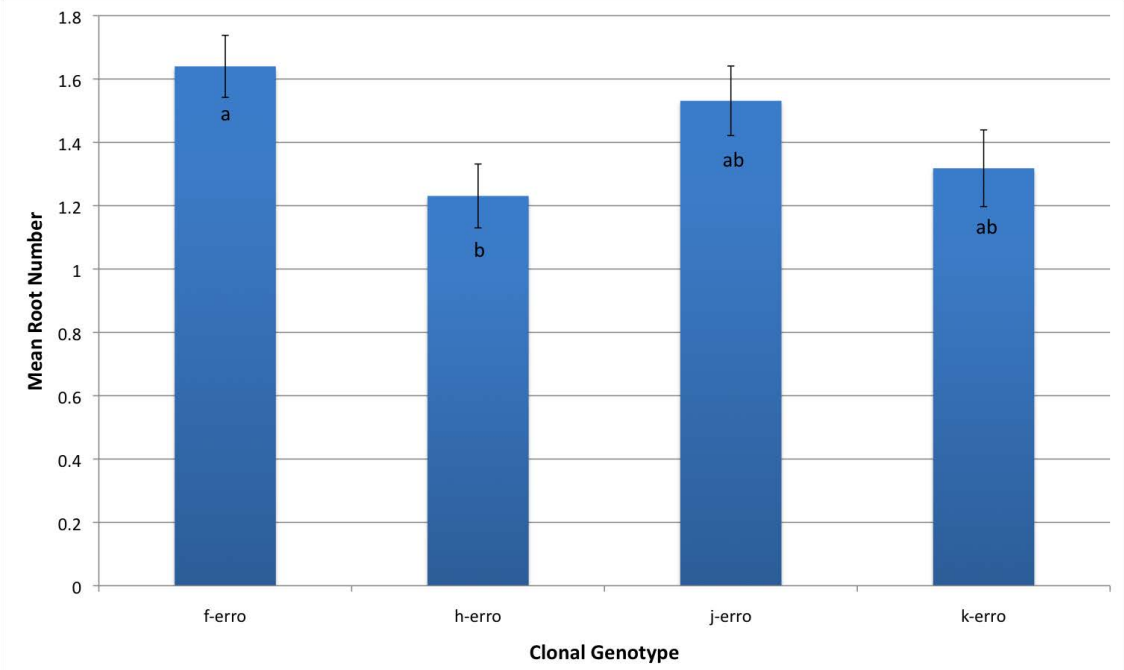

Figure 3.5 Mean number of roots per rooted cutting for *S. austrocaledonicum* for the four Erromango ('erro') clonal genotypes that produced adventitious roots.

Vertical bars denote standard errors for total percentage across all assessments. Clonal genotypes that share the same lower case letter are not significantly different at the 0.05 level

3.2.4.3 Mean root length in rooted cuttings

IBA did not have a significant effect on mean root length between the genotypes that rooted, and mean root length did not appear to correlate to rooting percentage. Similarly, genotype did not have any significant effect on mean root length. Propagation period did have an effect on mean root length per rooted cutting. Cuttings that rooted between weeks ten and 13 had significantly greater mean root length (18.8mm) per cutting per week, compared to those that rooted between weeks one and four (7.9mm), and weeks five and ten (3.5mm).

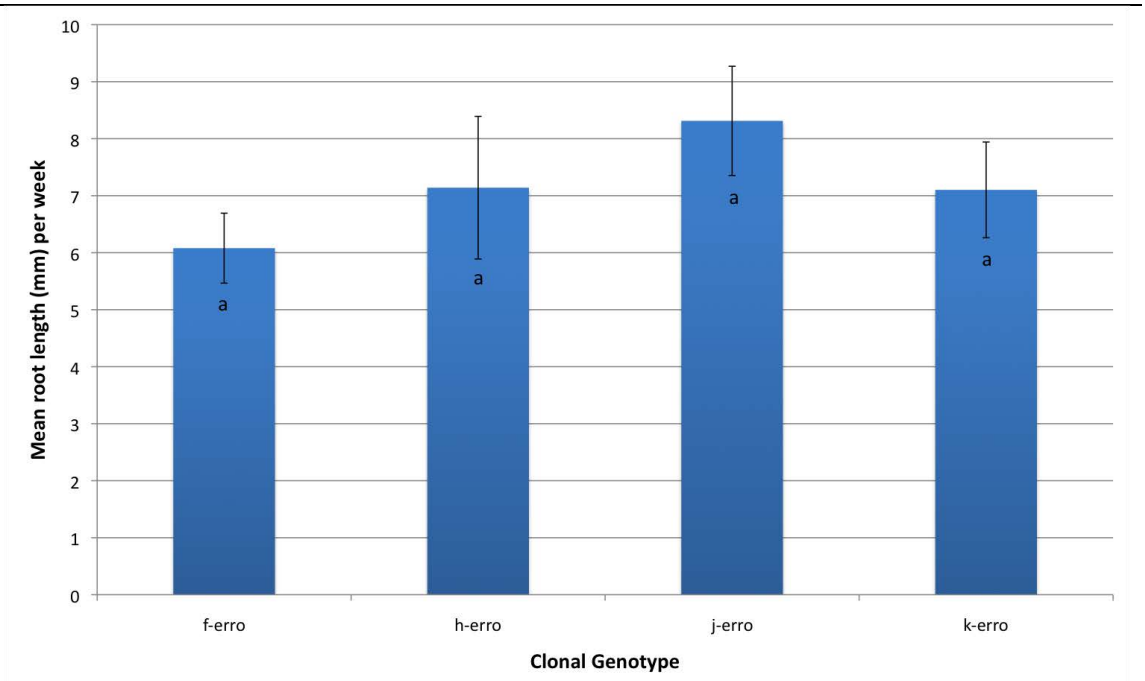

Figure 3.6 Mean root length per rooted cutting for *S. austrocaledonicum* for the four erro clonal genotypes that produced adventitious roots.

Vertical bars denote standard errors for total percentage across all assessments. Clonal genotypes that share the same lower case letter are not significantly different at the 0.05 level

3.2.5 Discussion

3.2.5.1 Effect of IBA on rooting percentage

IBA is well known for promoting the induction of roots on stem cuttings (Hartmann *et al.*, 2002), even with difficult-to-root tree species (Leakey, 1985). However, in this study, exogenous IBA application at two concentrations (4000ppm and 8000ppm) had no effect on the promotion of adventitious root induction in *S. austrocaledonicum* cuttings when compared with the control. This result is similar to that of the previous cutting experiment with *S. austrocaledoncium* (section 3.1). Babashpour Asl *et al.* (2012) recently reported similar results with semi-hardwood cuttings of the tropical species *Bougainvillea sp*., in which they found exogenous IBA application at three concentrations (2000, 3000 & 4000ppm) did not have any significant effect on rooting percentage compared with the control. Given this result

these authors concluded that *Bougainvillea* may be considered an easy-to-root species when propagated from cuttings. However, *Santalum* species have been described as being difficult to propagate from cuttings, since the capacity of sandalwood cuttings to develop adventitious roots decreases with ortet age (Thomson, 2006). The cuttings in this experiment were collected from ortets that were approximately two and a half years from seed germination. While further cutting experiments with more mature ortets would be recommended to examine its effect on rooting success, this research project only had access to seedlings hedges.

Alternatively, the lack of difference in rooting percentage between the concentrations of IBA used and the control may indicate that the rates applied could be below the minimum requirement for the species. In an examination of the effect of four IBA concentrations (0, 4000, 8000 and 16000ppm) in the cutting propagation of four tree species, Egbe *et al.* (2012) demonstrated that the optimum concentration was 16000ppm in three of these species (*Albizia zygia*, *Blighia welwitschii* and *Lophira alata*). The same IBA concentration (16000ppm) was found optimal for the cutting propagation of *Cordia alliodora* (Mesen *et al.*, 1997b). Other studies have established optimum IBA concentrations for several tropical tree species that is within the range of this study, including *Triplochiton scleroxylon* (4000ppm), *Milicia excelsa* (2000ppm) (Ofori *et al.*, 1996), and for *Citrus aurantifolia* (500ppm) (Bhatt & Tomar, 2011). These studies did, however, demonstrate a difference in response between concentrations at these lower levels. Despite the possibility of *S. austrocaledonicum* cuttings responding to IBA concentrations beyond 8000ppm, given the lack of response for any IBA treatments relative to the control it was considered more judicious to examine other variables including those related to cutting morphology and propagator environment.

Many smallholder farmers in Vanuatu currently have an insufficient supply of sandalwood seeds to meet their planting goals (Page, Tate, Bunt*, et al.*, 2012). The rooting success in non-mist propagators without the use of exogenous IBA for Erromango clones provides an option for these remote communities to 'bulk up' planting stocks using existing seedling material.

3.2.5.2 Effect of clone on rooting percentage

The effect of clone on rooting percentage is significant, notably between island provenances of Erromango compared with Tanna. In this case the clonal effect may not being considered as a 'stand-alone' factor, but rather could be an effect of the overall physiological effects including the propagation environment (Leakey, 2004a). The Tanna stockplants were managed identically to those of Erromango. The cuttings collected from both sources were similar in morphology (softwood) and preparation (leaf area, stem diameter and length). While the management and treatment of both stockplants and cuttings in this experiment were equivalent between the two sources, it is possible that the response due to island provenances was different. Further work may be required to determine the correct stockplant management and treatment for each genetically distinct seed source. The differences in rooting percentage between the four Erromango clones could also be due to sample size or other random factors affecting the stockplant and/or propagation environment. While recognising the value in optimising stockplant and cutting management for different seed sources, the low numbers of stockplants available for each clone during this study restricted potential statistical analyses, and therefore this project was not able to undertake that approach.

In the section 3.1 experiment, 70% of the Tanna clones remained alive but without roots after 113 days. In this experiment only 10% in the propagator could be

classified as alive without roots after 91 days. The difference in survival between these two experiments may be due to further ontogenetic aging of the stockplants, or physiological differences in the stockplants associated with seasonal differences in the growing environment. Tibbits *et al*. (1997) found that ontogenetic aging of *Eucalyptus nitens* seedlings from 3- to 12-months after germination may have caused a decline in rooting percentage in cuttings collected from them. In the previous experiment we suggested that ≥3 months might be required for Tanna cuttings to induce roots. In this experiment the final assessment was made at just over 3 months (13 weeks), and given the low number of surviving cuttings (10%) it was decided to conclude the experiment to allow the propagator to be freed up for other experiments.

3.2.5.3 Mean root number and length per cutting

For cuttings of *S. austrocaledonicum* with adventitious roots, IBA did not have any effect on mean number of roots or mean root length. This result contrasted with results of Sumbele (2012), who found that IBA significantly increased mean root number and mean root length in cuttings of *Treculia africana*. In this study, clone did not have a significant effect on the mean number of roots, nor for mean root length (Figure 3.6), in the cuttings that rooted.

3.2.6 Conclusions

The role of IBA as a stimulator for adventitious root induction and development has not been demonstrated for cuttings of *S. austrocaledonicum* used in this experiment. It is possible that optimum IBA concentration for this species lies in a range greater than 8000ppm. I suggest however, that given no response was found for any of the concentrations used in this study compared with the control it is more likely that cuttings collected from sandalwood seedlings do not respond to exogenous application

of IBA. Given this, I propose further experimentation to determine the effect of other cutting treatments, such as leaf area and available light on adventitious root induction in sandalwood.

Clonal and/or provenance variation in root induction was clearly demonstrated in this experiment, with seedlings sourced from Erromango producing cuttings with a superior performance to those from Tanna. Root induction was not recorded in four of the five Tanna ortets and 6% in the remaining ortet. Cuttings from Erromango ortets ranged between 63 and 92%. The promising results with cuttings collected from Erromongo seedlings in non-mist propagators without IBA suggests that this technique could be readily applied by smallholder farmers in Vanuatu to 'bulk up' stocks for establishing new plantings.

3.3 Study 3: Effect of leaf area, cutting type and genotype on induction of adventitious roots in stem cuttings of *Santalum austrocaledonicum*

3.3.1 Abstract

The effect of leaf area (400mm^2 and 800mm^2) and cutting type (apical, medial and basal) on the rooting percentage, mean root number and mean root length in two clones of *S. austrocaledonicum* was investigated. Cuttings were set in a non-mist propagator and assessed for rooting traits at six, ten and 13 weeks after propagation. No difference in rooting percentage was observed between the two leaf area treatments. However, leaf retention was found to positively influence the percentage of cuttings with adventitious roots. Cutting stem volume was not found to have any influence on any of three rooting traits measured. The differences in rooting percentage between the two genotypes used in this study indicate that genetic differences in responses to cutting propagation exist in *S. austrocaledonicum*.

3.3.2 Introduction

Leaves carry out essential biological functions that support plant growth and development. Carbohydrates that are vital for plant survival are produced in the leaves through photosynthesis, and translocated to other parts of the plant for growth. Soil-bound soluble nutrients are absorbed through the capillary action that is driven by the pulling force of transpiration at the leaf stomata. Leaf surface temperature is regulated through transpiration, and although this has a cost in terms of water loss, the rate of both water loss and temperature regulation is controlled through opening and closing of the stomata (Loach, 1977).

Leaves play an important role in the survival of and root development in plant cuttings. It has been found that maintaining the optimal amount of leaf area on a cutting will contribute to greater chances of the cutting developing a larger and better root system (Ofori *et al.*, 1996; Ngo Mpeck & Atangana, 2007). Similarly, the amount of leaves retained on cuttings during propagation has been found to be positively associated with survival and root induction (Tarragó *et al.*, 2005).

In this experiment the effect of leaf area (400mm^2 and 800mm^2) on rooting of cuttings from two clones of *S. austrocaledonicum* were investigated (e-erro and j-erro). E-erro was not used in the last experiment due to limited quantity of cutting material, but was selected for this experiment as it had the highest rooting percentage in Study One.

3.3.3 Materials and methods

Leafy stem cuttings were collected from potted hedges of two *S. austrocaledonicum* clones, j-erro (199 cuttings) and e-erro (177 cuttings). Cuttings were collected and prepared on 6th August 2007 from hedges of clones raised from

previous cutting experiments that represent a genetic age of 31 months (i.e. from original seed germination). Cuttings were prepared as either two- or four-node cuttings, with a total leaf area of 400mm^2 and 800mm^2 respectively. Cuttings were taken from the apical, medial and basal part of the stem, with the level of stem woodiness increasing from apical to basal. For apical cuttings, the foremost tip of the shoot was removed. The length and diameter of the cutting stem were measured and used to calculate cutting stem volume, which was then used as a covariate.

All cuttings were treated with 8000ppm indole-butric acid (IBA) dissolved in a 70% ethanol-based aqueous solution. Cutting bases treated with IBA were held in front of an electric fan to evaporate the ethanol before being randomly planted into the non-mist propagator. The rooting medium used was composed of equal parts vermiculite and perlite, with an air-filled porosity (AFP) of 45%. The medium was then treated with Fongarid (250g/kg Furalaxyl) immediately after setting of the cuttings, in order to prevent fungal outbreak.

During the propagation period cuttings were monitored regularly, and dead leaves were removed as required. Cuttings were assessed three times, at weeks six, ten and 13. During each assessment the number of rooted cuttings, the number of roots, the length of each root and the number of leaves retained on the cutting were recorded. The main effects and interactions of clone, leaf area and cutting type as well as cutting volume were determined using a general linear model (GLM) with cutting volume used as a co-variate. The data for the percentage of roots fitted a binomial distribution, and therefore a binomial model was used for this variable.

To determine the effect of cutting leaf retention during propagation, individual cuttings were allocated into groups as defined by the proportion of original leaves abscised at

the first assessment at week 6. A total of five groups were formed: 100% (all leaves retained in both 400mm^2 and 800mm^2 treatments), 75% (three leaves retained in the 800mm^2 treatment), 50% (one or two leaves retained in the 400mm^2 and 800mm^2 treatments respectively), 25% (one leaf retained in the 800mm^2 treatment) and 0% (no leaves retained in both leaf area treatments). Pairwise comparisons were made between each of the five groups using the equality test of two binomial proportions (Ott and Longnecker 2001).

3.3.4 Results

3.3.4.1 Effect of leaf area, cutting type and clone on adventitious root induction

The effect of leaf area on adventitious root induction was not statistically significant, with no difference in rooting percentage between 400mm^2 (75.2%) and 800 mm^2 (69.7%). Clone had a significant ($P<0.05$) effect on the percentage cuttings with adventitious roots, with j-erro (88%) significantly greater than e-erro (55%). Cutting type had a significant ($P<0.05$) effect on the rooting percentage, although a significant interaction between clone and cutting type was found. Both apical (100%) and medial (98%) cuttings had a greater rooting percentage than basal (62%) for j-erro. In e-erro medial (70%) cuttings had significantly greater rooting percentage than both apical (49%) and basal (47%) cuttings (Figure 3.7). The covariate cutting stem volume had no statistical effect on the percentage of cuttings with adventitious roots.

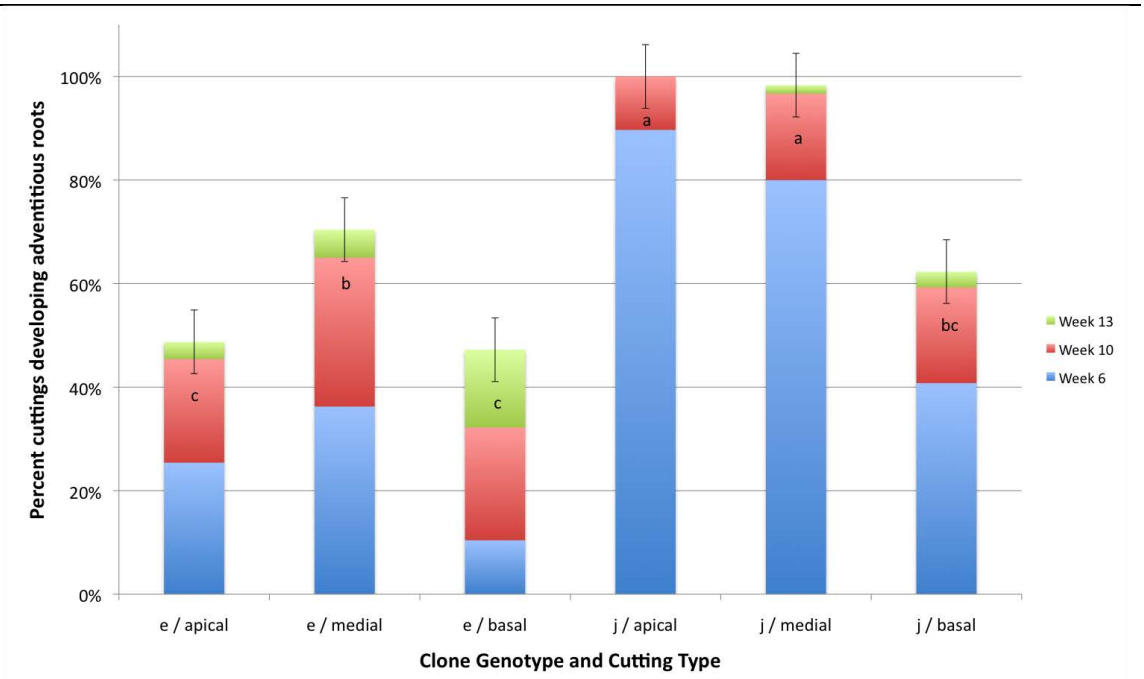

Figure 3.7 The cumulative rooting percentages for *S. austrocaledonicum* for apical, medial and basal leafy stem cuttings of e-erro and j-erro clones with original leaf areas of 400mm2 and 800mm2 during the three assessment intervals.

Vertical bars denote standard errors for cumulative rooting percentage across all treatments. Treatments that share the same lower case letter are not significantly different at the 0.05 level

The greatest percentage of rooting was recorded at the first assessment after six weeks (47%). A further 19% and 5% of cuttings were recorded as rooted by week ten and 13 respectively, resulting in a total rooting of 71% for the experiment. This means that 93% of all rooted cuttings in this experiment were recorded by the second assessment (week ten).

The proportion of leaves abscised by the cuttings within the propagator during the first 6 weeks had a significant effect on the percentage of cuttings with adventitious roots. Significant differences were found among all groups, with the greatest rooting percentage found for cuttings with no leaf abscission (85%) followed by quarter (71%), half (48%), three quarters (35%) and all (21%) leaves abscised (Figure 3.8).

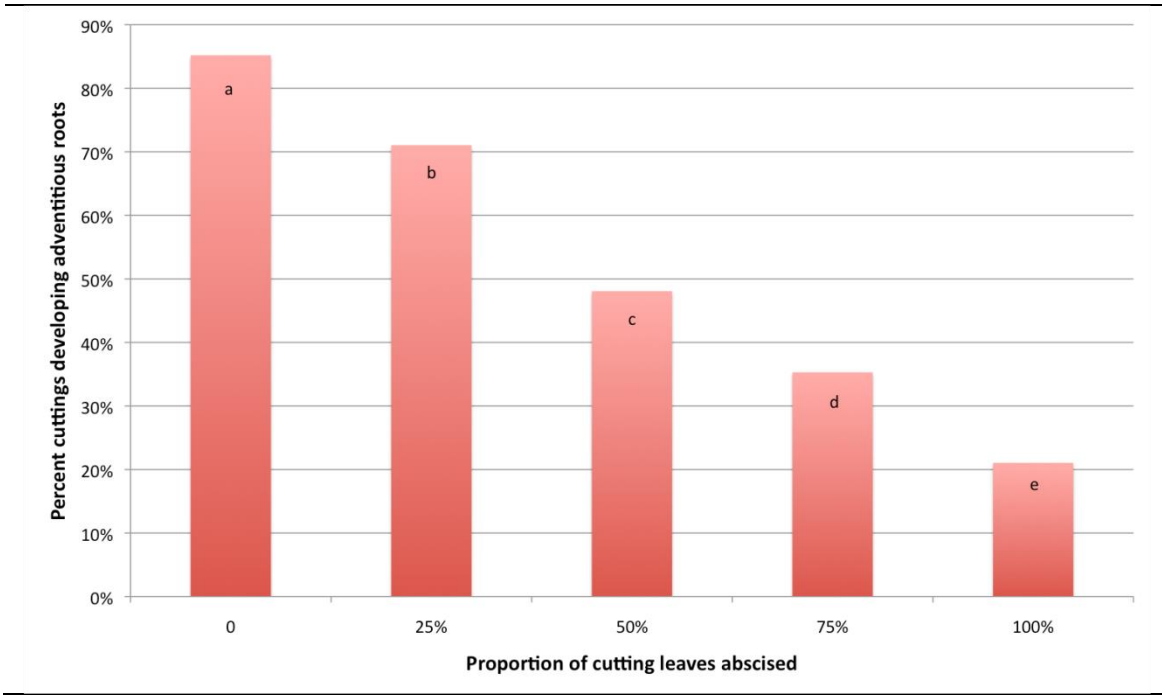

Figure 3.8 The rooting percentage of *S. austrocaledonicum* leafy stem cuttings with variable proportion of leaves.

Treatments that share the same lower case letter are not significantly different at the 0.05 level.

3.3.4.1 Effect of leaf area, cutting type and clone on mean root number

No statistical differences in mean root numbers were found between the two leaf area treatments (400mm^2 and 800 mm^2) or the three cutting types (apical, medial and basal) used in this experiment. Clone had a significant ($P<0.05$) effect on mean root number with j-erro (1.86) cuttings producing significantly more roots than e-erro (1.36) (Figure 3.9). The covariate cutting stem volume was found to have no effect on the mean root number.

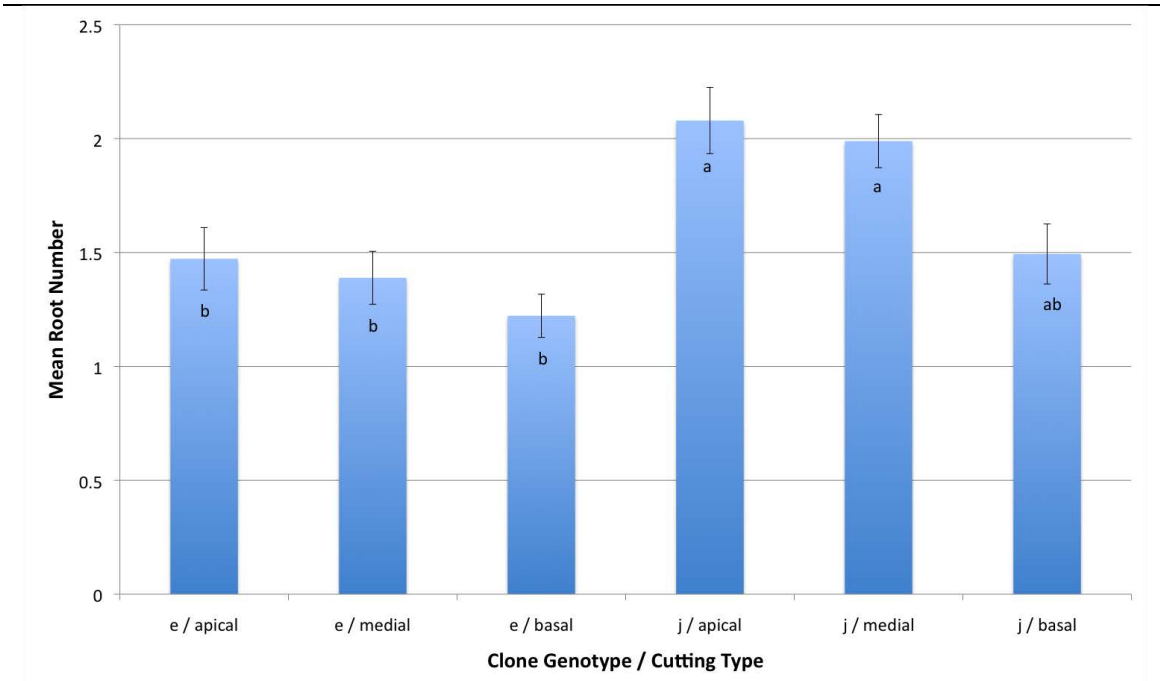

Figure 3.9 Mean root number for *S. austrocaledonicum* for apical, medial and basal leafy stem cuttings of e-erro and j-erro clones.

Vertical bars denote standard errors for mean root number across all treatments. Treatments that share the same lower case letter are not significantly different at the 0.05 level.

3.3.4.2 Effect of leaf area, cutting type and clone on mean root length

No statistical effect of leaf area (mean = coef. = 0.002, t = 0.001, P>|t| 0.146) or cutting type (coef. = -0.03, t = 0.66, P>|t| 0.963) was found on mean root length per rooted cutting (mm/week). Mean root length was 6.0 ± 0.27 s.e. and 7.2 ± 0.4 s.e. mm week^{-1} in cuttings with a leaf area of 400 and 800 mm^2 respectively. Mean root length was 6.3 ± 0.52 s.e., 6.6 ± 0.43 s.e. and 6.8 ± 0.34 s.e mm week^{-1} in soft, semi-hardwood and hardwood cuttings respectively. Statistical differences between the two clones was found for root length (coef. = 1.2, t = 2.52, P>|t| 0.014) with j-erro producing longer roots (7.1 ± 0.28 s.e. mm week^{-1}) compared with e-erro (6.1 ± 0.4 s.e. mm week^{-1}).

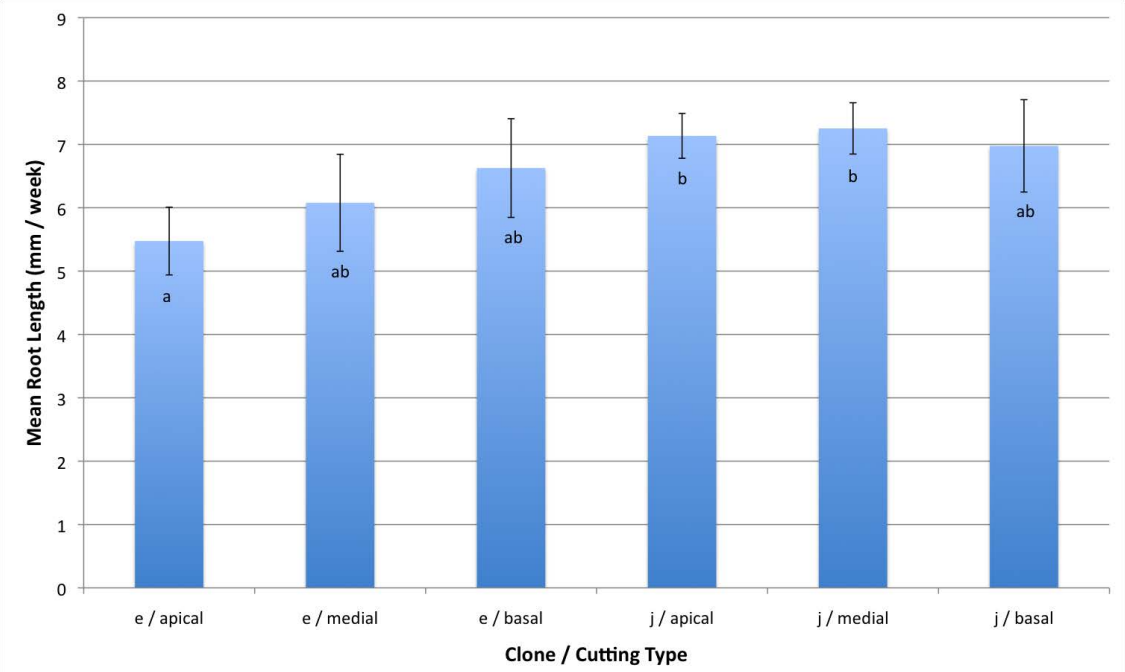

Figure 3.10 Mean root length for *S. austrocaledonicum* for apical, medial and basal leafy stem cuttings of e-erro and j-erro clones.

Vertical bars denote standard errors for mean root length across all treatments. Treatments that share the same lower case letter are not significantly different at the 0.05 level

3.3.5 Discussion

3.3.5.1 Effect of leaf area on cutting performance

The two leaf area treatments evaluated in this experiment had no discernable effect on any of the cutting response variables. It is possible that the two leaf area treatments (400mm2 and 800mm2) were within an acceptable range for the two genotypes evaluated in this study. It is clear however, that the proportion of leaves retained by the cuttings during propagation influences the percentage of cuttings that form adventitious roots. The results of this study did not determine whether leaf abscission was the cause of poor rooting or of physiological deficiencies in the cutting that also affected its capacity to form adventitious roots. In this experiment a 4-node cutting that retained 50% of its original leaf area had the same final leaf area as a 2-node cutting that retains all of its leaves. The rooting percentage of cuttings in the

former group was found to be significantly lower than the latter. This suggests that the provision of carbohydrate from the retained leaves is not the only factor affecting root induction. It is possible that some internal physiological deficiency within the cutting may have resulted in both leaf abscission and failure to root.

The positive effect of leaf retention on cutting success has been reported in other studies (Leakey & Courtts, 1989; Mesen *et al.*, 1997a). Studies have found that leaf retention is important for maintaining carbohydrates in propagated leafy stem cuttings (Leakey & Courtts, 1989; Ottosson & Welander, 1997; Tchoundjeu, Avana*, et al.*, 2002). Successful root initiation in cuttings of *Psidium guajava* was demonstrated in those that retained all their leaves during propagation, but those cuttings that lost all leaves failed to root (Santoro *et al.*, 2010). A strong correlation (r^2= 0.72) between leaf retention and root induction was recorded for cuttings of *Ilex paraguariensis* (Tarragó *et al.*, 2005). Rapaka (2007) demonstrated that leaf abscission was negatively correlated with the concentration of total nonstructural carbohydrates in the tissues of the cutting. Leaf abscission in *Portulaca grandiflora* was negatively correlated with pre-harvest leaf carbohydrate and stem starch concentrations (Rapaka *et al.*, 2007). These authors further demonstrated that internal carbohydrate concentrations in cuttings increased during the day. Leaf abscission in cuttings 48hrs after severance was complete in those collected at 8 a.m. and only partial 12 p.m. and 4 p.m (Rapaka *et al.*, 2007). In cutting experiments with *Vitis vinifera,* Thomas (2004) demonstrated that cuttings that retained their leaves during propagation had significantly greater rooting percentage (90-92%) compared with those where leaves were removed during propagation (2-4%).

Trueman (2013) demonstrated a positive correlation between rooting percentage and leaf retention in cuttings from two eucalypt hybrids. While auxin is important for

promoting root induction in cuttings, at high concentrations it can also contribute to leaf abscission (Trueman & Richardson, 2008), or reduce shoot growth (Hunt *et al.*, 2011) through its stimulation of ethylene production (Trueman & Adkins, 2013). Ethylene is a plant hormone controlling a wide range of physiological processes in plants, and its effect on cutting propagation is dependent upon sensitivity of tissues to ethylene at the time of propagation and is not consistent between studies (Tari & Nagy, 1996; Faivre-Rampant *et al.*, 1998; Ma *et al.*, 1998; Muller *et al.*, 1998; Currey *et al.*, 2013; Trueman & Adkins, 2013).

3.3.5.2 Effect of cutting type on cutting performance

The type of cutting had a significant effect on the percentage of rooting for both clones, although the effect was not consistent between them. In j-erro the percentage of rooting in apical cuttings was statistically equivalent to that in medial, but in e-erro they were significantly lower. In both clones, however, medial cuttings had a statistically greater rooting percentage than basal cuttings. Classifying cuttings by their relative position on the stem from which they were harvested may not take into account the possible physiological differences between stockplants, resulting in this inconsistent response to cutting type. However, given that the effect of cutting type on rooting was demonstrated in this study, further experimentation with this characteristic is required to assess its use as a reliable predictor of cutting performance on an individual clone basis. In both *Tectona grandis* (Husen & Pal, 2007) and *Dalbergia sissoo* (Husen, 2004), cuttings collected from the middle part of the stockplant stem had significantly greater rooting percentage compared with cuttings from both the upper and lower parts. Root induction in mini-cuttings of *Eucalypt globulus* hybrid clones was significantly higher in those collected from the apical compared with the medial part of the stem (Borges *et al.*, 2011; de Oliveira *et al.*, 2012).

Cutting type did not influence mean root number and length in either clone. Given the optimum performance of medial cuttings for the percentage of rooting across both clones, it is possible that these cutting types may provide acceptable results for routine propagation of this species.

3.3.5.3 Effect of clone on cutting performance

There was a clear difference between the two clones on percentage of rooting, mean number and length of roots in this experiment. The clone j-erro was statistically superior to e-erro for all these measures. This statistical difference remains even when we consider the optimum cutting type (medial) for each clone. The adventitious root induction of the clone j-erro in this experiment is consistent with Study 2, in which the j-erro rooting percentage was significantly greater than the three clones f-, h- and k-erro. However, it contrasts with Study 1, in which j-erro was an 'average' performer in terms of adventitious root induction. The effect of clone on rooting variation has been evident in several species such as *Tectona grandis* (Husen, 2013), *Pinus species* (Baltunis *et al.*, 2005; Shepherd *et al.*, 2005) and *Leucaena leucocephala* (Dick *et al.*, 1998).

3.3.5.4 Effect of cutting volume on rooting

Cutting volume was not found to have any influence on rooting percentage, mean root number or mean root length in *S. austrocaledonicum*. The results of this study contrasts with others studies that found a positive effect of cutting length (Leakey & Mohammed, 1985; Palanisamy *et al.*, 1997) and diameter (Palanisamy *et al.*, 1997) on rooting percentage.

3.3.5.5 Propagation period

The propagation period in this experiment may be defined as 10 weeks after the setting of cuttings, at which time 93% of all rooted cuttings had been recorded.

Limiting the propagation period to 10 weeks would result in five propagation cycles per year, whereas if 13 weeks is adopted, only four propagation cycles could be conducted. Economically, a shorter propagation period would be beneficial because more cuttings could be propagated during a given period. For instance, a total of five propagation cycles can be completed annually with a propagation period of 10 weeks, compared with four cycles using a 13 week propagation period. The length of the propagation period can be influenced by seasonal effects, possibly related to temperature at the time of collection and in the propagator (Hartmann *et al.*, 2002). Therefore further work may be required to quantify this in sandalwood propagation.

3.3.6 Conclusions

The study clearly demonstrated the importance of cutting leaf retention in the propagator that is independent of the original leaf area. Rooting percentage was positively associated with the proportion of original leaves retained during propagation. Given that the positive effect of leaf retention and no effect of leaf area on percent rooting, it is proposed that leaf abscission is a response to an unidentified physiological deficiency in the cutting. This deficiency also affects the cutting's capacity for developing adventitious roots.

Medial cuttings gave optimum performance for all three rooting traits across both clones. Significant interaction was found with cutting type and clone for each rooting trait. This interaction meant that, for e-erro clones, medial cuttings could be recommended, and for j-erro both apical and medial cuttings might be used in their propagation

The findings of this experiment demonstrated differences in response to cutting propagation between genotypes. Clone j-erro had superior cutting performance on all

three measures compared with e-erro. Rooting variation between the two clones used in this study reflected similar genotype-based variation observed in the previous two studies.

Cutting stem volume was not found to have any influence on rooting percentage, mean root number or mean root length in *S. austrocaledonicum*. The results of this study contrasted with other studies of woody perennials that found a positive effect of cutting length (Leakey & Mohammed, 1985; Palanisamy *et al.*, 1997) and diameter (Palanisamy *et al.*, 1997) on rooting percentage.

3.4 Study 4: Effect of available light, cutting type and clone on induction of adventitious roots in leafy stem cuttings of *Santalum austrocaledonicum*

3.4.1 Abstract

The effect of available light on the induction of adventitious roots in leafy stem cuttings in three clones of *Santalum austrocaledonicum* was investigated. A total of 960 cuttings were collected from potted hedges raised under 50% shade cloth. Cuttings were collected from apical and medial sections of stockplant stems and prepared, with each cutting having a total leaf area of 400 mm^2. Cuttings were treated with 8000 ppm IBA and set in four non-mist propagators with mean daily photon flux density levels of 116, 86, 56 and 48 $\mu mol\ m^{-2}\ s^{-1}$. Root induction in cuttings was assessed in weeks six, 10 and 13. Variation in mean daily temperature was found to be significant ($P<0.01$) among the four light treatments. A significant effect of irradiance was found for rooting percentage, but the effect was not consistent throughout the experiment since a significant interaction was found between irradiance and clone. While no clear response to irradiance emerged from this study it appears that, in a low light

environment, lower photon flux densities of 56 and 86 μmol^{-1} m^{-2} s^{-1} can be used effectively for routine propagation of *S. austrocaledonicum* cuttings.

3.4.2 Introduction

In leafy stem cuttings, carbohydrate production through photosynthesis is important in supporting the induction and development of adventitious roots (Costa & Challa, 2002). The level of photosynthesis and thus the capacity of stem cuttings to produce adventitious roots can be influenced by the level of irradiance in the propagation environment (Cameron *et al.*, 2005). However, higher light levels are not directly associated with improved root induction. Root induction in cuttings exposed to high light intensity may be inhibited, reduced or delayed due to the effects of water stress and/or stomatal closure with an associated reduction in photosynthesis (Loach, 1988a; Hartmann *et al.*, 1997).

Studies on the effect of light on cuttings during propagation have revealed variable responses between species. Mesen *et al.* (1997a) demonstrated rooting of *Cordia alliodora* cuttings is related to photosynthetic activity during propagation, and influenced by propagator environment, including the level of irradiance. In *E. grandis* light quality was found to have no effect in root induction, whereas exposure to far-red radiation was found to promote root induction in microcuttings of *E. globulus* (Ruedell *et al.*, 2013). The level of irradiance in the propagator was found to interact with IBA in the cuttings propagation of *Phaseolus aureus,* in which root production was enhanced under high irradiance and IBA and low irradiance without IBA. Root production in the cuttings propagation of herbaceous chrysanthemum (*Dendranthema* x *grandiflorum*) was elevated under propagator conditions of low irradiation (69 μmol m^{-2} s^{-1}) and high mean temperature (31°C) (Graves & Zhang, 1996). In contrast, root production in

fuchsia (*Fuchsia* x hybrida) cuttings was elevated under higher temperature regardless of the level of irradiance (Graves & Zhang, 1996). Given the optimum light intensity to promote the induction of adventitious roots in stem cuttings differs between species, it is important to evaluate this variable in little-studied species such as sandalwood.

In the current investigation, different levels of irradiance in the propagator were evaluated to determine their effect on the induction and growth of adventitious roots. The objective of the study was to determine the optimal light intensity (via different shade treatment(s)) for the promotion of adventitious root induction and development in *Santalum austrocaledonicum* leafy stem cuttings.

3.4.3 Materials and methods

Three clones of *S. austrocaledonicum* (j-erro, e-erro and f-erro) were raised in pots under 50% shade cloth. Leafy stem cuttings were collected from these clones and categorised as being from either apical or medial parts of the stem. A total of 960 double-node cuttings with a combined leaf area of 800mm^2 were prepared from three clones; j-erro (480 cuttings), e-erro (240) and f-erro (240). Cutting length and width of were measured and used to calculate cutting stem volume. All cuttings were treated with 8000ppm IBA (alcohol based) before being dried and set into one of four non-mist propagators, in a rooting medium composed of equal parts vermiculite and perlite (AFP 45%).

The four propagators were placed in a shade house under a 50% shade cloth, set at a height of 2.2 meters. Additional shade cloth of three different grades (25%, 50% & 70% shade) was fitted over 3 of the 4 propagators. These two levels of shading resulted in four light treatments within the propagators, defined by their mean daily photosynthetic photon flux density levels (116, 86, 56 and 48 μmol m^{-2} s^{-1}). The level

of photosynthetically active radiation (PAR) in each of the propagators was logged every 30 minutes using a PAR Sensor (Skye PAR Special SKP210) linked to a Unidata Prologger. Only PAR data from 18th January to 12th March was used to compare radiation levels between propagators, since technical difficulties were experienced after this period. Temperature was also monitored in each propagator using a Tinytag internal temperature data logger (Plus 2, TGP-4500), which logged the average temperature at 30 minute intervals over the entire propagation period.

The propagators were monitored daily. At the same time the water level was manually maintained at 1/3 the level of the lower rock substrate, and shed leaves were removed. Each time the propagator was opened for monitoring or assessment the cuttings were sprayed with a hand water-misting bottle, to maintain a high humidity within.

Cuttings were set from 28th January and 1st February 2008 (37 months old) and subsequent assessments were made on weeks 6, 10 and 13. Successfully rooted cuttings were transferred from the propagator and 'potted', with no further measurements included in this experiment.

In comparing the individual effects of the three treatments (clone, cutting type and shade) cumulative data was used across the three assessments (weeks six, ten and 13) for each of the three response variables (mean percentage rooting, mean root number and mean root length). Rooting percentage was arcsine transformed to ensure a normal distribution and equal variances. The effect of the treatments on the rooting characteristics of *S. austrocaledonicum* cuttings was evaluated using a General Linear Model (GLM) with the three treatments, and the pairwise interaction between them as the factors and the cutting length and width as co-variates. Pairwise differences

between the treatments were tested using the Tukey-Kramer method of multiple comparisons.

3.4.4 Results

3.4.4.1 Light intensity variation between the shade treatments

Light intensity was found to vary significantly ($P<0.01$) between all four shade treatments, with the highest mean light intensity found at 50% (116 μmolm^{-2} s^{-1}) followed by 50+25% (86 μmol m^{-2} s^{-1}), 50+50% (56 μmol m^{-2} s^{-1}) and 50+70% (48 μmol m^{-2} s^{-1}) (Figure 3.11).

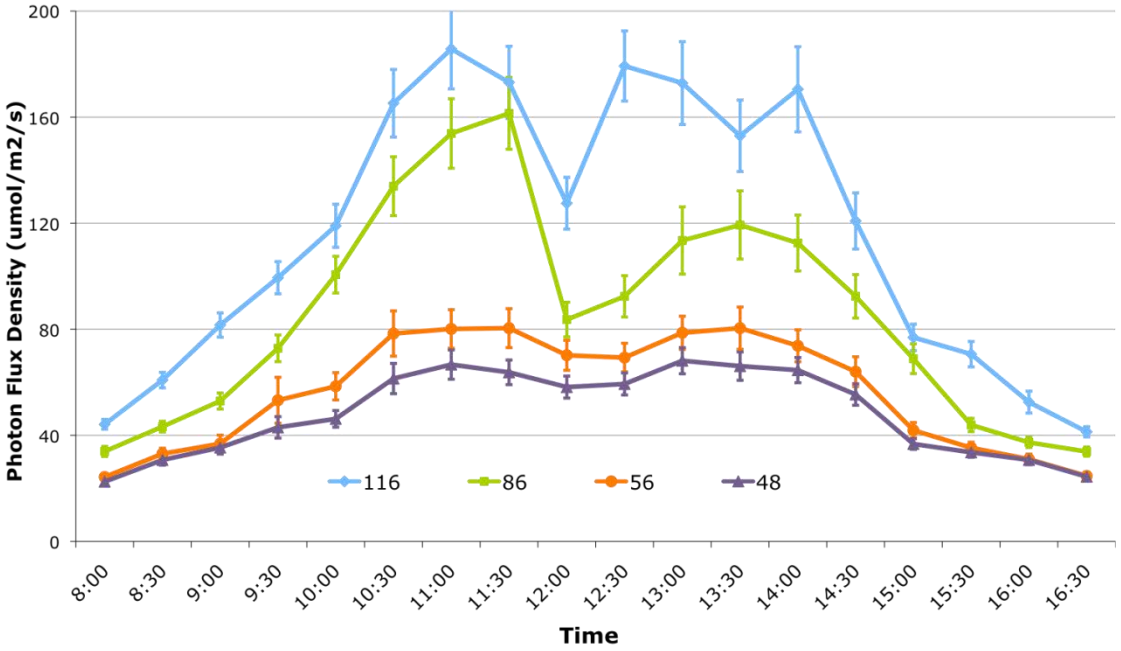

Figure 3.11 Mean photosynthetic photon flux density (PPFD) at 30minute intervals during the propagation period for each of the four propagators identified by their mean daily PPFD levels (116, 86, 56 and 48 µmol m^{-2} s^{-1})

3.4.4.2 Temperature variation between the shade treatments

The differences in light intensity between the propagators resulted in significant ($P<0.05$) temperature variation among them. The mean temperatures reflected the level of PAR in the propagator, with the highest mean daily temperature found in the 116 µmol m^{-2} s^{-1} treatment (26.4°C), followed by 86 µmol m^{-2} s^{-1} (26.0°C), 56 µmol m^{-2} s^{-1} (25.6°C) and 48 µmol m^{-2} s^{-1} (25.3°C). The level of sunlight in the propagator influenced the mean temperature, since no differences were found between them for daily minimum temperature (typically at night), but significant ($P<0.05$) differences were found for mean daily maximum temperature (Figure 3.12). This difference in maximum temperature resulted in a greater frequency of temperatures higher than 35°C in the propagators with higher light intensity. The temperature in 116 µmolm^{-2} s^{-1} treatment exceeded 35°C for a period of 156.5 hours, which was followed by 86 µmol m^{-2} s^{-1} (101.5 hrs), 56 µmol m^{-2} s^{-1} (59.5 hrs) and 48 µmol m^{-2} s^{-1} (12 hrs) light

treatments. Significantly greater mean daily temperature was found in the first two months (January and February) compared with the final three months (March, April, May) of the experiment (Figure 3.12).

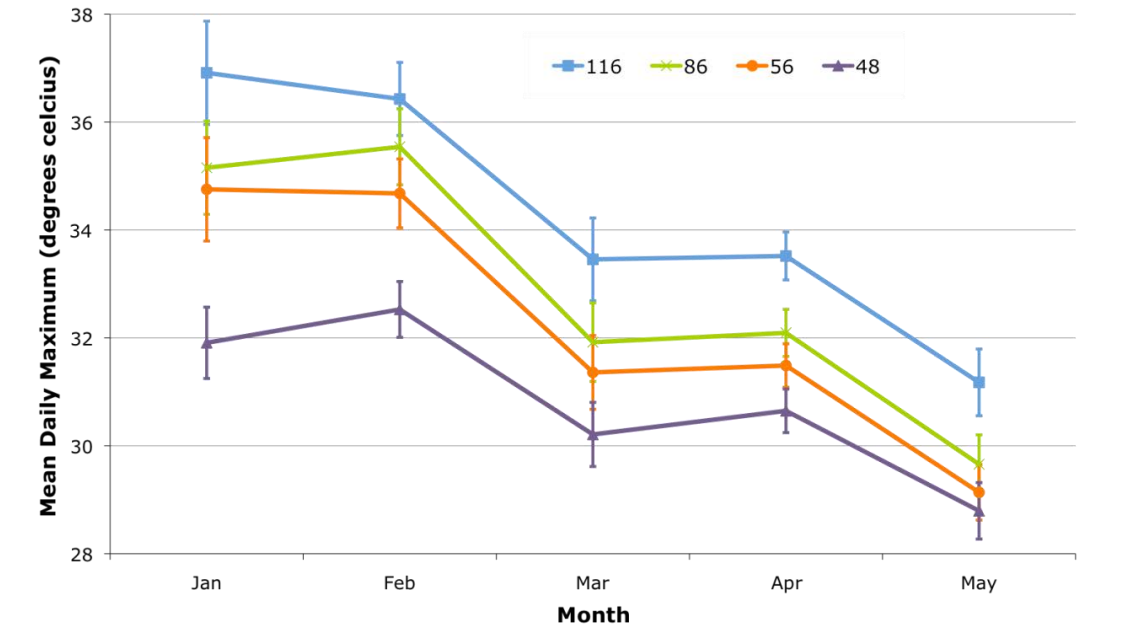

Figure 3.12 Mean daily maximum temperature (°C) between the four mean-daily-light treatments (116, 86, 56 and 48 µmol m^{-2} s^{-1}) for the months during the propagation period.

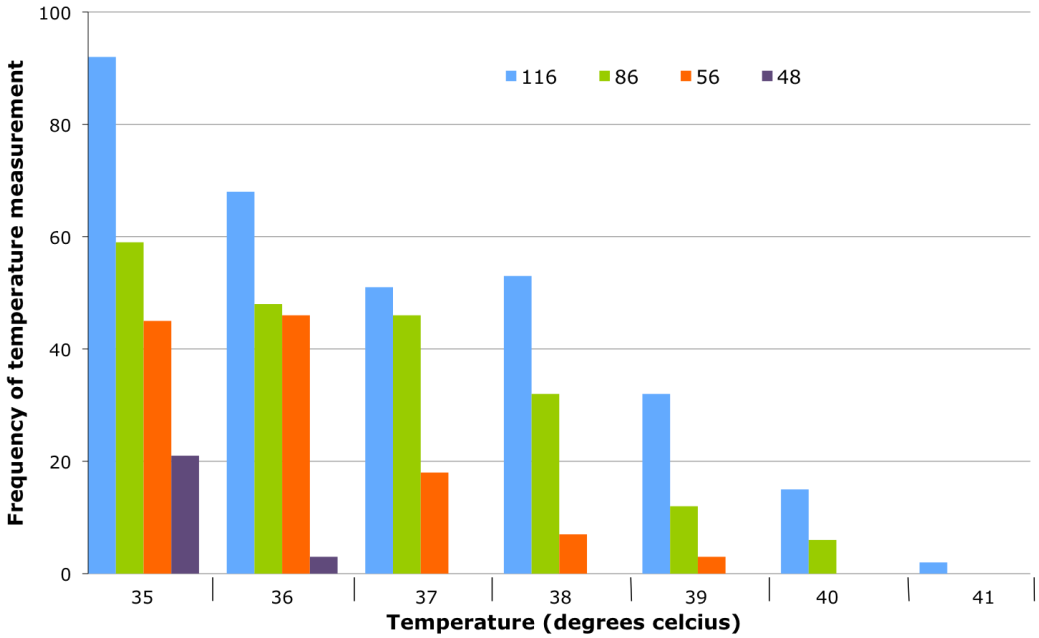

Figure 3.13 Frequency of recorded temperatures between 35 and 41°C for each light treatment (116, 86, 56 and 48 µmol m^{-2} s^{-1}).
Each observation represents the mean temperature for a 30 minute period.

3.4.4.3 Variation in root induction between assessments

A significantly ($P<0.05$) lower percentage of cuttings developed adventitious roots in week six compared with both weeks ten and 13, with no significant difference between the latter. No significant interaction was found between treatment combination and week of assessment, so therefore the effect of time in increasing the percentage of rooted cuttings was consistent among the individual treatment combinations. These results indicate that ten weeks in the propagator may be sufficient to allow cuttings with the capacity to develop adventitious roots to complete the physiological processes necessary for root induction. However, in this study we used the cumulative rooting percentages from the final assessment 13 weeks after setting the cuttings in the propagator.

3.4.4.4 Effect of clone, cutting type and irradiance on adventitious root induction

Significant differences in rooting percentage were found among the three clones used in this study, in which j-errro had a significantly ($P<0.01$) greater rooting percentage compared with both e- and f-erro clones. These results are consistent with those in previous experiments (Sections 3.2 and 3.3), when comparing j-erro with the clones e-, f-, h- and k-erro and the five tanna-derived clones. A significant interaction ($P<0.01$) between clone and each light treatment and cutting type was found. When the highest treatment combination within each clone (e-erro apical 48 $\mu molm^{-2}$ s^{-1}, f-erro apical 56 μmol m^{-2} s^{-1} and j-erro apical 56 μmol m^{-2} s^{-1}) was compared, no significant difference in rooting percentage was found between them.

Apical cuttings were found to produce a significantly ($P<0.01$) greater percentage of rooted cuttings compared with medial cuttings in both e- and f-erro

clones. A notable exception to this was the lack of significant difference between the rooting percentage between apical and medial cuttings in the e-erro clone within the 116 μmol m^{-2} s^{-1} light treatments. In the clone j-erro, no significant difference in rooting percentage was found between apical and medial cuttings.

No interaction was found between cutting type and shade treatment, so that the shade level for a given clone that maximised rooting percentage was consistent for both apical and medial cuttings.

A significant effect of irradiance was found, but given the significant interaction between shade and clone the effect was not found to be consistent throughout the experiment. For instance, in the clone j-erro a significantly ($P<0.05$) higher rooting percentage was found for 56 compared with 48 and 86 μmol m^{-2} s^{-1} light treatments for both apical and medial cuttings (Figure 3.14). In contrast there was no significant difference in rooting percentage between all shade treatments for 'f-erro' within each cutting type. A consistent rooting percentage between shade treatments was also found in 'e-erro', except for a significantly ($P<0.05$) lower percentage in 116 μmol m^{-2} s^{-1} compared with all three lower light levels (48, 56, 86 μmol m^{-2} s^{-1}) in medial cuttings (Figure 3.14).

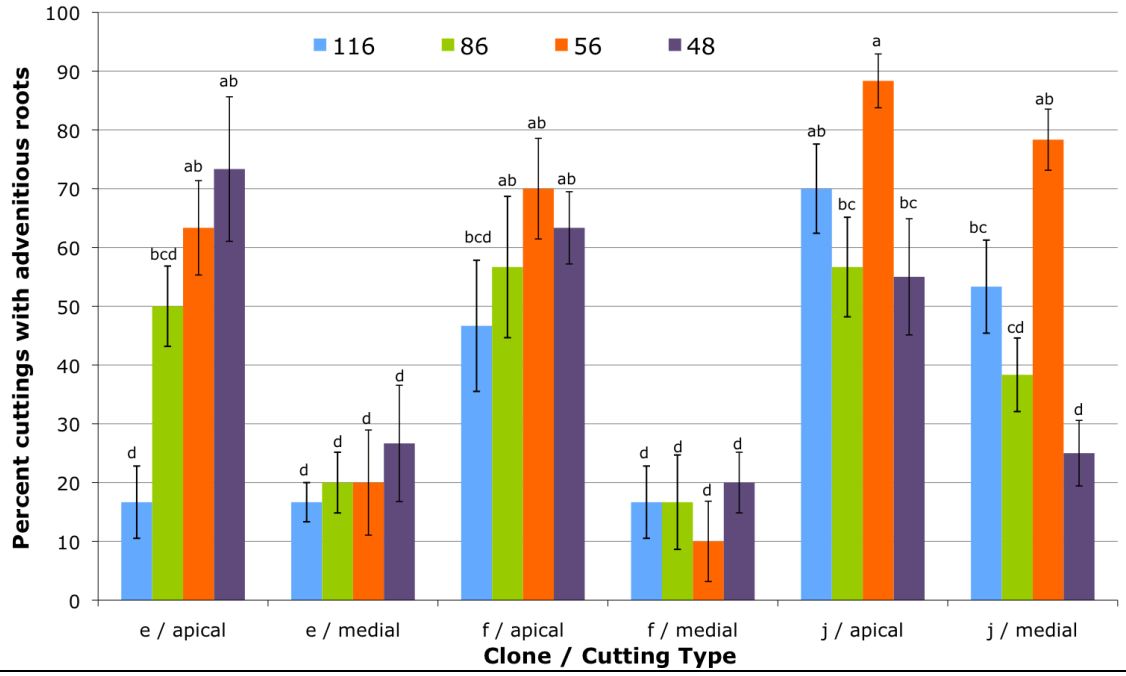

Figure 3.14 Final rooting percentage for two types of cuttings (apical and medial) across three clones of *S. austrocaledonicum* (e-, f- and j-erro) under 4 mean daily PPFD levels (116, 86, 56 and 48 µmol m^{-2} s^{-1}) after 13 weeks within the propagation environment.

Treatments sharing same lower case letters are not significantly different at the 0.05 level across all clone, cutting type and shade treatments.

3.4.4.5 Effect of clone, cutting type and irradiance on root number

The f-erro clone was found to have significantly ($P<0.05$) greater root numbers compared with the e-erro clone, while j-erro was statistically intermediate. A positive interaction was found between clone and shade treatments indicating that root numbers were not consistent between clones within a given shade treatment. No interaction was found between clone and cutting type.

No significant effect of cutting type on the mean number of roots was found. No interaction was found between cutting type and the light treatment, suggesting that equivalent root numbers could be found between cutting types within each light treatment. An exception to this was the f-erro clone, where apical cuttings in 48 µmol m^{-2} s^{-1} had a significantly ($P<0.05$) greater number of roots compared with medial cuttings grown under the same light treatment (Figure 3.15).

Irradiance had no significant effect on the mean number of roots per rooted cutting. An exception was the f-erro apical cuttings where those under 116 µmol m^{-2} s^{-1} had significantly fewer roots (1.4 roots) than 48 µmol m^{-2} s^{-1} (2.5 roots) (Figure 3.15). A significant ($P<0.05$) three-way interaction was found between clone, cutting type and shade treatment.

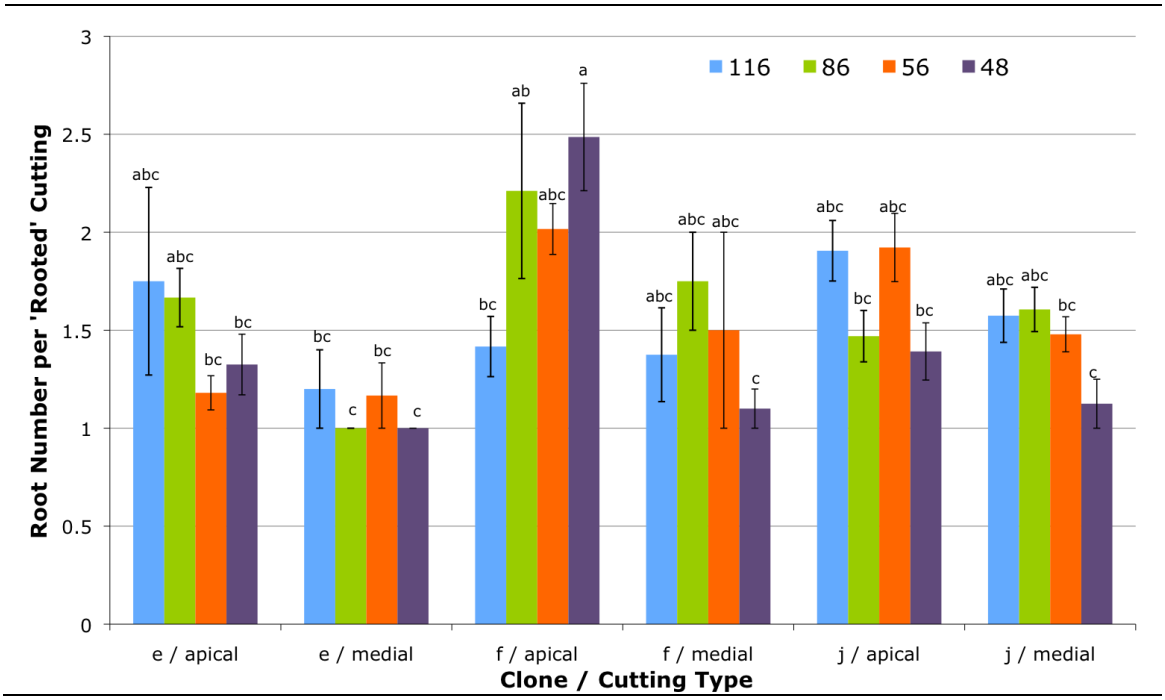

Figure 3.15 Mean number of roots per cutting for two types of cuttings (apical and medial) across three clones of *S. austrocaledonicum* (e-, f- and j-erro) under 4 mean daily PPFD levels (116, 86, 56 and 48 µmol m^{-2} s^{-1}) after 13 weeks within the propagation environment.

Treatments sharing same lower case letters are not significantly different at the 0.05 level across all clone, cutting type and shade treatments.

3.4.4.6 Effect of clone, cutting type and irradiance on root length

No significant differences were found in mean root length per cutting between clones or cutting types, indicating that root growth after induction was equivalent between the clones and cutting types under the four shade treatments. No significant interactions were found between the clone and cutting type treatments.

The irradiance treatment was found to have a significant ($P<0.05$) effect on the mean root length per cutting. The e-erro apical cuttings grown under 86 µmol m^{-2} s^{-1} had significantly lower mean root lengths than other e-erro apical cuttings grown under 48, 56 and 116 µmol m^{-2} s^{-1} light treatments (Figure 3.16). The j-erro medial cuttings grown at 86 µmol m^{-2} s^{-1} had significantly lower mean root length medial cuttings under 116 µmol m^{-2} s^{-1}. Under all other light treatments each clone had cuttings with roots of an equivalent mean length (Figure 3.16).

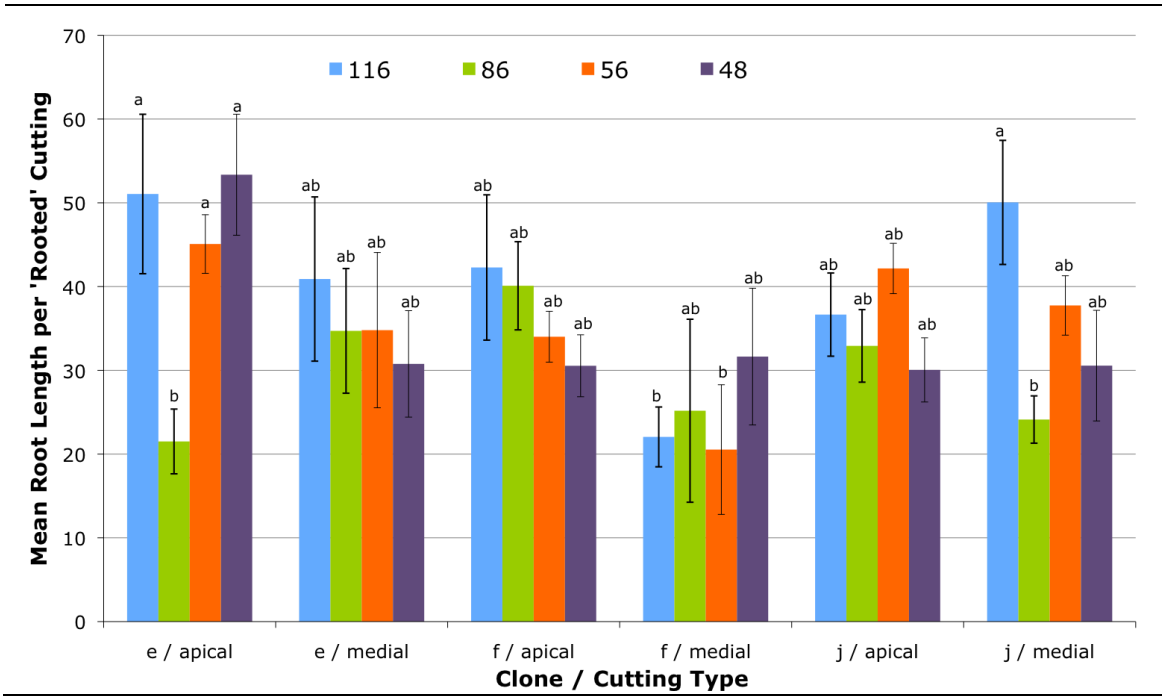

Figure 3.16 Mean root length for two types of cuttings (apical and medial) across three clones of *S. austrocaledonicum* (e-, f- and j-erro) under 4 mean daily PPFD levels (116, 86, 56 and 48 µmol m^{-2} s^{-1}) after 13 weeks within the propagation environment.

Treatments sharing same lower case letters are not significantly different at the 0.05 level across all clone, cutting type and shade treatments.

3.4.4.7 Effect of cutting length and width on rooting percentage, root number and length

Cutting length had no significant effect on mean rooting percentage, root number or length, when evaluated as a covariate. Furthermore, no correlation was found between cutting length with each of the rooting percentage, mean root number

and length across all clones. Cutting stem diameter had no significant effect or correlation on root number or length. Cutting stem diameter was found to be weakly correlated ($R^2 = 0.21$) with rooting percentage for the e- and f-erro clones. Furthermore, cuttings forming adventitious roots had a mean stem cutting diameter (1.11mm) slightly, but significantly lower than those failing to form any roots (1.17mm). No significant difference in cutting stem diameter however, was found between cuttings with or without roots within the apical or medial cutting types. Apical cuttings had a significantly ($P<0.05$) narrower mean cutting stem width (1.1mm) and significantly greater mean percentage rooted cuttings (61%) compared with the medial type (1.2mm & 33% respectively). The improved rooting capacity of apical cuttings compared with medial cuttings is likely to be the result of physiological differences (Agbo & Obi, 2007) rather than differences in cutting stem dimensions and leaf area as investigated in this study.

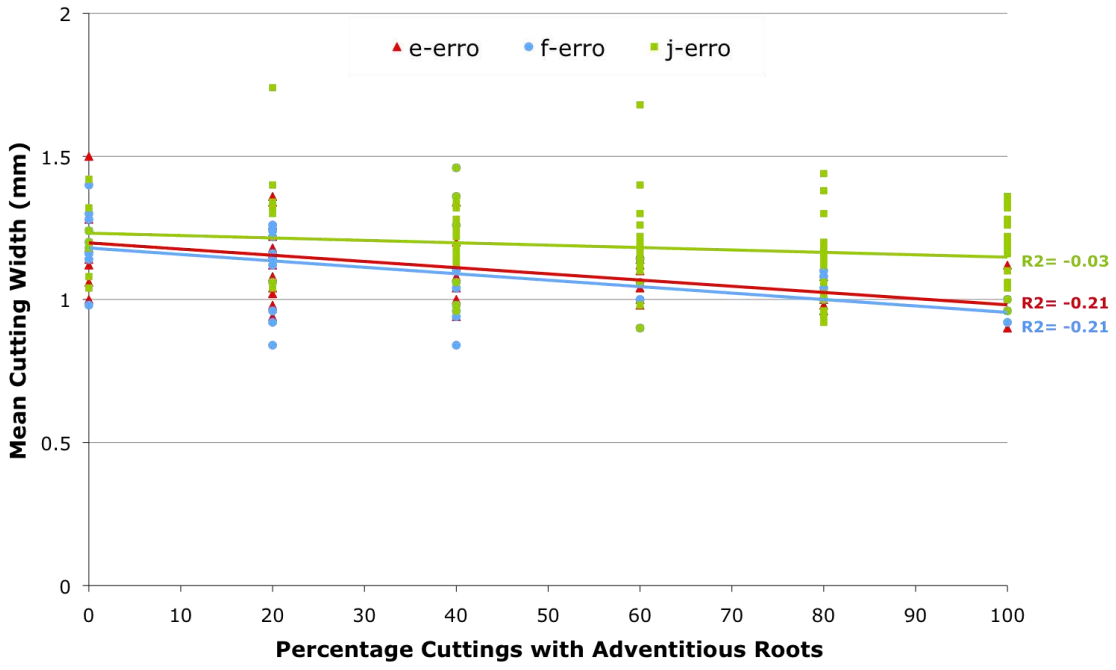

Figure 3.17 Scatterplot of mean cutting stem diameter and percentage cuttings with adventitious roots for each of the three clones (e-erro, f-erro and j-erro) evaluated.

3.4.5 Discussion

This study demonstrated that a species classified as 'difficult-to-root' can be successfully propagated by cuttings provided the conditions in the propagation environment are optimised for the particular genotype. The effect of a given shade treatment on rooting percentage, mean root number and length was variable among different genotypes of *S. austrocaledonicum*. This suggests that light levels need to be optimised for each clone to maximise the level of rooting success in cuttings propagation of this species.

3.4.5.1 Effect of clone on cutting performance

This experiment confirmed the superior performance for adventitious root induction of clone j-erro compared with e- and f-erro. Clone j-erro was intermediate for mean root number per cutting and there was no difference between the three clones for mean root length. This result is consistent with the clonal variation in root induction and development in the three previous experiments conducted in this chapter. It is also consistent with the variation in the cutting performance between genotypes across a wide range of species including *Arbutus unedo* (Metaxas *et al.*, 2008), *Camellia sinensis* (Lima *et al.*, 2013), *Colutea istria* (de Andres *et al.*, 2005), *Corylus avellana* (Contessa *et al.*, 2012) hybrid *Populus* (Stenvall *et al.*, 2004), *Leucaena* species (Shi & Brewbaker, 2006), *Tectona grandis* (Palanisamy *et al.*, 2009), and several hybrids and species of *Eucalyptus* (Nourissier & Monteuuis, 2008; Mankessi *et al.*, 2010; Brondani *et al.*, 2014).

The percentage of root induction in this study ranged from zero for several genotypes from Tanna, to a high of between 90 and 100% in specific treatments for genotypes from Erromango. Such an extreme range has also been demonstrated in

other species such as *Persoonia virgata,* in which one genotype failed to root but others achieved rooting of 90% (Bauer *et al.*, 1999). Similarly in *Cupressus sempervirens* the rooting percentage among clones ranged from 0 to 88% (Capuana *et al.*, 2000).

Marques (2002) identified quantitative trait loci for adventitious rooting capacity in four species of *Eucalyptus*. High narrow sense heritability was also found for adventitious rooting ability in *Eucalyptus globulus* (Araújo *et al.*, 1997; Ruaud *et al.*, 1999). These studies with eucalypts demonstrated that a plant's capacity for adventitious root induction is modified by its genotype, and the propensity for vegetative propagation may therefore be considered as a selection criteria. This is certainly an interesting area for further research in *Santalum*, as it would allow for routine mass propagation of selected genotypes. Leakey (2004b) suggested that differences between genotypes in their propensity for adventitious root induction is more likely due to genetic variability in morphological and physiological factors that influence root induction, rather than direct genetic control over root initiation. It is therefore considered important to continue research into the vegetative propagation of *Santalum* to improve our understanding of various physiological influences on root induction.

3.4.5.2 Effect of cutting type on cutting performance

Interestingly, apical cuttings were found to have a greater percentage of rooted cuttings compared with medial cuttings in e- and f-erro clones. This is in contrast with the previous experiment where the opposite was found for the e-erro clone. While this difference between experiments is important for clone e-erro, no such difference in rooting percentage between apical and medial cuttings was observed for the j-erro clone in both Studies 3 and 4. The biological mechanisms for this difference between the

experiments are not clear, although there could be some seasonal effects, since cuttings were collected in August in Study 3 and February in the current study. The classification of cutting types by position on the stockplant stem may need to be related to aspects of their physiological stage in order to provide a more objective classification of cutting type. Several studies have found seasonal effects on rooted cutting success. Loach (1977) found that cuttings collected during rainy seasons performed better than those collected in dry seasons, while the rooting percentage of juvenile cuttings of *Jatropha curcas L*. performed better when cuttings were collected during the monsoon (90.5%) than in the dry season (24.1%) (Bijalwan & Thakur, 2010).

3.4.5.3 Effect of irradiance on cutting performance

A significant effect of irradiance was found for rooting percentage, but given the significant interaction between irradiance and clone the effect was not consistent throughout the experiment. The irradiance treatment had no significant effect on the mean number of roots per rooted cutting. While the irradiance treatment was found to have a significant ($P<0.05$) effect on the mean root length per cutting, there was a significant interaction between the clones and no consistent pattern emerged. While no clear cutting response to irradiance emerged from this study, it appears that cuttings at lower photon flux densities (56 and 86 μmol m^{-2} s^{-1}), which were grown in propagators placed under 50% high-set shade with an additional 25 or 50% shade cloth put over the top of the propagator, could be used effectively for routine propagation of *S. austrocaledonicum* cuttings. In a practical application an equivalent light intensity may be achieved by placing propagators under 70% high-set shade.

3.4.5.4 Effect of cutting dimensions on rooting

While cutting stem volume is known to have a positive effect on rooting percentage in other species (Palanisamy *et al.*, 1997), our study found that cutting length did not correlate, and cutting stem diameter was weakly negatively (R2= -0.21) correlated with rooting percentage, suggesting that root formation in the species is more likely to be controlled by physiological effects than the stem volume of cuttings. Both cutting stem diameter and length had no statistical association with cutting root number or length.

3.4.6 Conclusions

Variable clonal responses in root induction and root number indicates that the propagation environment ought preferably to be optimised for each clone. This variation in cutting performance of clones has been consistent throughout the four experiments. Apical cuttings were found to have significantly greater percent cuttings with adventitious roots compared with medial in two (e- and h-erro) of the three clones. This result was in contrast with the better rooting found in medial cuttings for e-erro in the previous experiment. No difference in rooting percentage was found between the two cutting types for the third clone j-erro, which was consistent with the previous study. The level of irradiance in the propagator had a variable effect on rooting performance between clones. Generally cuttings of *S. austrocaledonicum* should perform well with a shade treatment that achieves a mean daily photon flux density of between 56 and 86 μmol m^{-2} s^{-1}, which may be most efficiently achieved by placing propagators under 70% high-set shade cloth, as measured previously with the PAR sensor.

3.5 Cutting propagation in *Santalum austrocaledonicum*

The cutting propagation studies have elucidated some basic requirements for successfully cloning *S. austrocaledonicum* by cuttings. The results demonstrated that IBA concentrations at 3000ppm, 4000ppm and 8000ppm did not have any significant effect on rooting percentage compared to control (0ppm), suggesting that either IBA had no effect on rooting or possibly that either concentrations lower than 3000ppm or greater than 8000ppm could be important to investigate further. *S. austrocaledonicum* cuttings will produce adventitious roots in suitable rooting media across a range of porosity (AFP 29%-42%); however, developing roots can be damaged in highly abrasive media (such as gravel) during cutting removal from the medium. The study also found that good management of cutting hedges is required to achieve high quality, less lignified softwood cuttings that root better than semi-hardwood and hardwood cuttings. Leaf retention during propagation is critical for success, since cuttings that shed all their leaves remain alive during the propagation period but only rarely produce roots. Light is important for root induction, and this study found that maintaining a shade treatment that achieves a mean daily photon flux density of between 56 and 86 $\mu mol\ m^{-2}\ s^{-1}$ increased rooting percentage compared to other shade treatments used in the study. The majority of cuttings produced adventitious roots by 65 days and the rooting percentage was reduced after that time, with the remaining cuttings either producing a small number of roots, or remaining rootless until death.

Chapter 4. Reproductive Biology of *Santalum*

4.1 Study: Floral phenology in five species of sandalwood: *Santalum album*, *S. austrocaledonicum S. lanceolatum*, S *macgregorii* and *S. yasi*)

4.1.1 Abstract

Systematic observations of floral morphology were made for five sandalwood species (*Santalum album*, *S. austrocaledonicum*, *S. lanceolatum, S. macgregorii and S. yasi*) to identify and describe the stages of flower development, their timing and duration. Similarities in both floral morphology and phenology were found between *S. austrocaledonicum* and *S. lanceolatum*. Both species have flowers of a similar size and both have green-white coloured tepals throughout the life of the flower. In contrast, the flowers of *S. album* and *S. yasi* were substantially smaller and the tepals were green-white in colour on opening, but rapidly changed to pink (19-24 hrs for *S. album* and 4-12 hrs for *S. yasi*) and dark red (25-53 hrs for *S. album* and 13-36 hrs for *S. yasi*). *S. macgregorii* is somewhat intermediate sharing a similar phenology with *S. austrocaledonicum* and *S. lanceolatum,* but a similar morphology with *S. album* and *S. yasi*.

The tepals of all five species were found to open rapidly (over 3-4 hours), typically during the morning. The tepals of *S. album*, *S. austrocaledonicum* and *S. yasi* were found to open completely (90^{o} in relation to the stigma), with the tepal tips recurving outward and away from the centre of the flower. In contrast, the tepals of *S. lanceolatum* and *S. macgregorii* appeared to open to approximately 45-60^{o} in relation to the stigma, with the tepal tips recurving inward towards the centre of the flower. No change in stigma morphology such as swelling or evidence of exudate was observed

after flower opening in any of the species. The style and stigma in *S. album, S. macgregorii and S. yasi* changed from white to red in synchrony with the change in tepal colour and after pollen shed.

Clear differences in flower longevity was found between the five species, with *S. album* opening for the longest period (7-9 days) followed by *S. yasi (*48 hrs), *S. austrocaledonicum* (24-48hrs), *S. lanceolatum* (12–24 hrs) and *S. macgregorii* (4-12 hrs). Pollen shed occurred immediately upon flower opening, and under conditions of open pollination most pollen grains are removed by the afternoon. Fertilisation and seed set was recorded after controlled pollination of the stigma immediately following flower opening in all three species. In *S. austrocaledonicum, S. lanceolatum* and *S. macgregorii* flowers, all of which are open for 24 hours or less, it is likely that stigma receptivity occurs during or immediately after pollen shed. While this may also be true for *S. album* and *S. yasi* it is possible that pollen grains remain viable until the stigma becomes receptive, possibly at the completion of flower and stigma colour change (52 and 24 hours respectively).

4.1.2 Introduction

The development of sandalwood cultivars suitable for agroforestry plantations requires three important components:

1) Genetic variation in the characters of interest for breeding,

2) Knowledge of its breeding system, and

3) Effective strategy to implement a breeding programme.

Significant variation in oil characters of interest has been found for *S. album* (Kumar, 2011) *S. austrocaledonicum* (Page, Southwell*, et al.*, 2010) and *S. lanceolatum*

(Page *et al.*, 2007) and aspects of the breeding system have been elucidated for *S. album* (Veerendra & Padmanabha, 1996; Rugkhla *et al.*, 1997; Kulkarni & Muniyamma, 1998; McComb & Jones, 1998; Ma *et al.*, 2006). A breeding strategy is likely to include open pollinated, controlled pollinated and clonal progenies. An efficient controlled pollination protocol for sandalwood will depend on detailed knowledge of a particular species floral morphology and development.

Flower opening, pollen-shed and stigma receptivity are important events in the development of a flower. Knowledge of the onset and duration of these events is important for undertaking controlled pollination, since it allows the correct timing of pollen collection, emasculation and pollination. The development of effective controlled pollination techniques and procedures is important to maximise successful fertilisation and subsequent seed set. The aim of the current study was to identify and describe the distinct morphological stages in the development of flowers and inflorescences in five species of sandalwood (*Santalum album*, *S. austrocaledonicum*, *S. lanceolatum*, *S. macgregorii* and *S. yasi*).

4.1.3 Materials and methods

4.1.3.1 Stages of flower and inflorescence development

Twice daily observations of individual inflorescences from three genotypes of each of *S. album and S. lanceolatum,* six genotypes of *S. austrocaledonicum* and two genotypes of each of *S. macgregorii* and *S. yasi* were undertaken to determine flower and inflorescence phenology. These observations were undertaken from the period of anthesis of the first flower, to petal fall and style desiccation of the last flower on a given inflorescence. Based on these observations, stages in the phenology of single

flowers were identified. Floral stages were measured relative to the day of anthesis, which was considered to be day zero on its development scale.

4.1.4 Results

Flowers of all five species occurred in terminal or axillary panicles consisting of >10 flowers. The corolla of all five species consisted of 4-, rarely 5-tepals, which together with the anthers, alternated with the hypanthial lobes. Anther filaments were short and dorsi-fixed to anthers that shed pollen along longitudinal slits. Trichomes were found at the base of each anther filament, extending in the floral tube and towards the back of the anthers (longer in *S. lanceolatum* and *S. austrocaledonicum*). The ovary was inferior to the floral tube, and once fertilisation had been affected, the floral tube abscised from the pedicel. The ovary swelled to become a single seeded drupe with a floral tube abscission scar at its top. In the absence of fertilisation, flower abscission occurred rapidly, approximately 1–2 days after flower closure (*S. austrocaledonicum, S. macgregorii* and *S. lanceolatum*) or floral tube desiccation (*S. album* and *S. yasi*).

4.1.4.1 Flower and inflorescence development in Santalum lanceolatum

The floral tube of *S. lanceolatum* was approximately 3mm long and the anther filaments, hypanthial lobes and tepals emerged from the top. The width of the flower from the tips of each tepal ranged from 5-7mm. Fragrant nectar was produced at the base of the floral tube. Flowers were found to open and close within 12-24 hours, often opening in the morning and closing by the evening of a given day (Table 4.1). All flowers on the inflorescence completed their opening and closing over a period of 7-14 days, depending upon prevailing weather conditions. The mean number of flowers per inflorescence was 11 and the mean number of days for each inflorescence was 11.5.

Anther filaments were short (1.0-1.5mm long) and dorsi-fixed to anthers (1.5-2.0mm long) that shed pollen along longitudinal slits. Trichomes (0.5-1.5mm long) were found at the base of each anther filament extending in the floral tube and towards the back of the anthers. Hypanthial lobes were typically yellow and alternated between anthers ranging from 1.0-1.5mm long.

The style was approximately double the length of the floral tube and the stigma was presented approximately 0.5-1.0mm above the top of the anthers. The stigma had 3 to 4 lobes and short papillae, but no change in stigma morphology such as swelling, colour change or evidence of exudate was observed. Flower abscission occurred rapidly (1-2 days) in flowers that fail to set fruit (Table 4.1).

Phenological stages of *S. lanceolatum* flowers may be simply described as opened or closed given that they performed this within 12-24 hours (Table 4.1), with little morphological changes in their reproductive structures during this brief period. Pollen shed occurred simultaneously as the flower opened (defined as anthesis) and, in the absence of sufficient pollinators, the flower can close again with apparently viable pollen remaining on the anthers. It is unclear whether the stigma was viable when the flower opens, but given that flower abscission in the absence of pollination occurred within 48 hours of closing, stigma receptivity was likely to be during the open phase or possibly just after the flower closed. Controlled pollination obviously needed to occur during the day of opening.

4.1.4.2 Flower and inflorescence development in Santalum album

While the general floral morphology of *Santalum album* was similar to that of *S. lanceolatum*, the flowers of *S. album* (a) had a longer 'life' – 7-9 days compared with 12-24 hours (Table 4.1), (b) changed colour from white to red rapidly after opening, (c)

had a smaller and less prominent stigma that did not extend beyond the height of the stamens (d) had fewer trichomes at the base of the anther filament, particularly within the floral tube and (e) had tepals that opened but did not close.

In the inflorescences evaluated, the mean number of days from flower opening to closing was 8.7 days, with the flower tepals opening white and remaining this colour for only a brief period (0.8 days), before gradually changing over 1.4 days from white to red and remaining red for the remaining 6.5 days, before either the flower or floral tube was abscised.

The floral tube was approximately 2.5mm long and the anther filaments, hypanthial lobes and tepals emerged from the top. The width of the flower from the tips of each tepal ranged from 5-6mm when the flower first opened, but as the colour of the tepals changed from white to red, the tips recurved. The length of anther filaments and anthers (<1.0mm and <1.5mm long respectively) were shorter than for *S. lanceolatum*. Trichomes (~0.5mm long) were found at the base of each anther filament extending only towards the back of the anthers. Trichomes at the base of the anther filaments were either minute or absent within the floral tube. Hypanthial lobes (0.5-1.0mm long) were red from flower opening and were shorter than in *S. lanceolatum*.

The style was approximately 3mm long and the stigma was presented at, or slightly below, the level of the top of the anthers (approximately 0.5mm away from each anther) when the flower opened. As the flower developed the anthers recurved away from the stigma, increasing the distance between the stigma and anther to approximately 1.0mm. The stigma had 3 to 4 lobes and short papillae, the stigma and style changed colour from white at opening to pink, concurrently with the colour change in tepals. No evidence of stigma exudate in *S. album* was observed during the

experiment. The ovary was inferior to the floral tube. The mean number of flowers per inflorescence was 28 flowers (range 20-40) and the mean number of days for the life of an inflorescence was 12.4.

4.1.4.3 Flower and inflorescence development in Santalum austrocaledonicum

The general floral morphology of *Santalum austrocaledonicum* was similar to that of *S. lanceolatum* with very few distinguishing features between the two. The main difference between these species was the longer flower life of *S. austrocaledonicum,* which has flowers that opened and closed within a 24-48 hour period compared with a 12-24 hour period for *S. lanceolatum* (Table 4.1). A flower from *S. austrocaledonicum* would typically open during the morning of a given day and close again during the afternoon of the following day. The tepals of *S. austrocaledonicum* opened more completely than *S. lanceolatum* where the tips of its tepals recurved outwards within 12 hours of opening before closing again 36 hours later. The mean number of flowers observed on an inflorescence was 15.5 and mean period of inflorescence was 12 days.

4.1.4.4 Flower and inflorescence development in Santalum macgregorii

The phenology of *Santalum macgregorii* flowers was similar to that of *S. austrocaledonicum* and *S. lanceolatum*, in that its flowers open and then close. The species however had the shortest flower life for an unfertilised flower among the five species studied, with an average flower life of 24 hours (Table 4.1). It was observed that once the flower closed, it could take an average of six hours for the closed flower to fall off the inflorescence. The flowers were observed to open at any time during the day from morning to afternoon. Despite the similarity of opening and closing *S. macgregorii* flowers changed colour from white to red, similarly to that of *S. album* and

S. yasi. The change in colour occurred just prior to flower opening, which continued to darken as the flower opened and closed. The mean number of flowers observed on an inflorescence was 23 and mean period of inflorescence was 12.2 days.

4.1.4.5 Flower and inflorescence development in Santalum yasi

Santalum yasi has the smallest flowers compared to other four *Santalum* species examined in this study. During anthesis the colour of the outer part of the tepal was light green, while the inner part was creamy white, similar to that of *S. album*. A few hours after opening, the colour of the tepals gradually changed from creamy white to pink, red then to dark purple. The colour of the style and the inner part of the flower changed simultaneously with that of the tepals.

After 12 to 24 hours of opening, the tepals recurved inwards (closing) but they never fully closed. No control hybridisation studies were conducted on the flower, but due to the short life of the flower (mean life of 2.2 days) (Table 4.1), it is anticipated that the stigma may be receptive during or immediately after anthesis and remain receptive for 24 hours after anthesis. The mean number of flowers observed on an inflorescence was 10 and mean period of inflorescence was 15.1 days.

4.1.4.6 Differences in floral phenology between five species of Santalum

Phenological and morphological differences were observed among the five species used in this study. These morphological characteristics are important because they provide basic but significant information relevant to successful artificial breeding of each species. These physical characters in each of the five species were assessed and described from floral anthesis to dehiscence, covering the period in which the flower opened and whether the flower closed after opening or remained open until it is shed.

4.1.4.6.1 Mean days of flower life

The mean days of flower life refers to the mean number of days in which the tepals open until the flower is shed (unpollinated flowers) or when the floral tube is abscised at the commencement of fruit development (pollinated flowers). In this study, *S. album* was found to have the longest flower life, with 8.7 days. This was followed by *S. austrocaledonicum* (2.46 days), *S. lanceolatum* (2.25 days), *S. yasi* (2.18 days) and *S. macgregorii* (1.03 days).

4.1.4.6.2 Mean days of flower open

Mean days of flower opening occurred from anthesis to the time in which the flower has closed or shed (in flowers that do not close). Similar to flower life, *S. album* had the longest period of flower opening (8.7 days) followed by *S. lanceolatum* with 0.735 days, *S. austrocaledonicum* with 0.67 days, *S. yasi* with 0.5 and finally *S. macgregorii* with 0.25 days.

4.1.4.6.3 Mean days of flower closing

S. album flowers were different from *S. lanceolatum*, *S. austrocaledonicum*, *S. macgregorii* and *S. yasi* flowers because when its flowers opened, the tepals were reflexed outwards and remained in that position for the life of the flower. In contrast to *S. album*, the tepals of the other four species opened with inward inflection of the tips of the tepals until the flower either totally closed, as in *S. lanceolatum*, *S. austrocaledonicum*, and *S. macgregorii* or abscised, as in *S. yasi*. The term closing is taken to mean that the tepals become inflected or recurved inwards, towards the centre of the flower until they are fully closed and the stigma is no longer available to pollinators. Flower closing was a feature exhibited by *S. lanceolatum*, *S. austrocaledonicum*, *S. macgregorii* and *S. yasi*. *S. yasi* had the highest mean days of

flower closing (1.68 days), followed by *S. lanceolatum* (0.52 days) and *S. austrocaledonicum* and *S. macgregorii* with 0.25 days each.

4.1.4.6.4 Mean days of flower closed

The mean number of days closed refers to the number of days that the flower bud has completely closed but remains attached to the flower inflorescence, before it either falls off (unpollinated) or the tepals abscise (pollinated). The species that closed their flowers completely were *S. austrocaledonicum*, *S. lanceolatum* and *S. macgregorii*. While the flowers of *S. yasi* began to close they were found never to completely close. In these species, *S. austrocaledonicum* had the greatest mean number of days for a closed flower to continue to be attached to the inflorescence, at 1.55 days. *S. lanceolatum* had 0.99 mean days for a flower to remain closed and *S. macgregorii* had the least mean time of floral closure at 0.35 days (Table 4.1).

4.1.5 Discussion

Given the brief life of the flowers in *S. austrocaledonicum, S. lanceolatum, S. macgregorii* and *S. yasi* the phenological variation was proportionally much greater than for *S. album*, such that the life of a flower in *S. lanceolatum* could vary by as much as 100% (i.e. 12 to 24 hours). In both *S. austrocaledonicum* and *S. lanceolatum,* no visual changes in stigma morphology were detected. The timing and duration of stigma receptivity requires further investigation, but it is likely that these species are either slightly protandrous (pollen shed before stigma receptivity) or pollen shed and receptivity occur simultaneously. This is proposed since pollen shed, particularly for *S. lanceolatum* and *S. macgregorii* occurred throughout the period where the tepals were open and the stigma was available for pollination. Furthermore the upper part of the style was abscised concurrently with the floral tube, so the stigma was only available

for pollination by 'large' insects during the opening of the tepals. In *Santalum* species, the most common pollinators were bees, flies, beetles, ants, butterflies and wasps (Jyothi *et al.*, 1991; Kulkarni & Muniyamma, 1998). Ants and flies, as well as thrips were commonly observed on the flowers of the three species in this study. Ants were often found to chew and remove the style at its base, although the purpose for this behaviour was not determined. From observations in this study it could be possible that small insects (such as thrips and ants) could penetrate the small openings in the tepals of *S. lanceolatum* and *S. austrocaledonicum* after the flower has closed. The frequency of such events and their influence on effecting pollination is not known.

The onset and duration of stages in the floral development in *S. album* was found to vary substantially between flowers on an individual. The rate of flower development, in *E. regnans*, can vary between flowers and seasons within a genotype, which may be strongly influenced by mean daily temperature (Griffin & Hand, 1979). Differences in the rate of flower development in *S. album* is likely influenced by variation in environmental factors such as temperature, but further investigation is required to examine its effects. It is likely that stigma receptivity in *S. album* occurs during the period of flower opening, since the stigma was observed to desiccate before floral tube abscission. Changes in stigma colour and shape after pollen shed may be used as a basis for determining the onset and duration of stigma receptivity.

Kulkarni and Muniyamma (1998) evaluated changes in stigma morphology of *S. album* and, while these authors did not directly measure stigma receptivity, reported that the presence of a shiny sugary drop on the stigmatic surface is likely to represent stigma receptivity. It was further reported that the greatest proportion of the stigma with this morphological feature was consistently observed on the day after flower

opening (Kulkarni & Muniyamma, 1998). No observations of any stigma exudate were observed in the *S. album* genotypes used in this study. However, further investigation of stigma receptivity and morphological changes may lead to visual associations between stigma receptivity and flower development stages, which could be employed in controlled pollination procedures in *S. album*. Stigma receptivity in both *S. spicatum* and *S. album* were reported to commence after the flower opened and attained a peak 2-3 days later (Rugkhla *et al.*, 1997). They further reported that pollen tubes grew more slowly in green compared with red coloured flowers, where they took 2 and 1 days to reach the ovary, respectively. Srimathi (1995) reported that the duration of stigma receptivity in *S. album* was between 20-48 hours, although the authors did not elucidate the timing of onset.

During observations of floral morphology and undertaking controlled crosses with three *Santalum* species (*S. austrocaledonicum, S. album* and *S. lanceolatum*), it was evident that substantial variation in pollen production could be found among the genotypes. Those with higher pollen production (Genotypes 02 25, 29, E5 and T1) were typically more successful in siring seeds when used as a male parent. Interestingly these were also relatively more successful seed bearing parents. This apparent variation in fecundity could explain the existence of the folklore 'man' and 'woman' varieties reported for *S. austrocaledonicum* in Vanuatu (Siwatibau *et al.*, 1998) and *S. magregorii* in Papua New Guinea (Gunn *et al.*, 2002). Further investigations of this breeding behaviour through replication of ramets is required, since this result may have been due to variation in the maternal resources, which resulted in lower general productivity of some compared with other genotypes.

Investigation into the life of a flower in the species has indicated a variation in duration of flower life, opening and closing among the four genotypes examined. The period that the flower remained opened is significant because it determines the period for effective pollination, either by insects of by controlled hybridisation in the species. Among the genotypes evaluated, it was observed that the Aniwa01 flowers have a much shorter life span compared with the remaining genotypes.

Table 4.1
Length (days) of floral phenology stages in five species of Santalum (S. austrocaledonicum, S. lanceolatum S. album, S. macgregorii and S. yasi).

	Species				
	S. austrocaledonicum	*S. lanceolatum*	*S. album*	*S. macgregorii*	*S. yasi*
Number of Inflorescences observed	6	4	3	2	2
Mean days of Inflorescence life	10.4	11.5	12.4	12.2	15.1
No of flowers observed	93	44	82	52	16
Mean number of Flower on each inflorescence	15.5	11	28	26	10
Mean days of flower life	2.46	2.25	8.7	1.03	2.19
Mean days open	0.68	0.74	8.7	0.39	2.19
Mean days closing	0.25	0.52	0*	0.25	1.69**
Mean days closed	1.55	1.00	0*	0.36	0*

* *Means that the flower remained open and not closed.* **Not all flowers entered the 'closing' phase, so this figure is nested within 'mean days open'

4.2 Study: Breeding behaviour of *Santalum lanceolatum* self-, intra- and interspecific cross-compatibility

4.2.1 Abstract

Controlled pollination using 13 genotypes of *Santalum lanceolatum* was undertaken to elucidate self-incompatibility, and intraspecific cross-compatibility in the species, and interspecific cross-compatibility with *S. album* and *S. austrocaledonicum*.

Santalum lanceolatum may be considered to have a facultative allogamous (incomplete outbreeding) breeding system. This study found variation between genotypes in the level self-incompatibility, where 20% were found to set seed following self-pollination, while the remaining 80% had no seed development with such pollinations. However, a significantly greater proportion of genotypes developed seed following intraspecific cross-pollination (62%), compared with self-pollination (20%). In genotype 2 in which sufficient self- and cross-pollinations were performed, no significant difference was found between them for the percentage of seed set. The seed set from self-pollinations were successfully germinated and the plants produced have been growing for 2 years without any substantial morphological distinction between inbred and outcrossed seedlings.

While total geographic isolation and significant morphological divergence exists between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum* this study found no indication of reproductive barrier(s) between them. No significant differences were found in the percentage of seed set among *S. lanceolatum* intraspecific crosses (7.5%) compared with reciprocal *S. lanceolatum* x *S. austrocaledonicum* interspecific crosses (7.6%). Germination of seed derived from intraspecific outcross pollinations was found to be low (41%), relative to interspecific pollinations with each

of *S. album* (114% - with many seeds producing two seedlings) and *S. austrocaledonicum* (70%). Therefore, while seed set from intraspecific outcross pollinations was greater than for reciprocal *S. lanceolatum* x *S. album* crosses (4.3%), no significant differences were found for the percentage of seedlings developed from these two pollination types (2.5% and 4.8% respectively).

The results of this study have implications for both the domestication of *S. lanceolatum* for its commercial production and for conservation of its natural stands. Genetic variations present within the high oil quality *S. album* and *S. austrocaledonicum* could be used for the improvement of *S. lanceolatum* and vice versa. However, inappropriate planting of one species within the natural range of another is likely to result in gene exchange among them and affect the genetic integrity of the natural populations.

4.2.2 Introduction

Santalum (sandalwood) is a genus of hemi-parasitic tree species occurring throughout south and south-east Asia, Australia and the Pacific Islands. The heartwood of several species produces valuable aromatic oil widely used in perfumery, medicines and incense. Throughout the world, sandalwood products are being sourced from declining numbers of natural stands, and the international price for natural sandalwood products continues to increase at rates well above inflation. Therefore, a significant opportunity exists to establish commercial sandalwood plantations and agroforests, in order to reduce pressure on wild stands, improve consistency of product supply and improve economic outcomes for smallholder farmers.

In Queensland, sandalwood (*S. lanceolatum*) has long been commercially exploited for its powdered heartwood used in funeral pyres and incense. Harvesting and

export to china of natural sources of indigenous sandalwood (*S. lanceolatum*) in Cape York began after 1900 and continued until the early 1930's, stimulated mainly by demand from China. Many of the European sandalwood-getters relied heavily on the local knowledge and labour of Aboriginal people in each area to find and harvest the trees (Wharton, 2005).

While little commercial harvesting continues on Cape York today due to the scarcity and wide distribution of the resource, indigenous communities are interested in re-establishing the sandalwood resource to support local enterprises. The lower quality of *S. lanceolatum* oil compared with other commercial sandalwood species such as *S. album*, *S. yasi* and *S. austrocaledonicum* has limited commercialisation of this species as cultivated sandalwood. However, with recent identification of high quality *S. lanceolatum* in Cape York (Page *et al.*, 2007), there is an opportunity to develop this resource for commercial agroforestry plantings.

The development of *S. lanceolatum* as a significant agroforestry crop will depend on the development of forms suited to commercial production, cultivars with high growth rates yielding high volumes of heartwood containing concentrated oils with high levels of α- and β- santalols. The implementation of a successful breeding programme for any sandalwood species will depend upon knowledge of its breeding system and its cross-compatibility with related species that are a source for potentially useful characteristics. Given also the continued exploitation of *S. lanceolatum* in Queensland, knowledge of its breeding system will assist those developing strategies aimed at conserving wild populations and establishing new plantings within its natural areas of distribution. Information on the breeding system and patterns of gene flow are important for planning germplasm collection, designing and managing seed orchards

and for maintaining genetic diversity in breeding populations. The objectives of the present study were to determine levels of self- and cross-compatibility within *Santalum lanceolatum*, and cross-compatibility of *Santalum lanceolatum with S. album* and *S. austrocaledonicum*.

4.2.3 Materials and methods

4.2.3.1 Plant material

The study used grafted plants produced from reproductively mature scions of *S. lanceolatum* and *S. album*. At the beginning of this study seedlings of *S. austrocaledonicum* were germinated and grown. The study was initially interested in the reproductive biology of *Santalum austrocaledonicum*, but the limited availability of reproductively mature specimens during the study period did not permit the detailed investigation of this species. The study therefore used *S. lanceolatum* as its focal species.

Thirteen genotypes of *Santalum lanceolatum* (genotypes 0, 1, 2, 5, 8, 10, 14, 16, 25, 27, 29, 30 and 31), collected from Cape York Peninsula, were used to examine self-incompatibility within, and intraspecific compatibility between them. Eleven of these genotypes (genotypes 0, 2, 5, 10, 14, 16, 25, 27, 29, 30 and 31) were also used in crosses to evaluate the reciprocal interspecific compatibility with three genotypes of *S. album* (E5, E7 and E8). Five *S. lanceolatum* genotypes (genotypes 2, 5, 14, 16, and 29) were used in reciprocal crosses with one genotype of *S. austrocaledonicum* (T1). Seed production in *S. lanceolatum* was examined in 182 unpollinated (isolated from open pollination to test spontaneous selfing), 232 self-pollinated, 241 outcross (between different genotypes) 1486 interspecific (1250 with *S. album* and 236 with *S. austrocaledonicum*) pollinated (Table 4.2). Genotype combinations and unique

pollinations are pollination treatments involving unique combinations of genotypes. For instance, a cross between genotypes 1 and 2 would be considered to be unique from a cross between genotypes 1 and 5.

4.2.3.2 Controlled pollination

Grafted clones of *S. lanceolatum*, *S. album* and seedlings of *S. austrocaledonicum* were grown in 300mm-diameter pots in a soilless potting medium in an insect-proof greenhouse with drip irrigation. Flowers were emasculated during anthesis using pointed forceps. The anthers removed during this process were either placed in small plastic vials and placed in a desiccator with silica gel or used immediately for pollination. All pollinations were made using pollen collected on the same day (i.e. pollen was not stored and used on subsequent days). Pollinations were carried out by applying the pollen-shedding anther to the stigma until pollen grains had adhered to the stigma. Individual inflorescences were pollinated with a single pollen source and each was tagged with details of the pollen donor.

Table 4.2
The number of genotype combinations (unique 'pollinations') and treated/pollinated flowers for seven different pollination types.

Pollination Type	Genotype Combinations	Flowers 'Treated'
S. lanceolatum unpollinated	7	182
S. lanceolatum self-pollinated	10	234
S. lanceolatum intraspecific	13	241
S. lanceolatum x *S. album*	20	820
S. album x *S. lanceolatum*	23	430
S. lanceolatum x *S. austrocaledonicum*	5	127
S. austrocaledonicum x *S. lanceolatum*	5	120
Total	**83**	**2143**

Pollinations were carried out during three separate flowering events in September 2007, December 2007 and February 2008. Flowers were left on the plants for approximately 8-10 weeks from pollination to fruit harvest. Fruits from each pollination category were collected, the flesh removed and the seed air-dried, before storage in sealed plastic containers at 4°C.

Germination of seed resulting from controlled pollination was undertaken in a seed raising mix with a 1:1 ratio of medium grade perlite and vermiculite. Seeds were placed under 50% shade and watered by an automatic irrigation system for 15 minutes per day. Seed germination was measured, as well as survival rate after they had been pricked into pots and grown for a period of 3 months. As no seedling mortality was recorded between initial germination measures and at 3 months, the data is presented as germination only.

Differences between the proportion of pollinated flowers developing into seed and seedlings between pollination types (i.e. unpollinated, self-pollinated, intraspecific out-cross pollinated etc.) and the proportion of unique pollinations developing seed and

seedlings were evaluated using an equality test of two binomial proportions (Ott & Longnecker, 2001), calculated by:

$$z = \frac{(\hat{p}_1 - \hat{p}_2)}{\sqrt{\frac{\hat{p}_1(1 - \hat{p}_1)}{n_1} + \frac{\hat{p}_2(1 - \hat{p}_2)}{n_2}}}$$

The two binomial populations are denoted by $\hat{p}_1 = \frac{y_1}{n_1}$ and $\hat{p}_2 = \frac{y_2}{n_2}$, where y_1 seeds/seedlings are recorded for the random sample of n1 pollinations from population 1, and y_2 seeds/seedlings are recorded for the random sample of n2 pollinations from population 2. The null hypothesis was rejected where the absolute value of the statistic z was greater than 1.645, as this represented a significance value of <0.05. This statistical approach was used because, although a sufficient number of pollinations per pollination type were performed, in some cases a low number of replicates or genotype combinations did not permit evaluation by ANOVA.

4.2.4 Results

4.2.4.1 Unpollinated flowers

No signs of fruit development were observed in any of the unpollinated flowers in this experiment. Flowers were shed towards the end of their expected 'life' (*S. lanceolatum* 12-24 hours). No floral-tube abscission, indicating fruit development, was observed and no seeds were set from any flowers of this treatment (Table 4.3).

4.2.4.2 Self-incompatibility in S. lanceolatum

Mean seed set per self-pollinated flower was 1.3%, which was significantly (P<0.05) fewer than the 7.5% of flowers in intraspecific cross-pollination (Figure 4.1). Seed set following self-pollinations occurred in genotypes 2 and 29, where 3.6 and 7.4% of self-pollinated flowers set seed from 55 pollinations combined. The

percentages for genotype 2 were not significantly ($P<0.05$) different from intraspecific cross-pollinations involving this genotype (used both as pollen donor or recipient) where 8.6% flowers set seed from 105 pollinations. No intraspecific cross-pollinations were performed using genotype 29, so a similar comparison between self- and intraspecific cross-pollinations for this genotype was not possible. No seeds were set from any of the remaining eight genotypes after a total of 179 self-pollinations.

Two of ten self-pollinated genotypes (20%) set seed in this experiment, which was significantly ($P<0.05$) lower than intraspecific pollinations, where 8/13 (62%) unique crosses developed seed (Figure 4.2). Likewise the percentage of unique 'crosses' developing seed within reciprocal interspecific hybridisations between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum* were significantly ($P<0.05$) greater (45% and 90% respectively) when compared with self-pollinated flowers (Table 4.3). A similarly low-level of self-pollinated genotypes developed seedlings relative to interspecific crosses, but no significant difference was found between self- and intraspecific cross-pollinations (Figure 4.2).

The percentage of self-pollinated *S. lanceolatum* flowers developing into seed was significantly lower than for all other pollination types. However, the percentage of self-pollinated flowers that developed into seedlings was not significantly different from intraspecific crosses among *S. lanceolatum* genotypes and also between *S. album* (♂) and *S. lanceolatum* (♀) and *S. lanceolatum* (♂) and *S. austrocaledonicum* crosses (♀). A significantly greater percentage of flowers developing into seedlings were found for each of the interspecific crosses *S. lanceolatum* (♂) x *S. album* (♀) and *S. austrocaledonicum* (♂) x *S. lanceolatum* (♀) compared with *S. lanceolatum* self-pollinated flowers.

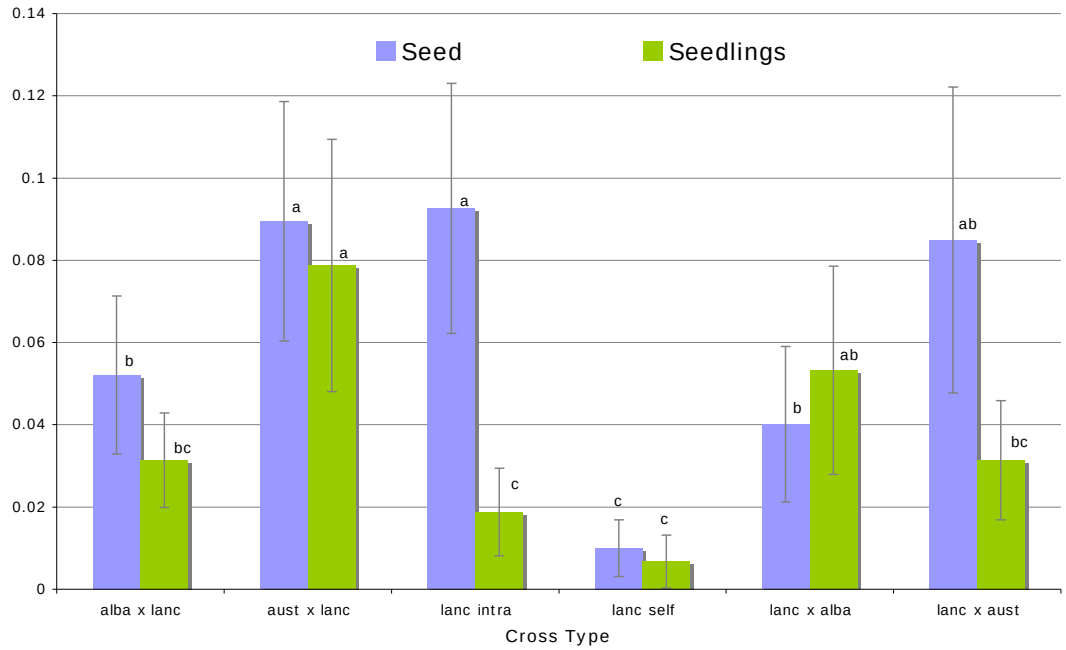

Figure 4.1 Number of seed and seedlings per pollinated flower for self and intraspecific pollinations in *S. lanceolatum* (lanc. self and lanc. intra respectively) and reciprocal interspecific pollinations between *S. lanceolatum* with each of *S. album* (lanc. ♂ x alba. ♀ and alba. ♂ x lanc. ♀) and *S. austrocaledonicum* (lanc. ♂ x aust. ♀ and aust. ♂ x lanc. ♀).

Vertical bars represent standard errors. Cross types sharing lower case letters are not significantly (P<0.05) different within either the seed or seedling response variable.

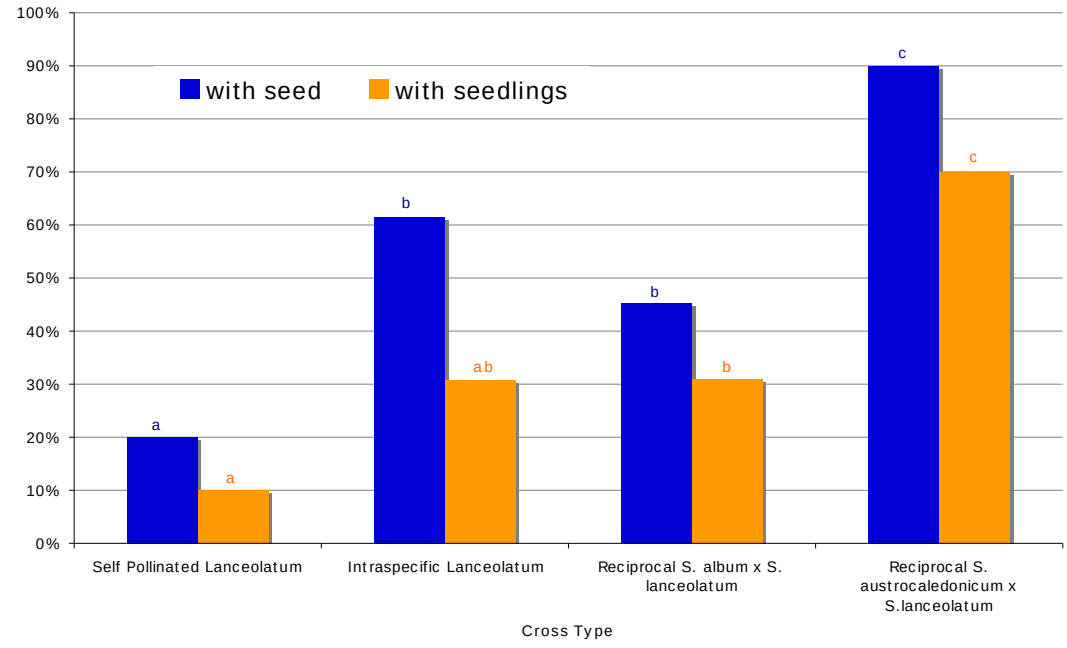

Figure 4.2 Percentage of unique pollinations (i.e. different self-pollinated genotypes or different genotype combinations among cross types) with viable seed and seedlings.

Cross types sharing lower case letters are not significantly (P<0.05) different within either the seed or seedling response variable.

4.2.4.3 Intraspecific cross-compatibility in S. lanceolatum

Of the 241 intraspecific crosses made in *S. lanceolatum,* only 7.5% and 2.5% of pollinations resulted in the production of seed and seedlings respectively. For those crosses representing greater than 10 pollinations, seed set ranged from 0% in 3 different genotype combinations (averaging 16 pollination for each) to 14.2% in crosses between genotypes 16 (♀) and 29(♂) (totaling 14 pollinations) (Table 4.3).

Only genotype 25 was used in over 50 intraspecific cross-pollinations each as a pistillate and pollen parent with at least 3 different genotypes. The mean percentage of seed set per pollination in this genotype was not significantly different between pistillate (4.8%) and pollen (5.4%) parent. No other genotype had a sufficient number of pollinations or was crossed with at least 3 different genotypes to permit such evaluation of differences in fecundity as a reciprocal parent for intraspecific crosses.

While the number of seeds developed per pollinated flower was significantly ($P<0.05$) greater in *S. lanceolatum* intraspecific crosses compared with self-pollination, there was no difference among these cross types for the number of seedlings per pollinated flower (Figure 4.1). A similar pattern was found between these two cross types for the percentage of unique pollinations that developed seed where intraspecific crosses were significantly ($P<0.05$) greater, but no statistical differences were found between these cross types for the percentage of unique crosses developing seedlings (Figure 4.2). No significant differences were found for unique crosses developing seed or seedlings between *S. lanceolatum* intraspecific and reciprocal *S. lanceolatum* x *S. album* interspecific. In contrast, a significantly ($P<0.05$) greater percentage of unique crosses were found to develop seed and seedlings in reciprocal *S. lanceolatum* x *S. austrocaledonicum* compared with *S. lanceolatum* intraspecific crosses (Figure 4.2).

4.2.4.4 Interspecific cross-compatibility between S. lanceolatum and S. album

Variation among the interspecific crosses between *S. lanceolatum* (♂) and *S. album* (♀) was found in the percentage of seed set per pollinated flower, ranging from 0–23% and from 0-16% in its reciprocal crosses (*S. album* (♂) and *S. lanceolatum* (♀)) for those crosses with greater than 10 pollinations (Table 4.3). Interestingly, 38% of the seeds developed from the former interspecific cross type resulted in two seedlings (double embryos) following germination. In crosses involving *S. album* (♂) and *S. lanceolatum* (♀), the percentage of seed producing two seedlings was 7.5%. No other cross type (self, intraspecific or *S. lanceolatum* x *S. austrocaledonicum*) had seed that produced two seedlings.

A significantly ($P<0.05$) greater number of seeds per pollinated flower was found following intraspecific pollination among *S. lanceolatum* genotypes compared with reciprocal interspecific crosses between *S. album* and *S. lanceolatum*. However, no significant differences were found in the number of seedlings per pollinated flower between crosses among *S. album* (♂) x *S. lanceolatum* (♀) and *S. lanceolatum* intraspecific pollinations. Furthermore, crosses among *S. lanceolatum* (♂) x *S. album* (♀) had a significantly ($P<0.05$) greater number of seedlings per pollinated flower than for *S. lanceolatum* intraspecific pollinations.

Significantly ($P<0.05$) fewer unique crosses were found to develop seed and seedlings in reciprocal *S. lanceolatum* x *S. album* interspecific crosses compared with reciprocal *S. lanceolatum* x *S. austrocaledonicum* crosses (Figure 4.2).

4.2.4.5 Interspecific cross-compatibility between S. lanceolatum and S. austrocaledonicum

In the present experiment only one genotype of *S. austrocaledonicum* (T1) flowered during the period of controlled pollinations. The flowering of this genotype coincided only with the flowering of five genotypes of *S. lanceolatum* (genotypes 2, 5, 14, 16, and 29). Therefore, in the evaluation of the compatibility between *S. lanceolatum* and *S. austrocaledonicum,* only reciprocal crosses between T1 with each of genotypes 2, 5, 14, 16, and 29 were possible.

Variation among the crosses between *S. lanceolatum* (♂) and *S. austrocaledonicum* (♀) was found in the percentage of seed set per pollinated flower, ranging from 4–23% and from 0-18% in the reciprocal cross (*S. austrocaledonicum* (♂) and *S. lanceolatum* (♀) (Table 4.3). No significant differences in the number of seed per pollinated flower were found between *S. lanceolatum* intraspecific crosses and each of the reciprocal interspecific crosses between *S. lanceolatum* and *S. austrocaledonicum*. The number of seedlings per pollinated flower for *S. austrocaledonicum* (♂) x *S. lanceolatum* (♀) cross was significantly ($P<0.05$) greater than both self- and intraspecific crosses within *S. lanceolatum*. The reciprocal interspecific cross (*S. lanceolatum* (♂) and *S. austrocaledonicum* (♀), however, was not found to differ from self- and intraspecific crosses.

Table 4.3

Percent seed set across all pollinations undertaken to elucidate the breeding system of Santalum (S. album, S. austrocaledonicum and S. lanceolatum).

No poll. = no pollination in plants isolated from insect vectors.

	S. album			*S. austro.*	*S. lanceolatum*												
Female	(E5)	(E7)	(E8)	(T1)	(L01)	(L02)	(L05)	(L08)	(L10)	(L14)	(L16)	(L25)	(L27)	(L29)	(L30)	(L31)	(L0)	No Poll.
Male																		
(E5)						8.7%	11.8%		2.8%	0.0%	6.0%	0.0%	16.7%	4.5%	0.0%	8.0%	40.0%	
(E7)						3.2%	0.0%	0.0%	0.0%	0.0%				0.0%	1.2%	0.0%	2.2%	
(E8)											0.0%		4.0%	8.3%				
(T1)						8.3%	10.5%			0.0%	7.7%			18.2%				
(L01)	0.0%		0.0%		0.0%						0.0%					0.0%		0.0%
(L02)	23.0%		2.5%	4.0%		3.6%					20.0%	9.0%						0.0%
(L05)	0.0%	0.0%		4.0%														
(L08)							0.0%	0.0%										0.0%
(L10)	0.0%	0.0%							0.0%									
(L14)	0.0%	0.0%		7.7%														
(L16)				3.7%							0.0%							
(L25)	1.1%				5.8%	4.1%						0.0%	4.3%					0.0%
(L27)	1.7%	0.0%										0.0%	0.0%					0.0%
(L29)	6.0%	0.0%	20.0%	23.1%							14.3%		0.0%	7.4%				0.0%
(L30)	24.6%		0.0%									6.5%			0.0%			0.0%
(L31)	0.0%		0.0%													0.0%		
(L0)															0.0%	23.0%		

4.2.5 Discussion and Conclusions

Resolving the nature of the breeding system in *Santalum lanceolatum* is important for planning appropriate breeding strategies for its domestication. Such knowledge is also important in interpreting the nature and extent of genetic variability in natural populations of the species, and would in turn lead to greater efficiency in the evaluation and use of this natural variation for plant breeding.

4.2.5.1 Unpollinated flowers of S. lanceolatum

In this study, no fruit or seeds were set following isolation of flowers and restricting pollination of *S. lanceolatum*. This result suggests that this species does not possess a capacity for the development of parthenocarpic fruit or clonal seed. This result is similar to that found in *S. album* in China, where no seeds were found in flowers isolated from open pollination by bags (Ma *et al.*, 2006).

4.2.5.2 Self-incompatibility in S. lanceolatum

The mean seed set per pollinated flower in *S. lanceolatum* was significantly greater following cross-compared with self-pollination, where seed set from cross-pollination was 5.8-times greater than from 'selfing'. This result indicates possible self-incompatibility mechanism(s) operating in this species. Rugkhla *et al.* (1997) proposed that both pre- and post- fertilisation self-incompatibility mechanisms were operating in *S. album* and *S. spicatum*. My study however, found that putative self-incompatibility mechanism(s) in *S. lanceolatum*, may either be incomplete, or subject to genetic variation between genotypes, given that seed set, was affected following self-pollination in 20% of genotypes tested. Furthermore, two self-pollination-derived seeds were successfully germinated and have continued to grow for a period of two

years without any indication of deleterious effects of inbreeding. Warburton (2000) found little to no sexual reproduction in natural populations of *S. lanceolatum* sens lat (now referred to as *S. leptocladum* Gand.) in Victoria due to pollen sterility in one and self-incompatibility or pistil dysfunction in other populations. The populations were found to consist of many ramets (derived from root suckers) of one clone, resulting from historical commercial exploitation. Combined with the findings of this study, this finding gives greater weight to the possibility that self-incompatibility mechanisms operate in *S. lanceolatum sens lat*, but that genetic variations in its expression exist within its natural populations.

These results are similar to those found by Muir *et al.* (2007) for *S. spicatum*, in which one family showed a high level of inbreeding, which was contradictory to the high mean outcrossing rate (95.2%). These authors proposed that flowering of this tree was non-synchronous with many other trees, resulting in higher inbreeding. This flexibility in breeding strategy would be an advantage to continental Australian species dispersing and colonizing many islands in south-east Asia and Pacific (Harbaugh & Baldwin, 2007). In *Santalum album,* Ma *et al*. (2006) reported 24% of flowers with geitonogamous (same plant and different flower) self-pollinated set seed.

In the present study all cross types (self-, intraspecific and interspecific) were carried out on a given individual ramet. Therefore it is possible that the reduced 'selfing' rate recorded in this study could be due to competitive interactions between flowers with 'outcross' and those with 'self' pollen and preferential maternal resource allocation to the most competitive. It would be of interest to evaluate the percentage seed set between these three cross types, where each type is restricted to an individual

ramet of a given genotype. This would remove any interaction effects that may have been operating in the present study.

Many plant species have made a successful transition from outcrossing to a self-fertile (Igic *et al.*, 2008), and this transition has been described as one of the most prevalent evolutionary trends in plant reproduction (Nasrallah *et al.*, 2004; Shimizu & Tsuchimatsu, 2015). In the present study, 20% of individuals in *S. lanceolatum* were self-fertile, while the remaining individuals were self-incompatible. This mixed breeding system is not uncommon among plants (Goodwillie *et al.*, 2005), with at least one-third of species reviewed by Barrett (2002) having a capacity for both selfing and outcrossing. The evolution of mixed mating may be driven by the benefits of outcrossing (heterozygocity and genetic fitness), together with selfing (reproductive assurance) in situations where pollen transfer between individuals is limited or mates are limited (Barrett, 2002; Ruan & da Silva, 2012). Goodwillie (2005) described three broad modes of mixed mating:

1) Populations contain both self-compatible and self-incompatible individuals,

2) Heteromorphic flower systems where individuals produced cleistogamous (purely selfing) and chasmogamous flowers (both outcrossing and selfing possible), and

3) Most commonly individual plant produce a single flower type, and fruits contain selfed, outcrossed or a mixture of progeny types.

4.2.5.3 Intraspecific hybridisation in S. lanceolatum

The mean level of seed set among crosses of eight genotypes of *S. lanceolatum* was 7.5% of pollinated flowers. Fruit set (and thus seed set, given a fruit is generally single seeded), from open pollinated *S. album* trees was less than 2-3% in China (Ma *et al.*, 2006) and 5.2% in India (Veerendra & Padmanabha, 1996). Rugkhla *et al.* (1997) reported a final fruit set of 1.3% in controlled intraspecific outcross pollination of *S. spicatum* in Western Australia. These authors also found a 10% fruit set in controlled outcrosses of *S. album*, which was similar to the 9.4% found by Kulkarni and Muniyamma (1998) in India. While Ma *et al.* (2006) found that 2-3% of open pollinated *S. album* flowers set seed, this was increased to 14% during artificial outcross pollinations. These results suggested that while increased seed set may be achieved using controlled pollination, several *Santalum* species produce an abundance of flowers but less than 10% of these typically develop into viable seed.

The significantly greater number of seeds set per intraspecific outcross and percentage of unique intraspecific pollinations (genotype combinations) developing seed compared with self-pollinated flowers, suggests a putative self-incompatibility mechanism. However, the low germination rate (40%) for intraspecific outcross derived seed resulted in no significant difference in the number of seedlings between intraspecific and self-pollinated flowers. Further replication of this work is likely to reveal the exact nature of the low viability among 'intraspecific seeds'.

4.2.5.4 Interspecific crosses between S. lanceolatum and each of S. album and S. austrocaledonicum

Despite total geographic isolation and significant morphological divergence between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum,* no reproductive barrier appears to exist between them due to the equivalent or greater

seedling production relative to the *S. lanceolatum* intraspecific cross. Seed producing two seedlings were found in crosses between *S. album* (♂) and *S. lanceolatum*(♀), and although this is not unusual in this genera, the level (7.5% of seed) was elevated compared with all other crosses in this study, and with *S. album* intraspecific crosses in controlled crosses in China where the frequency was (2.5%) (Ma *et al.*, 2006).

It appears that *S. lanceolatum* has particularly high cross compatibility with *S. austrocaledonicum*, but this result may be confounded by the use of only a single *S. austrocaledonicum* genotype (T1). It is possible that T1 may have a high specific combining ability with *S. lanceolatum,* and therefore greater numbers of genotypes would need to be used in crosses to determine if the results in this study accurately reflect the cross-compatibility between these two species.

These results, however, reflect similar findings with putative hybridisations between *S. yasi* and *S. album* in Fiji, with no apparent reproductive barrier or hybrid breakdown in early generations (Doran *et al.*, 2005; Bulai & Nataniela, 2007). Bulai (2007) further reported that spontaneous hybrids between *S. yasi* and *S. album* are now being produced in clonal seed orchards, and that these hybrids appear to have higher vigour, wider environmental tolerances and are less dependent on forming host associations. Rugkhla *et al.* (1997) found that no seeds developed after 1930 reciprocal controlled pollinations between *S. spicatum* and *S. album*, and reported that strong incompatibility mechanisms operated between pollen and style, and possibly in the developing zygote.

Doran and Brophy (2005) proposed that interspecific hybrids may provide the opportunity to improve the planted form of sandalwood, particularly given the good vigour of F1 hybrids between *S. album* and *S. yasi* observed in Fiji. Hybridisation

between *S. lanceolatum* and *S. album* may be used to incorporate important characteristics from each of these species into a cultivar for use in commercial plantations.

Combining characteristics such as straight form and fire tolerance from *S. lanceolatum* and high heartwood oil concentration and quality (%α- and β-santalol) from *S. album* in a cultivar may be possible, provided additive genetic effects predominate in the characters of interest (general combining ability). However, a more involved procedure of reciprocal recurrent selection would be necessary to combine the desirable traits from both species in cultivars if non-additive gene effects predominated in the F1 hybrids (specific combining ability) (Eldridge *et al.*, 1993).

Barriers to successful introgression were found to exist between *Eucalyptus crebra* and *E. melanophloia*, in which Drake (1981) found the hybrid population produced only 10% of the capsule yield of either parental species which, under natural selection, would put the hybrids at a competitive disadvantage. While segregating populations can be generated through artificial hybridisation of *Chamelaucium uncinatum* with each of *C. megalopetalum*, *Verticordia plumosa* and *V. grandis*, the resulting progeny of all crosses were infertile (Growns *et al.*, 2002), and therefore it was not feasible to carry out further breeding. In a comprehensive evaluation of the interbreeding between 15 species of *Leucaena*, Sorensson (1994) found that 77% of the possible two-way combinations produced successful hybrids, with one-third having unilateral incompatibility (i.e. cross only possible in one direction). The potential for hybridisation among related species of woody perennials may be reasonably commonplace (Sorensson & Brewbaker, 1994; Brewbaker & Sun, 1999), and has been

a feature in the domestication of many tree crops (Miller & Gross, 2011; Goldschmidt, 2013).

The high level of cross-compatibility between *S. lanceolatum* with each of *S. album* and *S. austrocaledonicum* indicates the likelihood that they are not widely divergent genetically and chromosomally (few chromosome structural differences), and thus the transfer of characters, even those under quantitative genetic control, would appear to be feasible from interspecific crosses. While the high cross-compatibility between these three species indicates the likelihood that they are not widely divergent genetically, it would be necessary to evaluate the fertility and seed production level of both their F1 hybrid and F2 progeny, because it is possible that genetic divergence between the two species may not be significantly manifest until these post-hybridisation stages.

The apparent lack of interspecific barriers between S. lanceolatum with each of S. album and S. austrocaledonicum also has implications for the conservation of their natural stands. Given its low relative value it is unlikely that *S. lanceolatum* would be introduced into areas of natural populations of *S. album* or *S. austrocaledonicum*. However, commercial plantings of *S. album* already have been established in some areas of Queensland within the existing range of natural populations of *S. lanceolatum*. It is very possible that gene flow will occur between the *S. album* plantings and the *S. lanceolatum* populations. It is unclear whether such hybrid progeny would have an advantage in these environments and persist beyond 1 or 2 generations. However, these considerations may need to be evaluated by those responsible for management of *S. lanceolatum* wild stands and improvement of *S. album* germplasm for commercial production.

Chapter 5. Discussion and Conclusions

Despite the economic importance of many sandalwood species, their cultivation has only commenced over last few decades (Sen-Sarma, 1977; Applegate *et al.*, 1990; Rai, 1990; Radomiljac, Shea*, et al.*, 1998; Annapurna *et al.*, 2004; Thomson, 2006; Forest Products Commission, 2007; Page, Tate, Tungon*, et al.*, 2012). This cultivation has been based primarily on wild-collected seed, with some consideration given to improvement and domestication (Kulkarni *et al.*, 1998; Bulai, 2007; McKinnell, 2008). This study has addressed key knowledge gaps in our understanding of propagation by vegetative cuttings in *S. austrocaledonicum* and reproductive biology across three commercial sandal species that can be used to inform their domestication.

Using a basic non-mist propagator it was demonstrated that *S. austrocaledonicum* can be readily propagated by leafy stem cuttings. Propagation factors that were not assessed, such as higher IBA concentrations and management of stockplants, may be worth evaluating for cutting propagation of Tanna-derived clones. An important factor that has not been emphasised in the study due to limited stockplant and study period, is the season as well as physiology of cuttings at the time of collection. The season in which cuttings are collected (Baltunis *et al.*, 2005; Agbo & Obi, 2007) and their physiology (Leakey, 2004b) have been reported to have a substantial effect on cutting success. In this study it is particularly evident e-erro, but also to some extent j-erro (Figure 3.8 and Figure 3.16) collected in August (2007) rooted better than those collected in February (2008). Season affects growth and the physiological status of a shoot, therefore a particular cutting type may be physiologically different between seasons. Improving our understanding of the

physiological status of cuttings could improve rooting percentage in more difficult-to-root clones.

It is of particular interest that cuttings taken from ortets derived from the island of Erromango outperformed those from Tanna. This finding is evidence that provenance-based variation may exist in the capacity for cuttings propagation in *S. austrocaledonicum*. Variation in cutting performance was also demonstrated between clones derived from Erromango, and our results suggest that the optimisation of cutting propagation is required on an individual clone basis. The intensive nature of optimising propagation for individual genotypes means that cutting propagation may only be commercially viable if there is a price premium for particular clones, in order to justify the extra expense of developing clone-specific protocols.

The successful utilisation of low-technology non-mist propagators for propagation of sandalwood is encouraging, since they would be suitable for application in remote areas of Vanuatu. They can be used in the 'bulking up' of planting material when seed supply is low and/or intermittent. This approach contrasts with the micropropagation of *S. album* (Bele *et al.*, 2012), which requires significant investment in infrastructure and capacity building. Cutting propagation may also be considered to be more 'accessible' for smallholder nurseries than grafting propagation, since the latter requires a comparatively higher amount of skill to utilise, as well as requiring seedlings to be used for rootstock.

The cutting propagation protocols developed in this study may be adapted to facilitate the establishment of clonal seed orchards comprised of productive but genetically distinct genotypes. This approach to sandalwood domestication can promote cross fertilisation among the most desirable genotypes, progeny testing,

recurrent selection and clonal propagation of plus trees. This method of tree improvement follows a proven approach to the domestication of other tree crops such as eucalypts (Zobel, 1992; Eldridge *et al.*), pines (McKeand & Bridgwater, 1998; Jayawickrama & Carson, 2000; Dungey *et al.*, 2008; Ahlinder *et al.*, 2014), poplars (Uniyal & Todaria, 2006; Stanton *et al.*, 2010) and acacias (Kamaluddin *et al.*, 1998; Harwood *et al.*, 2004; Hai *et al.*, 2008).

The domestication of sandalwood will also depend upon recurrent selection and propagation of outstanding individuals in each generation, resulting in a cultivated tree population that is phenotypically and genetically different from their wild progenitors (White, 1987). The generation of new progenies provides a resource from which new selections can be made. Establishing new generations can be implemented through either controlled or open pollination, which is informed by the species' breeding biology. *S. lanceolatum* was demonstrated to have a facultative allogamous (incomplete outbreeding) breeding system, with variation in the level of self-incompatibility between genotypes.

The current study shows that, despite the large geographical distance between the three *Santalum* species of this study, no clear reproductive barrier exists between them. This opens the potential for introgression of desirable traits from one species to another and/or the development of hybrid cultivars. *S. album* and *S. yasi* produce heartwood oils of the highest commercial value (Radomiljac & Borough, 1995), as do a limited number of populations of *S. austrocaledonicum* (Page, Southwell*, et al.*, 2010) and *S. lanceolatum* (Page *et al.*, 2007) and therefore they have the potential to be used to improve the oil quality of other species and populations. *S. lanceolatum* has the lowest commercial value of all commercial sandalwood species owing to its inferior oil

quality. However, it may offer other agronomic characters such as its thick protective bark (Page pers. com. 2010). Introgression of this character into other species would offer improved resistance to the bark sun-scald evident in *S. album* (Barbour *et al.*, 2010), a degree of fire tolerance for fire sensitive species *S. album* (Harisetijono & Suriamihardja, 1993) ; *S. yasi* (Jiko, 1993) and *S. austrocaledonicum* (Thomson, 2006; Page, Tate *et al*., 2012) and lower susceptibility to mechanical damage of the stem reported in industrial plantations of *S. album* (Barbour *et al.*, 2010).

Evidence exists that *S. austrocaledonicum* may have a propensity for early heartwood formation under favourable conditions, where at 16 years a tree can yield an average of 18-22kg (Page, Tate, Tungon*, et al.*, 2012) compared with 5.8kg for *S. album* (Brand *et al.*, 2012). This may result in a potentially shorter rotation of 15-20 years (Page, Tate, Tungon*, et al.*, 2012) compared with 25-30 years for *S. album* (Sen-Sarma, 1977; Doran *et al.*, 2005; Brand *et al.*, 2012). While much debate exists in the commercial sector as to the likely optimal rotation age it may be possible for introgression of traits related to heartwood development between species. This is certainly an important character since heartwood development heavily influences the commercial viability of planted sandalwood (Brand *et al.*, 2012).

The development of hybrid sandalwood cultivars to exploit the potential benefits of heterosis is another potential avenue for cultivated germplasm development. The productivity benefits of clonal deployment of hybrids has been clearly demonstrated with the success of F_1 hybrids eucalypts in Brazil (Rezende *et al.*, 2014) and South Africa (Arbuthnot, 2000; Duncan *et al.*, 2000) used for pulp production. The advantages of the hybrids include resistance to canker in Brazil, their amenability to clonal propagation and the short rotation age, which reduces their biological risk

(Dungey *et al.*, 2014). The fibre products of these clones is a direct result of the improved growth rates of the hybrids associated with heterosis. In sandalwood the marketable product is the heartwood, which is secondary to the growth of the tree. Heartwood formation was moderately correlated with stem diameter for cultivated *S. album* (R^2=0.88) (Brand *et al.*, 2012) and wild-grown trees of *S. austrocaledonicum* (R^2=0.70) (Page, Tate, Tungon*, et al.*, 2012) . Improving growth rates in sandalwood through hybridisation may therefore be expected to have a positive impact on heartwood yields.

Hybridisation of sandalwood species is a potentially desirable way of improving the productivity of cultivated sandalwood and the quality of its products. However any attempts at improving sandalwood through species hybridisation would need to operate in parallel with pure species domestication. This is important since although the allure of hybrids can be strong, owing to their high vigour, there may be incompatibility concerns that manifest in later generations. This issue was highlighted for *Eucalyptus* by Potts & Dungey (2004) who eloquently described "the outstanding success of selected hybrid clones has given a biased impression of the vigor of eucalypt hybrids and the strength of reproductive barriers in the genus. When full account is made of losses through the lifecycle, a picture of high incompatibility and inviability often emerges".

Apart from the potential biological issues of hybrid sandalwood they may also be undesirable where the genetic integrity of the species is important for cultural and/or marketing reasons. Sandalwood is a culturally significant product that has a long history of trade and consumption and the markets are distinct from the commodity nature of other forest tree products such as pulp and biomass. Consumers of

sandalwood are sensitive to differences in product qualities that are associated with different species. For instance the two major plantation companies in Australia (Santanol and TFS) are committed to producing Indian sandalwood, since its heartwood oil qualities are associated with a market premium (Tropical Forestry Services (TFS), 2015) (http://santanol.com/about-santanol/ accessed 7th April 2015).

In Pacific Island Nations such as Fiji and Vanuatu many industry participants view their indigenous sandalwood as having niche market opportunities, and a point of difference to the larger industrial plantations of *S. album*. The introduction of *S. album* into Fiji has already resulted in its hybridisation with the indigenous species *S. yasi* (Bulai & Nataniela, 2007). Therefore the introduction of exotic sandalwood species within the natural range of another will likely result in uncontrolled gene flow between them. This is likely to have significant implications on genetic structure, diversity and conservation of the local species (Ellstrand, 1992; Vilà *et al.*, 2000).

The value of a given mass of sandalwood heartwood is determined by the concentration and quality (especially levels of α- and β-santalol) of its oil (Brand *et al.*, 2012). The domestication of *S. austrocaledonicum* in Vanuatu is based on the selection and clonal propagation of wild trees with the highest levels of α- and β-santalols (Page, Southwell*, et al.*, 2010). This approach to tree improvement can potentially raise the overall oil quality and improve the species relative position in international markets. The quality of heartwood oil is not however a consideration during the domestic sandalwood trade between resource owners and buyers, whereby price is determined by quality and size of the heartwood. Heartwood quality is determined by the intensity of its colour and fragrance, which is related to oil concentration rather than specific levels

of santalols (although experienced buyers will seek out heartwood with higher santalol levels).

Combining conventional breeding with clonal propagation has been successfully demonstrated in several tree species for both commercial forestry plantations (Eldridge *et al*. 1993, Zobel 1992) and agroforestry purposes (Simons & Leakey, 2004; Asaah, 2012). Clonal propagation of elite selections has been used effectively for improving the productivity of industrial forestry crops such as *Eucalyptus* (Rezende *et al.*, 2014) and *Populus* (Ceulemans & Deraedt, 1999). Initial domestication of African (Ofori *et al.*, 1996; Shiembo *et al.*, 1996; Nketiah *et al.*, 1998; Negash, 2003) and South American (Mesen *et al.*, 1997a; Mesen *et al.*, 1997b; Mesen *et al.*, 2001) tree species through cutting propagation of selected trees has been implemented using non-mist propagators. Simons and Leakey (2004) discuss a participatory approach to domestication that relies on greater involvement of subsistence farmers in the implementation of the tree improvement process. A similar approach to the domestication of *S. austrocaledonicum* and *S. lanceolatum* can be used to improve the availability of planting material with high genetic quality. This can help sandalwood producers to improve the quality and value of their sandalwood woodlots and ultimately increase their competitiveness in the international market for sandalwood.

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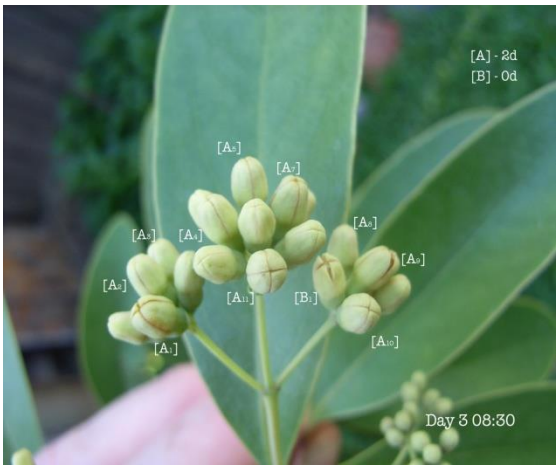
Appendix 1 Flower photos

Santalum lanceolatum




[A] - 2d
[B] - 0d
Day 3 19:00


[A] - 3d
[B] - 1d
Day 4 08:00

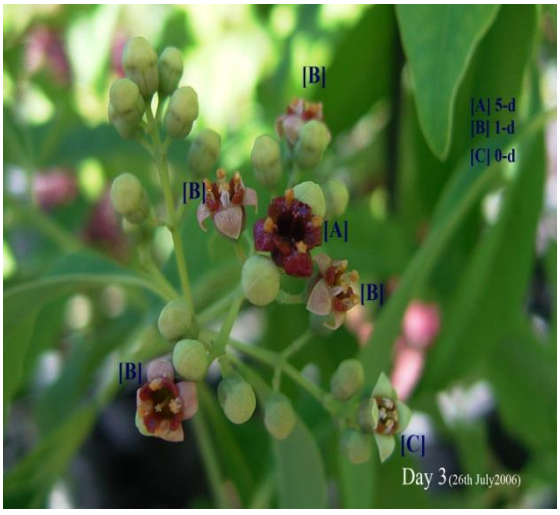

[A] - shed
[B] - 2d
Day 4 07:15

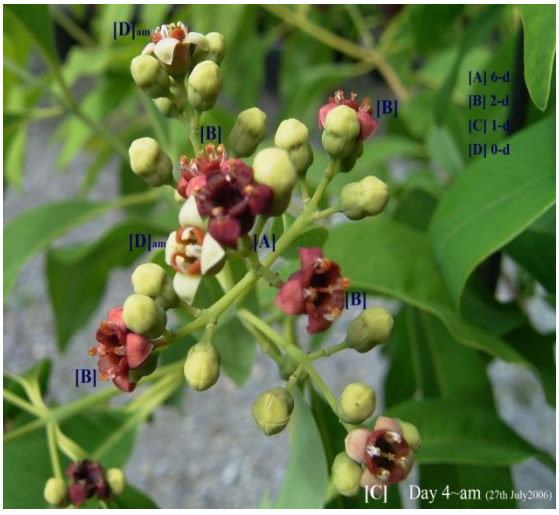

[B] - 3d
Day 5 09:00

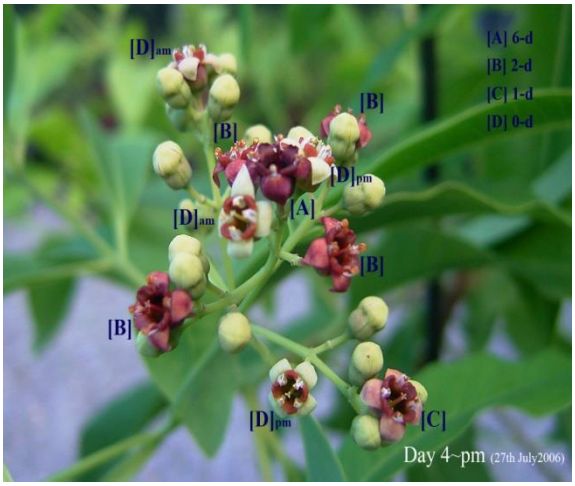

[B] - shed
Day 5 20:00

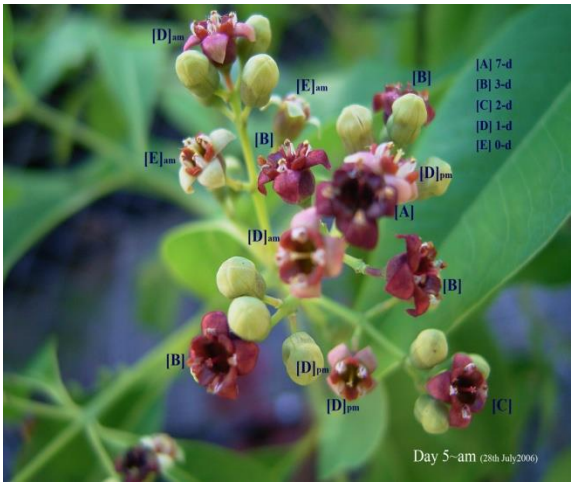
Santalum austrocaledonicum

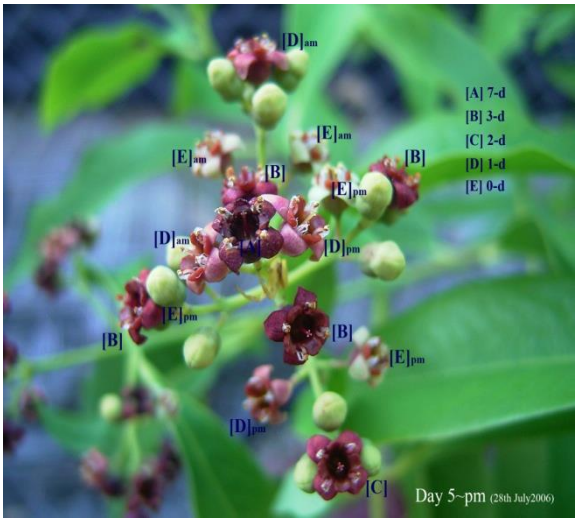
Santalum album



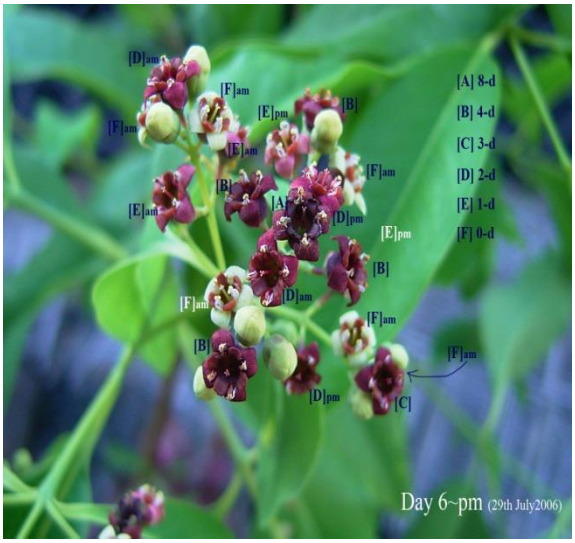


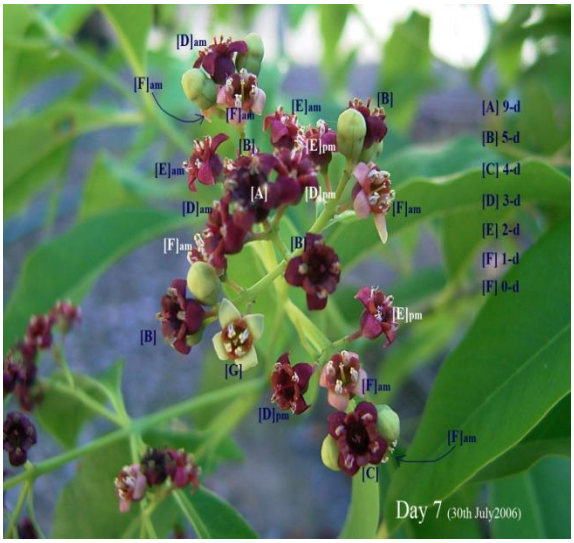

[A] 6-d
[B] 2-d
[C] 1-d
[D] 0-d
Day 4~pm (27th July2006)


[A] 7-d
[B] 3-d
[C] 2-d
[D] 1-d
[E] 0-d
Day 5~am (28th July2006)


[A] 7-d
[B] 3-d
[C] 2-d
[D] 1-d
[E] 0-d
Day 5~pm (28th July2006)

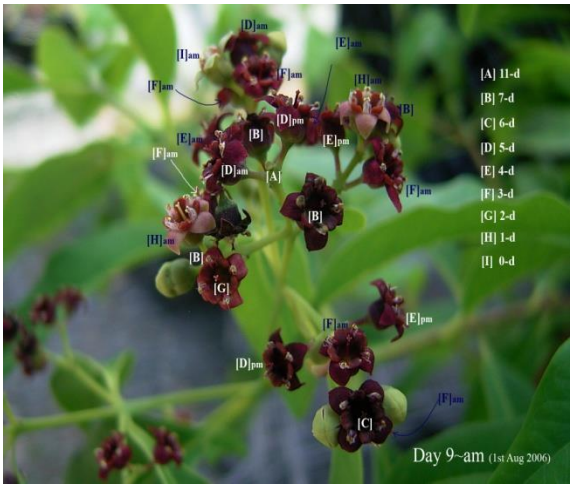

[A] 8-d
[B] 4-d
[C] 3-d
[D] 2-d
[E] 1-d
[F] 0-d
Day 6~am (29th July2006)

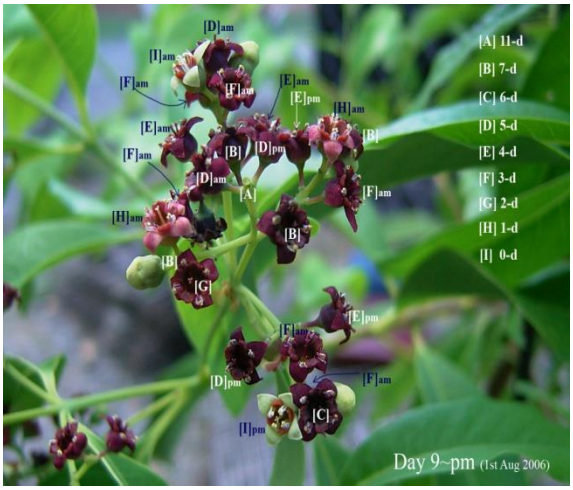

[A] 8-d
[B] 4-d
[C] 3-d
[D] 2-d
[E] 1-d
[F] 0-d
Day 6~pm (29th July2006)


[A] 9-d
[B] 5-d
[C] 4-d
[D] 3-d
[E] 2-d
[F] 1-d
[F] 0-d
Day 7 (30th July2006)


[A] 10-d
[B] 6-d
[C] 5-d
[D] 4-d
[E] 3-d
[F] 2-d
[G] 1-d
[H] 0-d
Day 8~am (31st July2006)


[A] 10-d
[B] 6-d
[C] 5-d
[D] 4-d
[E] 3-d
[F] 2-d
[G] 1-d
[H] 0-d
Day 8~pm (31st July2006)


[A] 11-d
[B] 7-d
[C] 6-d
[D] 5-d
[E] 4-d
[F] 3-d
[G] 2-d
[H] 1-d
[I] 0-d
Day 9~am (1st Aug 2006)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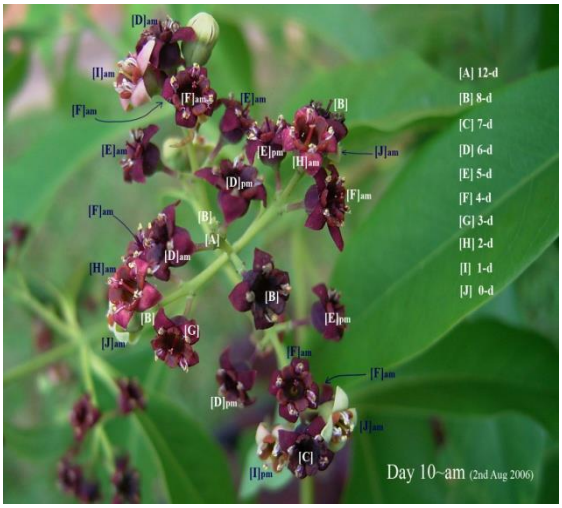
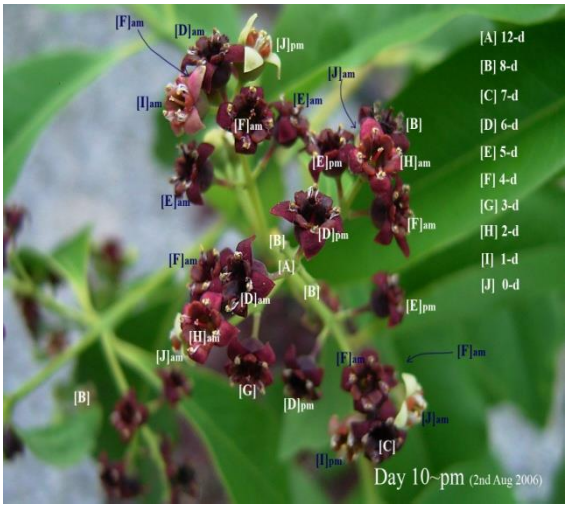
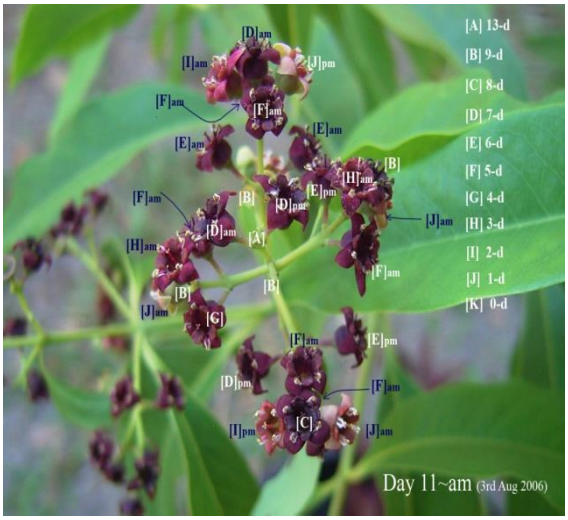
[B] 7-d
[C] 6-d
[D] 5-d
[E] 4-d
[F] 3-d
[G] 2-d
[H] 1-d
[I] 0-d
Day 9~pm (1st Aug 2006)


[A] 12-d
[B] 8-d
[C] 7-d
[D] 6-d
[E] 5-d
[F] 4-d
[G] 3-d
[H] 2-d
[I] 1-d
[J] 0-d
Day 10~am (2nd Aug 2006)


[A] 12-d
[B] 8-d
[C] 7-d
[D] 6-d
[E] 5-d
[F] 4-d
[G] 3-d
[H] 2-d
[I] 1-d
[J] 0-d
Day 10~pm (2nd Aug 2006)


[A] 13-d
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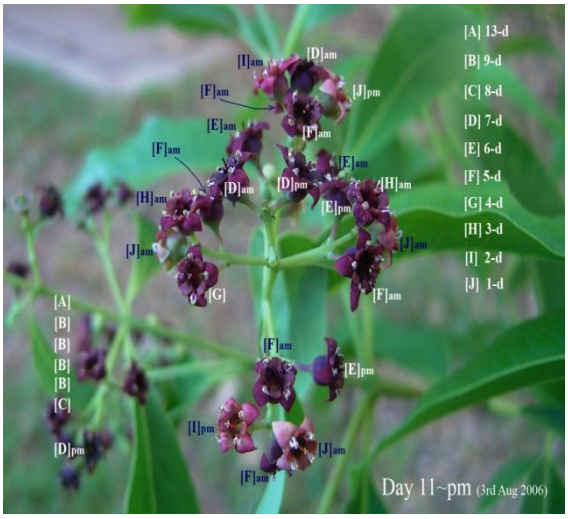
[C] 8-d
[D] 7-d
[E] 6-d
[F] 5-d
[G] 4-d
[H] 3-d
[I] 2-d
[J] 1-d
[K] 0-d
Day 11~am (3rd Aug 2006)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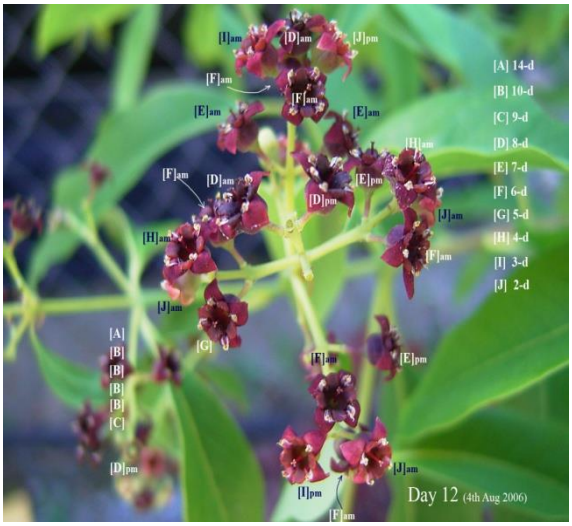
[D] 7-d
[E] 6-d
[F] 5-d
[G] 4-d
[H] 3-d
[I] 2-d
[J] 1-d
Day 11~pm (3rd Aug 2006)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[C] 9-d
[D] 8-d
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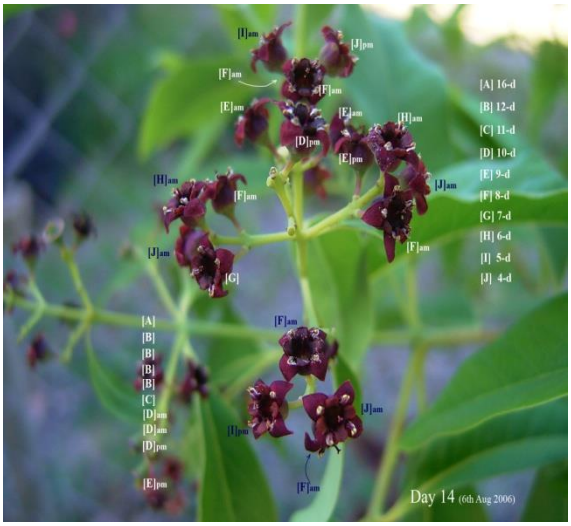
[F] 6-d
[G] 5-d
[H] 4-d
[I] 3-d
[J] 2-d
Day 12 (4th Aug 2006)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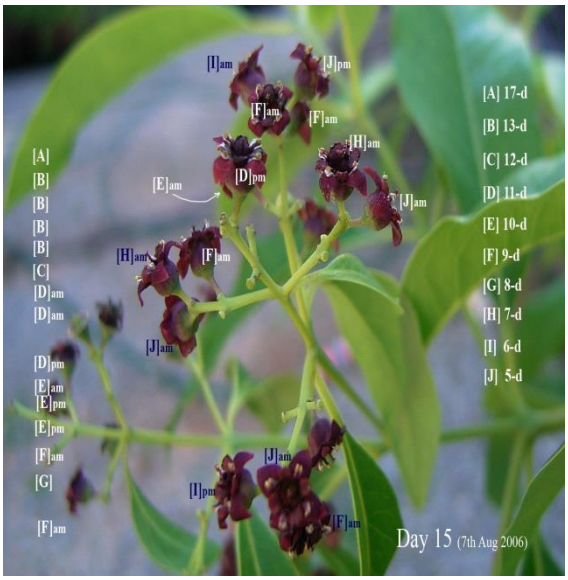
[C] 11-d
[D] 10-d
[E] 9-d
[F] 8-d
[G] 7-d
[H] 6-d
[I] 5-d
[J] 4-d
Day 14 (6th Aug 2006)


[A] 17-d
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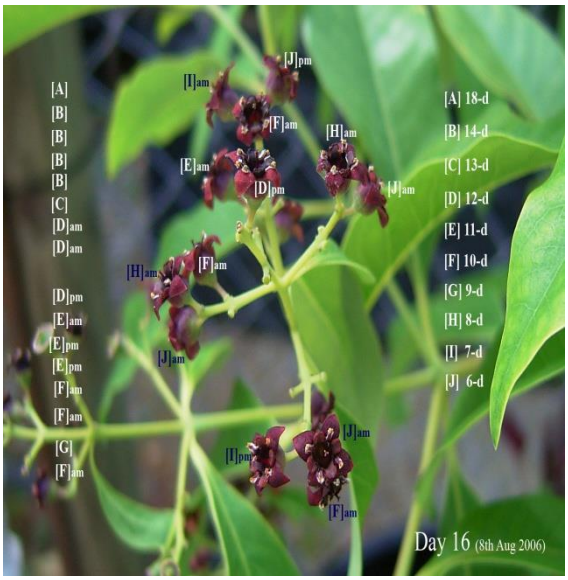
[D] 11-d
[E] 10-d
[F] 9-d
[G] 8-d
[H] 7-d
[I] 6-d
[J] 5-d
Day 15 (7th Aug 2006)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[D] 12-d
[E] 11-d
[F] 10-d
[G] 9-d
[H] 8-d
[I] 7-d
[J] 6-d
Day 16 (8th Aug 2006)

Santalum macgregorii


[C5, C6, C7] 0 days, just opened
[C3]
[A1]
[C4]
[C6]
[C7]
[B1]
[A3]
[C5]
[C2]
Day 3 (pm)


[A3] 3 days old
[B1] 2 days old
[C1-C7] 1 day old
[D1, D2] 0 days old
[D2]
[C3]
[C4]
[D1]
[C7]
[C6]
[C1]
[A3]
[B1]
[C5]
Day 4 (am)


[A1] falls off
[B2] falls off
[C2-C7] 1 day old
[D1-D3] 0 days old
[D2]
[C3]
[C4]
[D1]
[C7]
[C6]
[D3]
[C2]
[C5]
Day 4 (pm)


[C2-C7] 2 days old
[C1-C3] 1 day old
[C3]
[D2]
[C4]
[C7]
[C6]
[D3]
[C5]
Day 5 (am)


[C2-C7] 2 days old
[D1-D3] 1 day old
[E1-E4] 0 days old
[C3]
[E1]
[D2]
[E2]
[D1]
[E3]
[C4]
[C7]
[E4]
[D3]
[C5]
[C2]
Day 5 (pm)


[D1, D2] 2 days old
[D2] falls off
[E1-E4] 1 day old
[F1-f3] 0 days old
[E1]
[D1]
[E2]
[E3]
[F2]
[D3]
Day 6 (am)

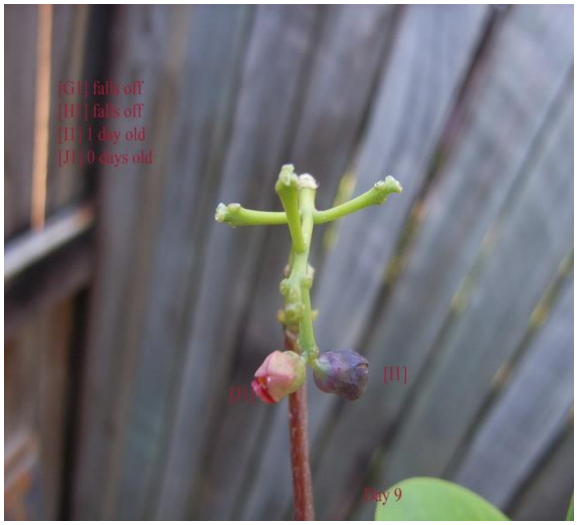

[D1, D2] 2 days old
[D2] falls off
[E1-E4] 1 day old
[F1-f3] 0 days old
[E1]
[D1]
[E2]
[F3]
[E3]
[F2]
[E4]
[D3]
Day 6 (pm)

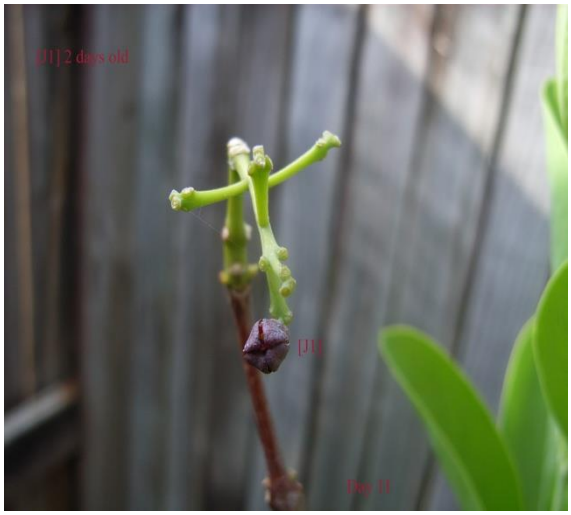

[D1]3 days old
[E1-E4] 2 days old
[F1-f3] 1 day old
[G1] 0 days old
[E1]
[G1]
[F3]
[D1]
[E2]
[E3]
[F2]
[E4]
[F1]
Day 7 (am)


[G1]
[F3]
[H1]
[E4]
Day 7 (pm)

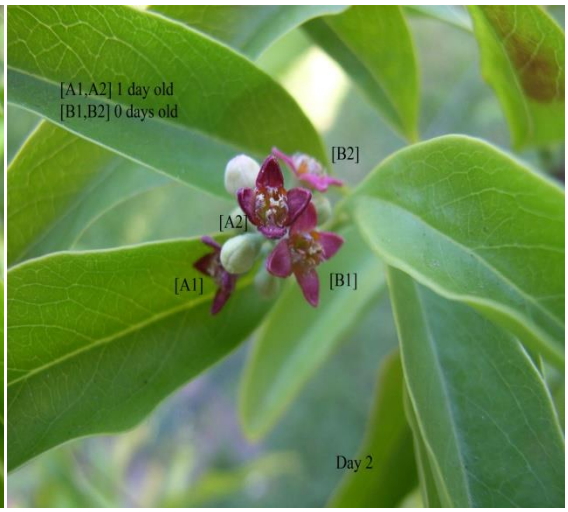

[G1]
[F3]
[H1]
[I1]
[F1]
Day 8 (am)


[F1, F2] fall off
[G1]
[H1]
[I1]
Day 8 (am)

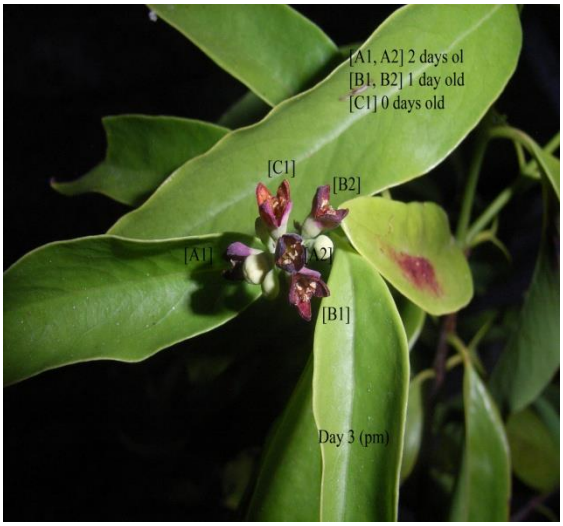

[G1] falls off
[H1] falls off
[I1] 1 day old
[J1] 0 days old
[I1]
Day 9

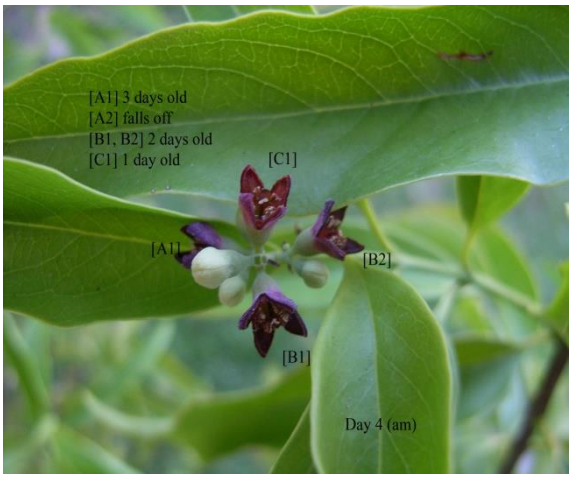


Santalum yasi




[A1, A2] 2 days ol
[B1, B2] 1 day old
[C1] 0 days old
[C1]
[B2]
[A2]
[B1]
[A1]
Day 3 (am)


[A1, A2] 2 days ol
[B1, B2] 1 day old
[C1] 0 days old
[C1]
[B2]
[A1]
[A2]
[B1]
Day 3 (pm)


[A1] 3 days old
[A2] falls off
[B1, B2] 2 days old
[C1] 1 day old
[C1]
[A1]
[B2]
[B1]
Day 4 (am)

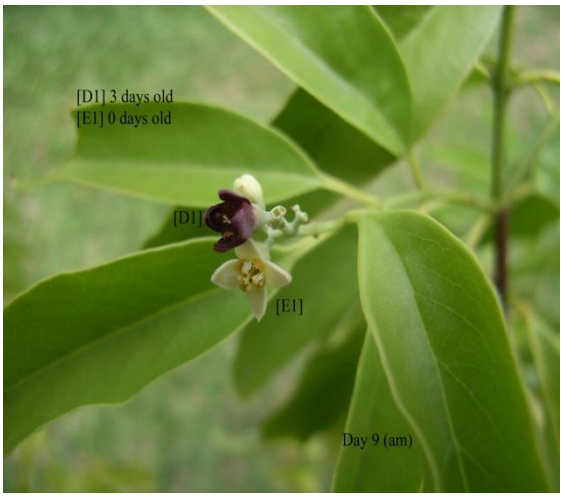

[A1] falls off
[B1, B2] 3 days old
[C1] 2 days old
[C1]
[B2]
[B1]
Day 5 (am)


[A1] falls off
[B1, B2] 3 days old
[C1] 2 days old
[C1]
[B2]
[B1]
Day 5 (pm)


[B1, B2] falls off
[C1] 3 days old
[C1]
[B1]
Day 6

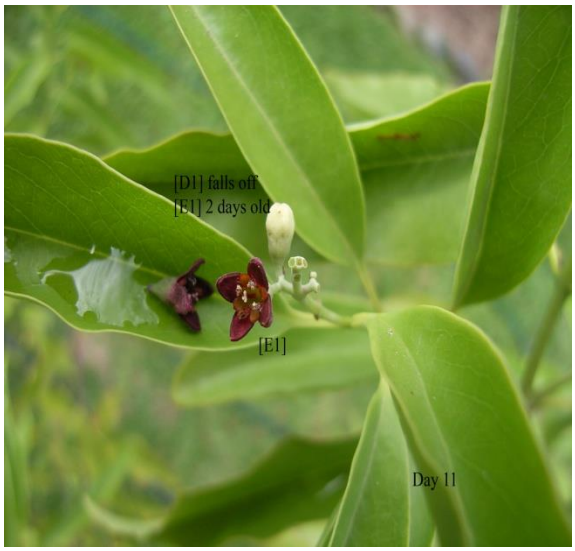

[C1] falls off
[D1] 0 days old
[D1]
Day 7

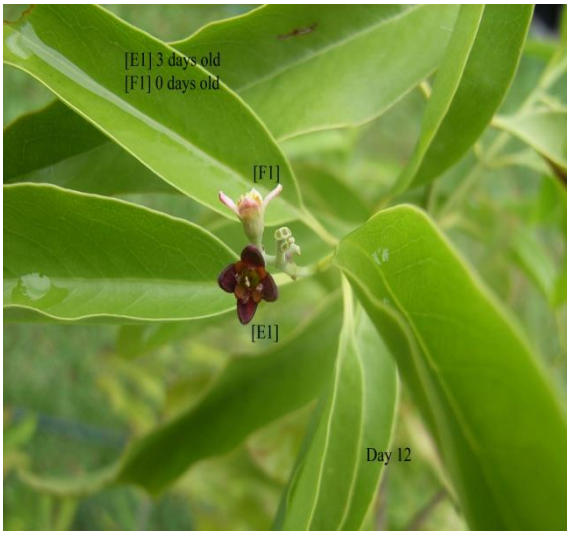

[D1] 1 day old
[D1]
Day 8


[D1] 3 days old
[E1] 0 days old
[D1]
[E1]
Day 9 (am)

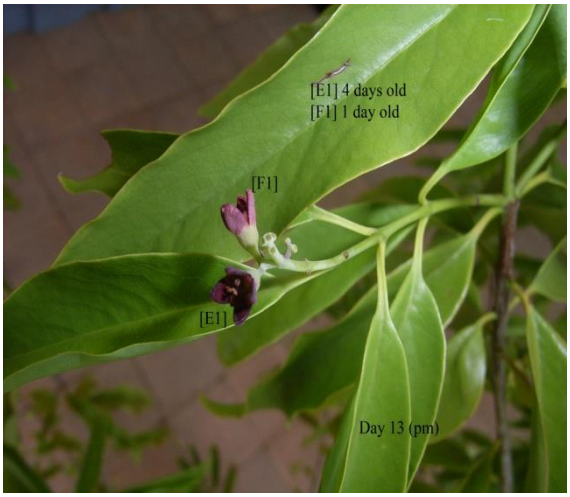

[D1] 3 days old
[E1] 0 days old
[D1]
[E1]
Day 9 (pm)


[D1] 4 days old
[E1] 1 day old
[D1]
[E1]
Day 10

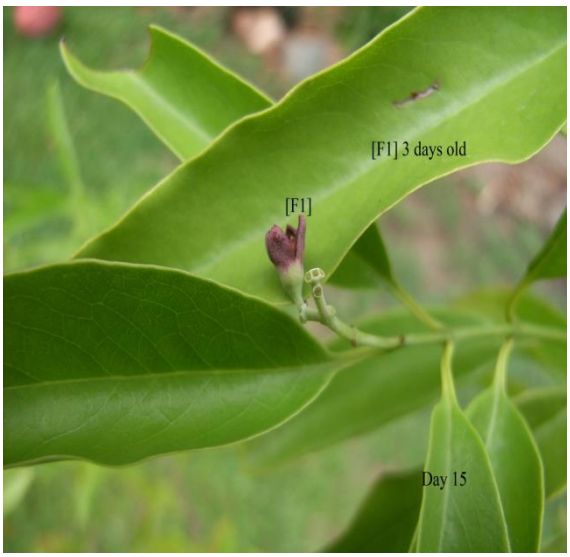

[D1] falls off
[E1] 2 days old
[E1]
Day 11

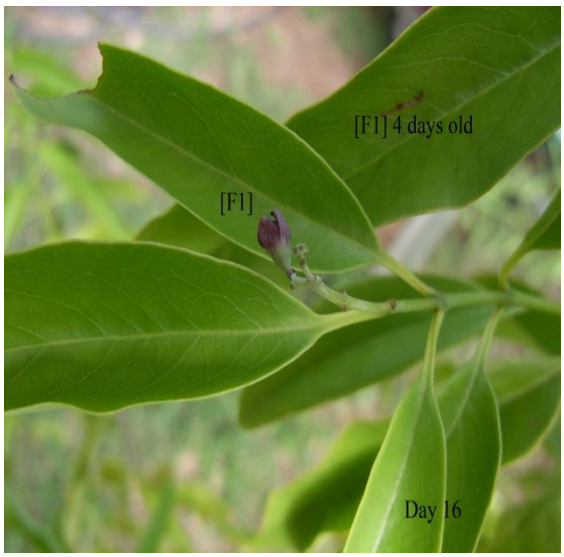

[E1] 3 days old
[F1] 0 days old
[F1]
[E1]
Day 12

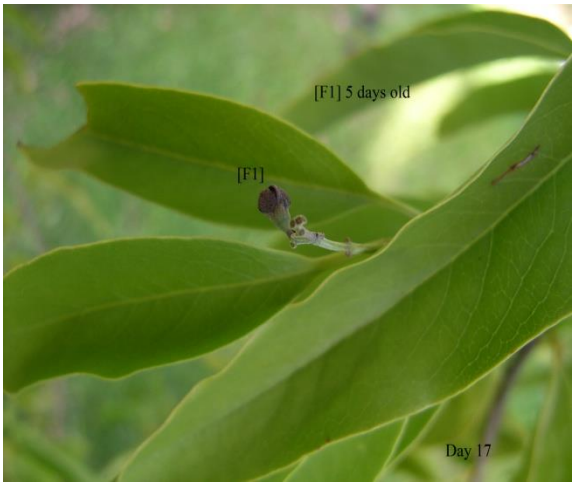

[E1] 4 days old
[F1] 1 day old
[F1]
[E1]
Day 13 (am)


[E1] 4 days old
[F1] 1 day old
[F1]
[E1]
Day 13 (pm)


[E1] falls off
[F1] 2 days old
[F1]
Day 14


[F1] 3 days old
[F1]
Day 15


[F1] 4 days old
[F1]
Day 16


[F1] 5 days old
[F1]
Day 17


All flowers have fallen off
Day 18