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Growth physiology and productivity of cultivated *Aquilaria crassna* Pierre ex Lecomte (Thymelaeaceae) in tropical Australia and its reproduction biology

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Abstract

Agarwood is a highly prized, resin-infused fragrant wood that has been used since early history in both secular and religious practices. Agarwood is produced mainly by species of the genus *Aquilaria.* The high demand for agarwood has resulted in overharvesting of the natural population. Growing *Aquilaria* in plantations seems a sensible approach to supply the market and meet consumer demands. Several initiatives, including the establishment of plantations, are in place in the countries of origin of the species to supply cultivated agarwood. In tropical Australia, a research plantation of *Aquilaria* species was established to determine the biological and commercial viability of production in an area beyond its natural range. Research was conducted in this plantation as part of this dissertation to improve our understanding of floral biology and the breeding system of *Aquilaria crassna*, morphological characters that differentiate species of *Aquilaria*, the environmental, physiological and leaf- morphological characters that influence tree growth and productivity. This knowledge (theoretical and practical) can inform methods, techniques and tools for its cultivation.

In this study, analysis of leaf outlines using elliptical Fourier descriptors was used successfully to distinguish morphological variability and discriminate between three species of *Aquilaria.* Flower anthesis of *Aquilaria crassna* occurred at evening and early morning, flowers stay open up to 3.5 days. Stigma is receptive when flowers are fully open for one day. Hand-pollination experiments showed that *A. crassna* is self-compatible and that there is no pollen limitation for fruit production in its new environment. Hybrids between *A. crasnna* and *A. baillonii* were possible.

Physiological and morphological leaf-traits were a useful predictor of tree productivity. Isotopic composition of carbon ($\delta^{13}C$) in leaf dry matter explained 35% of the variability in diameter. Predictors that explain growth in *Aquilaria* are $\delta^{13}C$, $\delta^{15}N$, petiole length, number of new leaves produced per week and specific leaf area. CO_2 assimilation increased linearly with PFD peaking at PFD of 1000 $\mu mol\ m^{-2}\ s^{-1}$ (A_{max}). Relative leaf chlorophyll content (determined using a SPAD meter and expressed as leaf greenness) correlated positively with the rate of CO_2 assimilation and % of leaf nitrogen. Leaf greenness index could be used by *Aquilaria* growers to maximize productivity in *Aquilaria*. This study provides the evidence that support the cultivation of *Aquilaria* spp. outside its range of distribution.

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Chapter 1. Introduction

1.1 Background

Despite a long history of global agricultural development, people still rely on natural forests to a varying extent for a range of products including timber, fibre, medicine and other non-wood forest products (Belcher *et al.*, 2005; Sunderlin *et al.*, 2005). Combined with rapid growth in global human population, this dependence has led to increased demands and degradation of natural forests, including the genetic erosion of key species (Ledig, 1992). The cultivation of previously wild trees is one approach to reduce our reliance on natural sources and limit further forest degradation (Andersen *et al.*, 2008; Paquette & Messier, 2010). The degree of success in this approach will depend on the development of both reliable cultivation practices and productive domesticated forms (Simons & Leakey, 2004). This process is already underway for many cultivated trees, which have become an important alternative source for the goods and services provided by natural forests (Bauhus *et al.*, 2010; Carle & Holmgren, 2008).

The area under 'planted forest' as of 2010 is estimated at 264 million ha (this represent 6.6% of the total area under forest cover worldwide). The use of species introduced from outside their natural range is commonplace and represents 83% of all planted forest (FAO, 2010). Just as international food supplies are comprised of a limited number of highly domesticated species, planted forests may also encompass a few species from genera such as *Pinus* L. (Pinaceae), *Eucalyptus* L'Hér. (Myrtaceae), *Populus* L. (Salicaceae), *Acacia* Miller. (Fabaceae, Mimosoideae) and some species of the Verbenaceae family (Jagels, 2006). While it is recognised that these genera are of great significance to international timber supply, other non-timber forest products that have been severely depleted also require development. It is within this context that interest in the cultivation and domestication of a whole range of tree species is increasing (Leakey *et al.*, 2012). For instance, research has been conducted with Brazil nut trees (*Bertholletia excelsa* Humb. & Bonpl.*)* to increase the density of this species in secondary forest through enrichment planting of seedlings in different arrangements (Peña-Claros *et al.*, 2002). Cultivation of peach palm (*Bactris gasipaes* Kunth.) crops under a range of management regimes (from agroforestry to industrial plantation) has expanded to provide an alternative and sustainable source of palm heart to wild stands (Yokomizo & de Farias Neto, 2003). The cultivation of several species of plants for the extraction of high-value essential oils is also emerging (Budiadi *et al.*, 2005; Lubbe & Verpoorte, 2011; Shackleton *et al.*, 2007; Zuzarte *et al.*, 2010).

As human population increases so too will the need for planted forests. This increase in demand will encompass many minor forest products extracted unsustainably from forests. It is only in more recent times that tropical trees have been the subject of domestication efforts (Evans, 1999; Leakey *et al.*, 2012; Simons & Leakey, 2004). While there are a myriad of potential candidates, this thesis will focus on developing basic biological knowledge to support the domestication of *Aquilaria* Lam., species. The focus on this species was due to the excessive overharvesting of agarwood, leading to many species in the genus *Aquilaria*, being listed as threatened or endangered (IUCN, 2013).

Species of *Aquilaria* produce an exquisite fragrant resin known as agarwood, aloes, gaharu and eaglewood (Jensen, 2004; Soehartono & Newton, 2000). The resin is formed in the heartwood in response to an injury, insect or microbial invasion or a pathogen attack (Persoon, 2007). The genus *Aquilaria* (Thymelaeaceae) includes 21 species (The Plant List, 2013) endemic to north-eastern India to southern China, Cambodia, Laos, Vietnam, Thailand, Malaysia, Indonesia, Philippines and Papua New Guinea. The habit of different species range from shrubs to trees and species occur in both low- and mid-altitude forests (from sea level to up to 1000 m). In Malaysia, *Aquilaria* species can be found in a variety of soils ranging from very fertile alluvial soils to wetland soils with periodic flooding (Faridah-Hanum *et al.*, 2009). Estimates of tree density of *Aquilaria crassna* Pierre ex. Lecomte (dbh $\geq$ 10 cm) in natural population at two locations (Vietnam and central Thailand) ranged from 1.7 trees ha^{-1} to 4.9 trees ha^{-1} (Jensen & Meilby, 2012; Zhang *et al.*, 2008).

As a response to the increasing pressure to find sustainable sources for agarwood, there has been an increasing research focus over the last decade across several fields. There is an emerging body of research on techniques to artificially induce agarwood formation in cultivated trees (Liu *et al.*, 2013; Mohamed *et al.*, 2014b; Pojanagaroon & Kaewrak, 2003; Zhang *et al.*, 2012). There is also increasing activity in the field of molecular genetics such as identification of genes related to agarwood formation (Gao *et al.*, 2012; Wong *et al.*, 2012; Xu *et al.*, 2013), characterization of polymorphic microsatellite of *A. crassna* for forensic applications (Eurlings *et al.*, 2010) and the analysis of wood for the diagnostic of chromones to differentiate cultivated and wild agarwood. (Espinoza *et al.*, 2014). Research activity into the chemical qualities of agarwood is also increasing particularly with respect to the presence and concentrations of individual chemical constituents (Li *et al.*, 2015; Naef, 2011; Nor Azah *et al.*, 2014; Yang *et al.*, 2014). Mad Amin *et al.* (2012) used imaging processing to correlate the colour properties of agarwood with agarwood of different prices (an expression of quality), which supports the use of colour when grading and pricing agarwood. Other areas of research include the study of incense smoke (factor of indoor air pollution) and its possible effects on human health (Cohen *et al.*, 2013; Chang *et al.*, 2007; Navasumrit *et al.*, 2008). In comparison with the numbers of studies in current areas of agarwood research, few recent studies have been published on taxonomy (Kiet *et al.*, 2005; Yinzheng *et al.*, 2007), ecology (Saikia & Khan, 2013; Saikia & Khan, 2014a; Soehartono & Newton, 2001b), demography (Zhang *et al.*, 2008) and silviculture (Soehartono *et al.*, 2002; Xiao-xia *et al.*, 2010) of agarwood trees.

The high prices paid for agarwood products have stimulated interest in its cultivation with smallholders, private investors, entrepreneurs, government and local organizations (Hoang Ha & Nghi, 2011). Cultivation of *Aquilaria* offers an opportunity for establishing a sustainable commercial source of agarwood and reducing the harvesting pressure in natural populations (Nakashima *et al.*, 2005; Persoon, 2008). Over recent times plantings of *Aquilaria* species have been established in Laos, Bangladesh, Indonesia, Cambodia, Malaysia, Vietnam and Australia (Barden *et al.*, 2000; Donovan & Puri, 2004; Page & Awarau, 2012; Rahman & Khisa, 1984; Tran *et al.*, 2003).

The performance and productivity of these plantations have not been reported and it is most likely plantings are based on wild-collected seed. The agronomic and silvicultural management of the plantations include weed control, fertilization, pruning, induction of agarwood formation and harvesting. Trees are planted at different densities, for example, Xiao-xia *et al.* (2010), reported that *A. sinensis* trees growing in Hainan, were planted at 1650 or 2400 trees ha^{-1}. To induce agarwood formation in cultivated *Aquilaria* trees, traditional and commercial induction techniques have been practiced in several producer countries (Liu *et al.*, 2013; Ng *et al.*, 1997; Pojanagaroon & Kaewrak, 2003). The technique seeks to activate the immune system of the trees and initiate the production of the fragrant resin. Normally one single straight stem is preferred (Lok & Zuhaidi, 2016). A minimum diameter is required to carry out the induction or inoculation of the trees. For example, in Indonesia trees are inoculated when they have reached a minimum of 20 cm of diameter. In Hainan, inoculation is performed in strong or old trees of at least 30 cm diameter at height breast. Lok and Zuhaidi (2016) suggested that trees with stem diameter at breast height of 10 cm or more are ready for inoculation. It is expected that after two year or more of the inoculation, trees will be harvested and will produced agarwood of different quality and oil (Turjaman & Hidayat, 2017).

In Australia, the modest research estate consists primarily of *A. crassna* from Vietnam and smaller introductions from other locations in Southeast Asia possibly involving other species of *Aquilaria*. While the results of these early plantings indicate that *A. crassna* has good productive potential in tropical north Queensland, knowledge about performance of individual species, physiology and breeding systems is required to develop an informed tree improvement programme. This study describes the floral morphology, stigma receptivity and breeding system of *A. crassna* growing in a cultivated environment outside of its range of natural distribution. Also, this study may provide links between morphological leaf-productivity traits and tree growth performance as a tool to aid in the selection of superior genotypes; and investigate the suitable environmental conditions (light and nutrition) in which *A. crassna* maximize productivity.

1.2 Aim

The aim of this research is to develop knowledge in key areas of *Aquilaria* biology that supports improved practices for its domestication and cultivation. These areas of biology include a basic understanding of the (i) morphological characters that differentiate species of *Aquilaria*, (ii) leaf functional traits relationship with tree growth and productivity, (iii) environmental influences on photosynthesis, and (iv) floral biology and breeding systems. The specific objectives of the research were to

- Define the species currently under cultivation through morphometric analysis.
- Describe the relationship between leaf functional traits and tree growth in *A. crassn*a.
- Define relationships between light, nitrogen and chlorophyll with photosynthesis in *A. crassna*.
- Describe the floral morphology and phenology in *A. crassna*.
- Determine the predominant breeding system operating in *A. crassna*.

1.3 Research questions

1. What are the morphological characters or combinations that discriminate among putative *Aquilaria* species present in the Australian research estate?

2. What are the physiological and morphological leaf traits or combinations that could explain tree growth, expressed in diameter, in *A. crassna* and assist in the selection of good trees?

3. What influence does the intensity of solar radiation; relative leaf nutrients and chlorophyll content have on the rate of CO_2 uptake in *A. crassna*.

4. What are the main phenological stages of flowering in *A. crassna* and what is the variation in expression and duration of each stage?

5. What is the relative level of self- and cross- compatibility in *A. crassna*, and its inter-specific compatibility with each of *A. baillonii* and *A. subintegra*?

1.4 Research approach

Plant domestication is an evolutionary and sequential process where plants adapt to the conditions of cultivation (Kupzow, 1980; Vaughan *et al.*, 2007). The contemporary process of tree domestication is typically conducted through the characterization of morphological variation in existing wild and planted sources and selection of desirable individuals based on traits of interest (Neale, 2007; Wiersum, 1997). Contemporary plant improvement is based on the genetic processes of evolution and domestication but defined by clear objectives involving selection and propagation of superior, provenances, progenies and genotypes for subsequent generations (Eldridge *et al.*, 1993; Hancock, 1992; Simmonds, 1979). Tree performance in terms of growth rate is also of interest in this research. To meet the aim of the thesis and improve the understanding of the species' to assist its domestication, it was necessary to confirm the species growing in north Queensland research estate. Species were evaluated based on the botanical descriptors available for the species, and then elliptical Fourier analysis applied to determine how leaf outlines conform to species delineation.

Leaf functional traits (LFTs) provide a method to evaluate how the plants are adapting to the conditions where they are growing (Bussotti & Pollastrini, 2015). LFTs can be used to analyse growth and productivity through the assessment of leaf attributes linked to physiological processes. These leaf attributes include morphological and physiological traits. Examples of leaf functional traits uses that relate to growth or productivity include carbon isotope ratios (Correia *et al.*, 2008; Xu *et al.*, 2000) and other morphological and chemical leaf traits (Possen *et al.*, 2014; Santiago & Wright, 2007). To evaluate tree growth in *A. crassna*, isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) and other chemical and morphological leaf traits were measured to determine any relationship with growth and a selection of leaf traits scored and screened to assist in the selection of superior individuals for further evaluation and propagation.

Knowledge and understanding of the focal species' reproductive biology is essential for domestication, since it will influence methods and strategies used to affect genetic improvement over both the short- and long-term (Acquaah, 2007; Eldridge *et al.*, 1993). A plant breeding system including aspects of flower morphology, flowering phenology, pollen flow and incompatibility systems, will influence potential gene flow between individuals and populations (Richards, 1997). Knowledge of a species floral morphology and phenology will determine the methods for carrying out detailed studies of its breeding system and operational controlled breeding. Observations of the sequence of flower morphology (anthesis to flower fall) will help to determine the lifespan of the flowers and the timing and duration of important events such as flower opening, pollen shedding, and stigmatic receptivity. In this study, sequence of flower development was recorded using digital camera as has been used to document others aspect of reproductive biology (Mawdsley *et al.*, 2013). Detailed information on flower development is important to identify the period when the sexual organs are ready for the exchange of gametes, therefore activities and techniques of controlled pollination can be appropriately managed and developed for the tree improvement programme.

Each experimental chapter was written as a paper to facilitate the production of manuscripts for journal submission. An edited version of chapter 2, 4 and 5 has been submitted for publication.

1.5 Summary of chapters

1.5.1 Chapter 2

To give the thesis an historical context a review of the past use, importance and trade of agarwood from ancient to modern times is presented in Chapter 2. This chapter presents the position that agarwood hold in ancient cultures, its cultural, religious and trade value, and how this has led to the depletion of the wild resource and the consequent need for cultivation and domestication. Details of its early use were examined throughout the history of human civilizations. Chapter 2 culminates in a discussion of the contemporary demand, trade of agarwood and initiatives to attain sustainability in both producer and importers countries.

1.5.2 Chapter 3

The aim of Chapter 3 was to identify morphological characters that discriminate between the putative species growing in the north Queensland research estate. This is important for the rapid identification of species to support the development of silvicultural techniques and domestication strategies. Three species (*A. crassna*, *A. baillonii* and *A. subintegra*) were initially identified by their distinguishing reproductive characters and then examined for a range of vegetative morphological characters. Leaf outline analysis using elliptical Fourier descriptors was found to be an effective tool to reliably discriminate among the three species planted in the estate.

1.5.3 Chapter 4

The aim of Chapter 4 was to assess whether leaf-productivity traits, as measured by $\delta^{13}C$ and nitrogen isotope ($\delta^{15}N$) and another six leaf attributes, are associated with tree growth. The leaf attributes considered in this analysis were: 1) rate of leaf lamina expansion in length (Lexp, mm day^{-1}), (2) number of new leaves produced in a shoot; and for the single visibly largest leaf in the canopy the following traits: (3) petiole length (PeLen, mm) and width (PeWid, mm), (4) Leaf area (LA, cm^2) measured using a leaf area meter (CID 203 handheld laser leaf area meter- CID Bioscience Inc, Camas, WA, USA), (5) Specific leaf area (SLA, cm^2 g^{-1}), (6) carbon and nitrogen content.

1.5.4 Chapter 5

The aim of Chapter 5 was to determine the nature of relationships between the rate of photosynthesis with each of (i) intensity of solar radiation, (ii) relative leaf nutrients and (iii) chlorophyll content in *A. crassna*. Leaf chlorophyll was determined using a hand-held chlorophyll meter: SPAD-502 meter (Konica-Minolta, INC, Osaka, Japan).

1.5.5 Chapter 6

This chapter comprises two important aspects of the reproductive biology of *A. crassna*. In the first part of the study, I define the main stages of flower development in *A. crassna* and determine the timing and duration (phenology) of each stage. This was achieved through the analysis of a sequence of images taken twice daily. As part of the flower phenology study I determined the timing of onset and duration for stigma receptivity as related to the different stages of flower development. In the second part of the study I conducted controlled pollination experiments to determine the level of self- and intra specific compatibility within *A. crassna* as well its interspecific cross-compatibility with *A. baillonii* and *A. subintegra.*

1.5.6 Chapter 7

Chapter 7 presents the summary and outcomes of the study.

Chapter 2. History of use and trade of agarwood

2.1 Abstract

Among the world's aromatics, agarwood is the most highly esteemed, with fragrant properties that appeal to the very basis of human spirituality and psychology/consciousness. Agarwood has unique animalistic and 'other fragrant properties' that interact with human olfactory senses in a way that defies conventional language. As a result of these desirable properties, agarwood has been a heavily traded aromatic throughout history, and used across many cultures as a spiritual, opulent, and/or aphrodisiac item. Details of its use are recorded in several important historical and religious texts. For instance, agarwood is cited several times in the Old Testament as a fragrant substance; it was one of the preferred scents of the Prophet Muḥammad and, incense used in Tantric Buddhist rituals. In China agarwood was used in the crafting of the incense seal that also served as time-measurement. In India agarwood was considered one of the most important aromatics and cited in several treatises including texts regarding sexual experience. In current times, agarwood remains as an important element of many societies supporting cultural/folkloric traditions for incense and traditional medicine as well as more contemporary uses in perfumery. Its global trade includes more than 40 countries. People's reverence and demand for agarwood has led to significant declines in its natural sources. Efforts to obtain sustainability of wild resources have been attempted in most countries where it grows and is manufactured.

2.2 Introduction

The use of aromatics and spices has been a conspicuous element in many cultures and life practices (Turner, 2004). Their rarity through history often elevated them to a noble or mystical status. Aromatics and other spices have been synonymous with wealth, exclusivity and luxury. Frankincense and myrrh are well known examples of fragrant resin highly demanded and precious to the people of ancient Egypt, Mesopotamia, Greece, Rome, China and India (Groom, 1981). The earliest recorded use of plant aromatics is found in the four Vedas of ancient India. These texts are proto Hindu, written between 1500 and 1000 BCE and include descriptions of already sophisticated uses of aromatics. The spiritual significance of aromatic plants was highlighted in these ancient texts and included the bestowment of fragrances in plants by the 'spirit of life' (Prâna) (Bloomfield, 1897), use of fragrant smoke for spiritual connection with heaven and as offerings for divine worship (Griffith, 1896). The secular use of fragrances was also prominent, where they were used to scent the people and houses of noble classes (Griffith, 1896; Keith, 1914) as well as for their natural healing properties (Keith, 1914).

Plant aromatics are typically derived from volatiles resulting from secondary plant metabolism (Rehman *et al.*, 2016). Plant volatiles perform biological and/or ecological functions for the plants that produce them (Duradeva *et al.*, 2006; Holopainen, 2004). The chemical composition of plant volatiles varies widely between species, but are mainly derived from terpenoids, phenols, benzenoids, fatty acids and amino acid derivatives (Duradeva *et al.*, 2006; Morris, 1984). Essential oils may be defined as plant volatiles that are perceivable to people as a fragrance or flavour and many are valued by people for their bioactive health-promoting and/or fragrant properties (Bakkali *et al.*, 2008; Murbach Tales Andrade *et al.*, 2014; Seow *et al.*, 2014; Shaaban *et al.*, 2012). Terpenoid, and less so, phenylpropanoid- derived volatiles are those most commonly used as essential oils (Rhind, 2014). These oils are produced by a wide range of plants within organs such as leaves, reproductive structures, stems and roots (Bakkali *et al.*, 2008; Sangwan *et al.*, 2001), although volatile aromatic oils are also derived from a limited number of animals (i.e. musk deer, the sperm whale, civet cats) (Morris, 1984). The term ‘plant aromatics’ may be used generically to encompass all forms in which essential oils are extracted/consumed by people, such as incense, physical and chemical extracts, maceration in oils, fats or water, distillation, perfumes, among others.

Aromatics have innumerable uses including embalming and medicinal practices (i.e. aromatic fumigation), perfume, religious purposes, public ceremony, aphrodisiac, spiritual and philosophical healing, funeral rites, seasoning of food, and as inspiration in poetry (Classen *et al.*, 1994; Turner, 2004). For medicinal purposes, more than 732 plants (not only aromatics) have been listed as being used in medicinal practices covering more than 1000 uses (Pennacchio *et al.*, 2010). One of the most remarkable examples took place in ancient Greece, when Hippocrates of Cos (460-377 BCE) saved Athens (c 430 BCE) from a plague by fumigating its surroundings with aromatics (Pinault, 1986). In Europe until mid- 18th century a mix of juniper berries, wood and rosemary were used to disinfect the air and prevent plagues (Rhind, 2014). Indigenous Australians use fragrant smoke to strengthen newborn babies and their mothers during the birth and postpartum (Conservation Commission of the Northern Territory *et al.*, 1993). Species such as *Erythrophleum chlorostachys* were commonly burnt in the maternity area and other species such as acacias and eremophilas were inhaled to induce lactation (Conservation Commission of the Northern Territory *et al.*, 1993). Fragrant smoke also has been used for religious, ceremonial or magical purposes (as incense). At least 400 plant species have been reported for this purpose. For example, in Mesoamerica incense from *Bursera*, *Protium* and *Hymenaea* species was burned during human sacrifices to the god of rain (Pennacchio *et al.*, 2010). For fragrance, flavouring and preservation 239 species has been listed (Pennacchio *et al.*, 2010). There are other examples of uses of plant-derived smoke that are not considered in this review as they lie beyond the scope of it, such as recreational, pest control, toxic, and veterinaries.

While many hundreds of species produce aromatic essential oils, some such as agarwood are particularly revered for their aromatic, historical, and religious significance. Agarwood is a resin-infused fragrant wood (also known as aloeswood, eaglewood, gaharu, jinkoh) that is derived principally from tropical trees of the genera *Aquilaria*, *Gyrinops*, *Aetoxylon* and *Gonystylus* (Thymelaeaceae) (Gunn *et al.*, 2004). Historically *Aquilaria* species have been the primary source for agarwood and are distributed westward from India (Bengal and Assam) to the island of New Guinea and southward from tropical China (Kwantung, Yunnan), to the Lesser Sunda Islands (Ding Hou, 1960, 1964). Records of agarwood use date back at least c 1400 BCE (Old Testament books, and Hindu epic text *Mahābhārata*). In this review the use and trade of agarwood (only derived from trees of the genus *Aquilaria*) from ancient to contemporary times is examined to contextualize and highlight the reasons for its high esteem and value. The terms agarwood, aloes and aloeswood are used in this review to refer to the resin infused wood of species from the four main genera.

2.3 A review of the history of use and trade of agarwood

2.3.1 Etymology

Agarwood, described as aloë in classical literature, has several names that are derivations or loans of ancient languages and vary according to the languages/dialects of the traders and places where was used or known. The many terms by which agarwood is referred to in religious and historic texts add to the confusion about its true history. In ancient texts the word aloe may refer to agarwood, typically as lignin aloes, or products of the arborescent succulent species *Aloe* which produces the medicinal bitter paste. It seems that agarwood has its origin in Dravidian (ak^{h}Vl-u-) or Indic language (i.e. Sanskrit, Prakrit) (Greppin, 1988). In Sanskrit agarwood was known as *agāru* and *aguru* [‘meaning the non-floating wood’(Miller, 1969)]; the Prakrit form is *agaru* and *Pali agalu*, all of them represents the word aloë and *agalochon* (Greek *agallochum)*. The word agarwood in Greek (*agallochum*), Hebrew (aḥāloth), and Arab (ālūwwa) are loanwords from the Indic names (Greppin, 1988; Schafer, 1963; Yule & Burnell, 1903). In Arabic medico-pharmacological literature agarwood is reported as *ūd* a synonymous for (*a*) *ġālūğan* borrowing through Greek *agallochum* (*âgáloxon*) which is a loanword from Sanskrit (Kahl, 2011). The main Malay name is kayu *găharu* or ‘gaharu wood’ a corruption of *agallochon (*Miller*,* 1969*)*. In China agarwood is referred to as, *ch’en hsiang* (‘the sinking incense’, derived from the Sanskrit) (Miller, 1969), or *kilam* and *bac* that means the ‘fragrant plant’ (Mathews, 1974). In Japan it is known as *Jinkoh* (also means ‘the sinking incense’) (Brechbill, 2012). In European languages, such as Portuguese agarwood is known as *aguila* or *pao d’aguila*, in French the wood is recognized as *d'aigle*, and in English as eaglewood. The latter taken from Medieval Latin (*aquilaria*) which is derived from the Greek (*agallochum*). The Sanskrit word for incense is known as agar-Bhatti, which is derived from the word for agarwood (*agāru*) (McKenna & Hughes, 2014).

2.3.2 Agarwood and other spices in Egyptian era

Egyptians were possibly the first civilisation to record the use of aromatics with paintings depicting the use and trade of frankincense in the Temple of Dayr el Bahri in Upper Egypt (c 17th century BCE) (Howes, 1950). The *Eber Papyrus* (c 1600 BCE) contains the procedure for up to 100 aromatic blends (*Ancient egyptian medicine: The Papyrus Eber*, 1930). Incense and perfumes were well known by ancient Egyptians as described by Theophrastus (484-425 BCE) and Pliny (23-79 CE). Theophrastus described the composition of Egyptians unguents, which were made of several ingredients, including cinnamon and myrrh, but agarwood known as aloes, does not seem to have been used in this period (Lucas, 1930). Moreover, there is no record of the use agarwood in the Egyptian art of embalming (31 plant species has been mentioned to be used during the process) (Baumann, 1960; Koller *et al.*, 1998). M'Clintock and Strong (1867) wrote however that the Greek historian Herodotus reported that aloeswood was used by the Egyptians for embalming dead bodies. Serpico and White (2000) argued that the resins used in Early Dynastic Egypt (13-14th BCE) were not the Arabian frankincense or myrrh; they were resin from species of the genus *Pistacia* (determined after analysis of pottery using chromatography/mass spectrometry), which was probably mixed with other volatile scented products. Gannal (1840) asserted that the Egyptians used myrrh and bitter aloes during embalming for their powerful effect in resisting putrefaction, rather than for purely their fragrant properties. While the timing of the first use of agarwood (aloeswood) and other foreign spices has not been determined, it is clear that aromatics were important products in early times. Many of the aromatics used were of imported origin, and while the Sumerians were possibly the first civilisation to engage in ocean-mediated international trade (3000 BCE), the Egyptians began sailing the Red Sea and the coast of east Africa as early as 2500-2400 BCE, trading manufactured goods in exchange for slaves, ivory, gold (from Ethiopia & Somalia), spices (from India via Yemen) and aromatics (frankincense and myrrh from Yemen) (Kearney, 2004).

2.3.3 Agarwood in Greek and Roman periods

The people of Ancient Greece may not have made routine use of incense in their rituals (Groom, 1981), but the practice was possibly adopted around the 6th BCE, as evidenced by the use of frankincense by the Greek philosopher Pythagoras to help him to prophesise (Dannaway, 2010). Also around this time Pythagoras brotherhood, a secret society, religion and political group and educational institute, burned only incense in their offerings to the gods (Classen *et al.*, 1994). The Greek also used incense at public festivals, procession and at ceremonies for the oracle (Groom, 1981). It is recognized that Herodotus (c 485-425 BCE) knew about myrrh, frankincense cinnamon and other spices as Arabian produce traded by the Phoenicians. According to Strabo (c. 64 BCE to c. 25 CE), Eudoxus of Cyzicus, made two trips to India (c 120-110 BCE) on behalf of Ptolemies to buy spices and other luxuries (Strabo, 1930).

Romans adopted practices and other cultural customs (including uses of plants) from other kingdoms brought under their control (i.e. Greek, Egyptian, and Phoenician). Among the aromatics used in ancient Rome were rose, lily, sweet flag, narcissus, imported pepper, cinnamon, spikenard, aloewood and fragrant grasses exported from India (Morris, 1984). Dioscorides in his book *Materia Medica* (65 CE), described the healing properties of sweet aloe-wood (and other spices) (Miller, 1969). Aloe-wood was mentioned in several recipes used to promote healing of wounds and as anti-inflammatory. With the annexation of Egypt by the Roman Empire (30 BCE), the Romans controlled many overland and ocean trade routes that directly connected the Mediterranean with Africa, southern Arabia (Egypt) (Miller, 1969) and India (Heldaas Seland, 2011) and the Phoenician ports of the Southern Mediterranean littoral. Rome commercial ties reached China, it has been reported that around *c* 284 CE a Roman and probably an Egyptian ‘embassy’ gave a tribute to the Emperor of China comprising 30,000 rolls of thin agarwood (Warmington, 1974). Cosmas Indicopleustes, a spice merchant, in his *Christian Topography* (written *c.* 550 CE), mentioned imports of silk, aloeswood, cloves, clove-wood, and sandalwood sailing from China and south-east Asia to Ceylon in the sixth century BCE (Indicopleustes, 2010 translation of c 550 CE book).

2.3.1 Agarwood in religious texts

2.3.1.1 Hindu text

The Sanskrit epic narrative *Mahābhārata* [text describing the period 1493 BCE-1443 BCE of Indian history according to Iyengar (2003)] contains the description of the use of scent/fragrance as the story unfold to describe human pleasure, luxury and wellbeing (Rhind, 2014). Agarwood was cited in various parts of the text, as a display of wealth, a tribute and welcoming. In the first book the people of the ancient city of *Khandavaprastha* received the Madhava (warrior) and other tribes by, among other things, filling every part of the town ‘with the sweet scent of burning aloes.’ (Book 1, Section CCXXIII). Also in Book 1 the use of agarwood for displaying status and wealth was well accounted in the description of an amphitheatre on the outskirts of King Drupada's capital of Kamapilya, which was ‘enclosed on all sides with high walls and a moat (and) scented with black aloes and sprinkled all over with water mixed with sandal-paste and decorated with garlands of flowers’ (Book 1, Section CLXXXVII). The mansions that surrounded this amphitheatre were also ‘rendered fragrant with excellent aloes’; and the ‘exalted sovereigns’ that inhabited the mansions were ‘possessed with the desire of excelling one another’ and ‘all adorned with the fragrant paste of the black aloe’ (Book 1, Section CLXXXVII). Also in second book was the detailed description of the mansions of monarchs, which ‘hung over with garlands of flowers and perfumed with excellent aloes’ (Book 2 Section XXXIII). Also in the second book after the Bharatas people conquered the Mlechchha tribes, the vanquished were made to pay tributes of a great many valuable items including fragrant goods of sandalwood and aloes (Book 2 Section XXIX). This detailed description of the use of aromatics and specifically fragrant agarwood, demonstrates the cultural importance and significance of aromatic in ancient times.

2.3.1.2 Christian scriptures

Agarwood is referenced several times in the Old Testament of the Christian Bible, firstly where Balaam describes God’s vision of Israel and likens their settlements as being ‘like aloes planted by the LORD’ (Numbers 24:6). Also in the Old Testament, the noble and seductive importance of agarwood is presented in Psalm 45:8, where recounting a king’s preparation for marriage it refers ‘All your robes are fragrant with myrrh and aloes and cassia’. The seductive power of aromatics including agarwood is further described in Song of Songs 4:14 rejoicing the sexual attraction between two lovers, where one praises the other ‘Your plants are an orchard of every kind of incense tree, with myrrh and aloes and all the finest spices’. Similarly, in Proverbs 7:17, there is an invitation by a seductress to her lover; ‘I have perfumed my bed with myrrh, aloes and cinnamon’. In the New Testament, the spiritual significance of agarwood is clearly outlined in the gospel of John (20-:39-40), where Jesus’ body was anointed with a mixture of myrrh and aloes following his crucifixion. However, there are some scholars that argued that the aloe mentioned in this passage referred to medicinal *Aloe* and not the aromatic agarwood (Crosswhite & Crosswhite, 1984; see Greppin, 1988 for etymologycal discussion and uses of the word to describe two different plants). This may be based on the fact that both myrrh and aloes were also used in the embalming procedures of ancient Egyptians (Crosswhite & Crosswhite, 1984; Gannal, 1840; Grindlay &

Reynolds, 1986). Although there are some inconsistencies in the use of the word aloes, it is widely accepted that term aloes referred to ‘fragrant spice (not a bitter plant) used as a perfume’ (Browning, 2010; Zohary, 1982). Therefore, the aromatic aloes as described in the Old Testament may not be confounded with bitter aloes (Balfour, 1866; Rimmel, 1865; Schoff, 1922), with many authors recognizing it as the East Indian tree agarwood (Balfour, 1866; Greppin, 1988; Harbaugh, 1855; McKenna & Hughes, 2014).

2.3.1.3 Buddhist texts

In several Buddhists texts it is possible to find references to the use of aromatics. For instance, in the Jātaka tales, that are extensive literary works about the Buddha Birth stories (~4th Century BCE) (Pierce, 1969). Agarwood is mentioned in vol VI no 542, the use of fragrance by women in the story that tells of a King’s attempt to enter the world of the gods through ritual sacrifice of his most treasured possessions, including his sons, daughters and wives. When the sacrifice was prepared, the sons were taken to the sacrificial pit, and there the royal ladies and other women of the town adorned ‘with aloes, sandalwood, valuable gems and silk robes’ paid their respects to the son(s) Canda-Suriya, before going off to the king's sacrifice (*The Jātaka or stories of the Buddha's former births*, 1907). This use of agarwood and sandalwood in combination with other valuable items (gems and silk) during a very important occasion demonstrates that they were being used as valuable fragrant products during ancient times. In the *Mahayana Mahaparinirvana Sutra* (Nirvana Sutra Buddhism) the use of aromatics is mentioned in the introductory chapter that describes a succession of events/teachings when Buddha was about to enter Nirvana. In one description, it is mentioned the use of fragrant wood in the cremation of *Tathagata's* (Buddha) body, ‘people each held in their hands tens of thousands of bundles of such fragrant wood as sandalwood, aloes, goirsa sandalwood, and heavenly wood’. Also, aloe was used as fuel in stove to prepare meals for the Buddha and the Sangha (monks). In the *Vimānavatthu* text (‘*Stories of celestial mansions*’), a collection of 85 poems on the happiness of persons reborn in heavenly realms and on the worthy deeds that led to this reward (Khuddaka Nikaya), there are several references of the use of fragrance/ointments. In poem 35 (7), The Seventh: Sesavati’s Mansion (Sesavatīvimāna), during the funeral ceremony of the venerable Captain of Dhamma (Sāriputta), aloe, sandalwood and similar woods were used a part of the pyre of a hundred cubits high (*Vimāna-Vatthu of the Khuddhaka Nikāya Sutta Pitaka*). In the examples mentioned above, it is clear that the use of fragrant wood were part of Buddhist tradition of any kind.

2.3.1.4 Islamic texts

In the Qur'an there are some references to aromatics but none specifically devoted to agarwood. In the Sūrah 55 (Ar-Rahman) the first section describes the abundance of gifts that Allah has bestowed on men including life itself (55:3), speech (55:4), fruits and dates (55:11) and grains, fodder and fragrant plants (55:12). Inclusion of fragrant plants among this list of gifts so fundamental to life, demonstrates the spiritual reverence for these resources. In Sūrah 83:26, where those who refrain from fraudulent temptations will be rewarded in Paradise, which includes access to a range of resources including fragrant musk.

In several Hadith (reports of the words, actions or tacit approval of the prophet Muḥammad) (Lucas, 2008), agarwood is referred as a certain type of Indian incense (i.e. Ūd Al- Hindi or aloes). Allah's Messenger was quoted (by Abu Huraira) describing Paradise where, among many wondrous things, agarwood would be used in their censers (Sahih al-Bukhari, 3327). The use of a certain type of Indian incense (i.e., Ūd Al-Hindi) as a medical treatment was recommended by the Prophet Muḥammad, as containing seven types of remedies, including one for a swollen uvula and pleurisy (Sahih al-Bukhari, 5692, 5693; Sunan Abi Dawud, 3877). Agarwood was also cited as relieving very common ailments, where the prophet said concerning a Muhrim whose head or eyes hurt: 'Let him smudge them with aloes'(Sunan an-Nasa'i, 2711). Agarwood was used in the important practice of fumigation/purification where Nafi' reports on Ibn Umar fumigating with aloeswood either by itself or mixed with camphor (Sahih Muslim, 2254).

The Prophet Muḥammad counsels men and women in the use of fragrance; he advised that men should bath and perfume themselves for the Friday sermon at the mosque, and women may use perfume at their homes but not at the mosque, and both men and women could perfume themselves during sexual intimacy (Thurkill, 2009). It is known that the Prophet preferred the scent of aloes or aloes and camphor (Sahih Muslim, 2254). The burning of incense in a mosque was practiced by Umar the second caliph (around this time became a common practice). There is no record of what was burned, but it is believed that was aloes or aloes and camphor (the preferred aromatics of the Prophet) (Ergin, 2014). In the book of *Friday Prayer,* the use of perfume by men is recommended if it is available.

2.3.2 Agarwood in the Middle East

Agarwood was one of the revered aromatic and is mentioned in most of the ancient documents regarding medicine and perfume usage and other Arabic literature (Zohar & Lev, 2013). For example in al-Kindī's (801-807 CE) *Book of the Chemistry of Perfume*, five recipes for perfume are written specifically for agarwood. In Ibn Māsawahī's (777–857 CE) book *On Simple Aromatic Substances* agarwood was categorized in the groups of principals perfumes (most of them imported from other regions) (cited in Zohar & Lev, 2013). Two compound perfumes named *nadd* and *ghāliya*, regarded as expensive and exclusive, contained musk, aloes and amber. In a collection of stories written during the Abbasid Caliphate known as *Arabian Nights*, many exotic commodities were recorded, including baskets of aloes and many kinds of perfumes (*The arabian nights. English selection*).

Many Arab tribes were involved in trading activities before the pre-Islamic era, with commercial connections with people from north Africa, west coast India, southeast Asia and China (Hourani, 1951; Morris, 1984). For example, the Nabateans, a nomadic Arab tribe were a link between 'Arabia Felix' (Yemen) and the Mediterranean coast and controlled the trade routes of incense, perfume and spices between c 25 BCE and c 4th or 5th century CE (Groom, 1981; Zohar, 2003). During the Sasanian (Neo-Persian) Empire (224-651CE) vibrant overland and ocean trade routes existed with India and southeast Asia, with the latter becoming increasingly active in trading spices, drugs, and aromatics. A commercial network emerged in the Java Sea, during the 2nd & 3rd Centuries CE, based on exchange of aromatics such as agarwood and sandalwood and spices including cloves (King, 2015). An early account of Arab visits to the Indian coastline are found in the book *Akhbār al-Ṣīn w'al-Hind* written by Sulayman Tajir (851 CE) where he describes *Sarandip*, an island on the Bay of Bengal, and its main products gold, rubi, aloeswood and the sea full of pearls and conch (cited in Islam, 2010). Arabs and Indians were prominent traders in the Indian Ocean between the 7th and 11th Centuries CE, which is confirmed by archaeological evidence (Flecker, 2001; Stargardt, 2014). There are records of an Ibādite merchant (Abu-'Ubay-dah) of Umāni origin who went to China during the middle of eighth century and purchased agarwood (aloes) (Hourani, 1951). Much trade between Arab and Chinese traders during this time also occurred in the Strait of Malacca particularly for luxury goods including pearls, ivory, coral, glass, fabrics and a plethora of fragrant products (agarwood, ambergris, benzoin, frankincense, myrrh, costus, dragon's blood and rosewater) and spices (cloves, nutmeg, asafoetida) (Chaudhuri, 1985). Records from the thirteenth century pointed that trade of aromatics were coming from Muslim people established in Indonesia (known at that time as Island of the Southeast Asia). In the account of Ibn Baṭṭūṭa's (1304-1377) travel to Southeast Asia around 1345, he referred to the region as the source of the incense benzoin and aromatic aloeswood (Feener & Laffan, 2005).

2.3.3 Agarwood in India

As with many aromatics, the use and trade of agarwood has had a rich history in India and has been considered one the most valuable, mentioned in poetry, trade, educational and medical manuscripts. The *Arthaśāstra* (c. 320 BC) for instance, was a treatise of economic policy, governance, military affairs and international relations of the Mauryan Empire (Liebig, 2014) and provided details of important natural and traded products (Zumbroich 2012). In this text aromatics featured as one of the 'precious articles to be received in the treasury', with aloeswood and sandalwood being described as the finest aromatics (cited in Zumbroich, 2012). In the texts of Kāmasūtra (3rd century CE), Nāgarasarvasva and others treatise dealing with pleasure (end of the First millennium CE), the use of perfumes and unguents (among others aesthetics practice) were essential for the sensual experience (Ali, 2011). In the *Kāmasūtra Gandhayukti*, the technique of making perfumes, it was one of the 64 arts to be learned by men and women (Penchala *et al.*, 2008).

Aromatics, including agarwood, were the ingredients widely used in the making of fragrances (McHugh, 2011; Penchala *et al.*, 2008). Evidence of the importance of aromatics in Tamil India is presented in the poetic work of *Cilappatikāram* of the time (c 5th CE), where it mentioned that scent of sandalwood, aloeswood, musk and civet emanating from the city of Madurai (capital of the Pandya kingdom) could be detected by travellers from afar (Pathasarathy, 1994). The *Harshacharita* is a fictionally embellished biography of Indian emperor Harsha written by the Sanskrit writer Banabhatta. In Chapter VII an offering to a king was made and among a plethora of valuable gifts included 'volumes of fine writing with leaves made from aloe bark, thick bamboo tubes containing mango sap and black aloes oil, and bundles contained in sacks of woven silk and consisting of black aloe dark as pounded collyrium' (Banabhatta, 1987). While it is not unusual to find agarwood included in long lists of valuable items as offerings to important people, the use of the bark of agarwood for writing demonstrates an alternative non-fragrant use for this tree. The *Gandhasara* (500 and 1000CE), suggests that aromatics lead to the attainment of the three aims of human life, namely 'religious merit, worldly prosperity and sensual enjoyment' (Rhind, 2014).

India has been of commercial interest to many civilizations since early times. Repeated visits to India by Greek traders were reported following the original voyages of Herodotus (c. 485-425 BCE). During Roman times when Alexandria was central to regional commerce, commodities from

India, Arabia, Egypt and Ethiopia converged (Banerjee, 1921). The products traded from India at this time were varied and included silk, precious stones, spices and aromatics including aloes (Prasad, 1977). Merchant ships, using the Indian monsoon current to ply the coast of India, facilitated the trade of luxury products and an increase in the importation and consumption of eastern goods in Rome (Warmington, 1974). In 65 CE the Roman Emperor Nero expended large amounts of oriental spices (probably cinnamon and cassia) at the funeral of his second wife Poppaea Sabina. Benjamin of Tudela, who travelled to India in the 12th century CE, mentioned India, as an exporter of silk cloth, cinnamon, ginger and others sort of spices found in abundance in South India (Samaddar, 1911). Sandalwood, agarwood, musk, camphor, and saffron persist as central ingredients in Indian perfumery from the 9th to 11th eleventh centuries CE (McHugh, 2012) In later times, during the turn between the 18th and 19th centuries, the extraction of agarwood was a customary activity in various part of Assam, with much of it being from Calcutta and then exported to Turkey, Arabia, Persia and Europe (CSIR, 1953).

2.3.4 Agarwood in China

The development of trade routes linking the Mediterranean to East Asia in the early centuries CE marked the start of long period of trade between north India and north China. Luxury goods including coral, pearls, precious stones, glassware, incense and perfume from India were traded along these routes for Chinese silk (Liu, 1994). The first use of incense in China has been placed around the 1st century CE, and considered a direct influence from Indian culture (Buddhism) (King, 2007). The burning of incense has been part of both spiritual and secular life in China. Records of incense/aromatic use can be found in the oldest known official pharmacopeia (*hsin hsiu ppen ts'ao c* 659 CE), which contains a list of prominent components (aloeswood, frankincense, cloves, patchouli, elemi, and liquidambar). Aromatics were also discussed in *Ch'ing i lu* (translated as 'records of unworldly and strange things') compiled by T'ao Ku. The 'incense seal', a sophisticated form of incense developed during the 8th century CE, burned during Tantric Buddhist rituals, is believed to be crafted mainly with agarwood (Bedini, 1994). Agarwood is also one of the twenty-four ingredients required in a recipe for incense seal for use in Buddhist worship (Kao Lie, Tsun sheng pa chien, c 1570 quoted in Bedini, 1994). The incense seal also served as time measurement in both China and Japan, where incense tablets or sticks were marked with the time divisions at certain intervals and put in a container or tray where the seal-character (shape of the incense trail when burned) would form. The burning of the tablet or stick would indicate the passage of the time (Bedini, 1963). The prominence of agarwood in these important spiritual and time-keeping products demonstrates the reverence for it in China. In the third Century CE, aromatics from Southeast Asia were sophisticated luxuries for the Chinese ruling elite. The governor of Jïngzhou in the late third century was reported to have finely powdered agarwood as fine as dust scattered over an ivory bed. His favoured serving girls were requested to step on it, with rewards (pearls) for those who left no footprint and punishments (ordered to lose weight) for those who disturbed the powder (Gungwu, 1958). By the 4th century CE aloeswood and cardamom were imported from Vietnam and Cambodia to China (Keay, 2006).

Schafer (1963) provides a very detailed account of exotic products imported into China during the three centuries of the T'ang Dynasty (A.D. 618-907). These include many plant products, animals and minerals, as well as spiritual and manufactured goods. Over this period aromatics were sourced from a large number of native plants as well as imported products, although the latter were always more valuable and coveted (Schafer, 1963). Schafer describes that ‘women and men of the upper classes lived in clouds of incense and mists of perfume’. The emperor Hsϋan Tsung (847 CE), promulgated a decree, which encompassed a range of provisions including that he will only examine memorials and petitions, after he had washed his hands and burned incense. Schafer (1963) also described how a Chinese prince of the 8th century CE would speak to guests only after he had aloeswood and musk in his mouth. Extravagance is found in Han His-tsai practice, he burned incenses in his garden (full of flowers) to produce an exquisite blend of aroma, a different incense for a different flower, for example, aloeswood with bramble, musk with magnolia, and sandal with michelia. A ‘gallery’ build from agarwood by one of Hsuan Tsung’s minister’s containing sandalwood railings and walls rendered with the fragrance of musk and frankincense was used as an extravagant display of wealth and power.

In the official pharmacopeia of T’ang agarwood, frankincense, cloves, patchouli, elemi and liquidambar were the six essences most used by blenders of aromatics (Schafer, 1963). Aromatics were a recurrent subject in literary works (Bedini, 1994). For instance, in poetry the phrase ‘hundred-blend aromatics’ is commonly used, which is a paste formed with several ingredients, including resins (i.e. agarwood, sandalwood). Agarwood is also reported as been used to perfume buildings, and to scent the garments of particular courtesans (Schafer, 1963). Agarwood also had a prominent status in T’ang period Chinese medicine, prepared as an ointment for external applications and purportedly as a decoction in wine to treat many different internal ailments, to expel evil spirits, and to cleanse the soul. Agarwood similarly had a spiritual dimension in China, and of the many tributes provided to emperors of the T’ang, one includes a detailed description of a ‘mountain of the myriad of Buddha’, which was a ten foot high carving of agarwood and adorned with jewels comprised a verdant mountain scene that included a setting of buildings and images of all the Buddhas (Schafer, 1963). The religious significance of agarwood was further demonstrated when in 871 Emperor I Tsung granted high seats to the monks of An-Kuo Temple that were 20 feet tall, framed in sandalwood and agarwood and used by lecturers on the sutras (Schafer, 1963).

Vibrant international trade occurred during the last four centuries of the first millennium CE, due to the concurrent emergence of a unified China under the Sui dynasty and the peaceful expansion under the Tang Dynasty (618-907) and the rise of the Islamic dynasties of Umayyad of Damascus (661-750), and Abbasids (749- 1258) (Villiers, 2001). This combined with advances in shipbuilding and navigation in both civilisations opened up ocean trade between China, India and Arab centres in the middle east (Kearney, 2004; Villiers, 2001). Persian and Arabs sailed to Canton where they traded a wide range of aromatic goods for porcelain, silk and copper coins, and T'ang goods spread as far west as Constantinople (Kearney, 2004). In 916 Abü Zaid, wrote that the centre of the trade in agarwood, sandalwood and camphor was Kaläh, (believed to be either on the west coast of the Malay peninsula or Tenasserim Coast) which was a protectorate of the Sri Vijaya in Sumatra (Villiers, 2001). By the late 11th Century CE, large volumes of agarwood and sandalwood were traded into China by southeast Asian merchants such as the 2950 kg (4,890 catty) of aloeswood recorded from Jiaozhi (northern Vietnam) in 1063 (Villiers, 2001). Stargardt (2014) reports that Chinese merchants were still taking advantage of the lucrative trade in agarwood in Southeast Asia during the mid- to late 12th Century CE and were exploiting agarwood sources in the Kra Ecotone of South Thailand, which was proximal to the port in Satingpra. Satingpra settlement is believed provided a wide range of incense (fragrant) plants and among them, four of the high-grade quality incenses that fetched great prices and were highly consumed in Chinese market (Stargardt, 2001). In the Quanzhou cargo (a ship discovered and studied by archaeologists), the most precious content was incense wood, found in 12 of the 13 compartments and weight 2400 kg. Other exotic goods were inventoried, including small quantities of (Arab?) frankincense (6.3 g), ambergris from Somalia, and dragon's blood (Stargardt, 2001). Yoshnobu (1983) confirmed that in Sung times, Chinese were eager to obtain spices and incense from abroad, and aloeswood was the preferred among incense woods.

Chinese trade presence in Southeast Asia and Indian Ocean was also prominent during the Ming Dynasty of the 1400s. At this time, China had a significant navy and a large number of private merchant ships trading with Korea, Japan, Southeast Asia, India and east Africa. For example, the Chinese imported several plant products for medical purposes from Sumatra, such as, pepper, ginger, medicinal herbs, camphor, frankincense and sulphur (Kearney, 2004). From Jihha and Dhafur the Chinese purchased frankincense, myrrh, aloe and medicines (Kearney, 2004). Contact with Western merchants is reported through the history of China, commencing during the T'ang dynasty, where Greeks, Arab, Indians, Persians, Japanese frequented the empire, and continuing to much later the beginning of direct trade with Europeans (16th century and beyond) (Fitzgerald, 1961).

2.3.5 Agarwood in Japan

The use of aromatics in Japan came during the sixth century CE, and like in China, with the arrival of Buddhism. The first written record of the use of fragrant wood is found in the Nihongi: (Chronicles of Japan when Emperor Kimmei (549-571 CE) commissioned the carving of two Buddha from camphor wood that was found floating in the sea by one of his operatives (Atahi). The first Japanese written record of agarwood was also found in the Nihongi when in 595 CE a large billet of agarwood drifted ashore on the island of Awaji. The islanders noticed in wonder that it produced a strong fragrance when used as firewood, and later presented it to Empress Suiko (Aston, 1896). Early mixtures of aromatics burned in Japanese Buddhist ceremony included *jinkoh* (the best incense: agarwood), sandalwood, cloves, cinnamon and camphor. A recurrent story about incense use in Japan refers to *Ranjatai* a gift from the China Court to the Emperor Shômu (724-748), it is assumed this piece of wood came from aloes (Brinkley, 1902). Small bits of the wood were removed occasionally but only in prosperous occasion (Bedini, 1994). During the Nara period (710-794 CE), the burning of incense became a secular activity (Gatten, 1977). Kneaded incense blends from T'ang (618-907 CE) was a favourite in Japan, the exported product from China contained aloeswood, sugar and plum meat (Schafer, 1963). Incense ceremonies (kōdō) began to emerge during the Heian Period (794 to 1185 CE) and were emblematic practices of aristocrats of Heian Period (794 to 1185 CE) and where aromatics were the centre of attention. In this ceremony the participants discriminated and judged different aromatics, including pine and cedar or the precious exotic agarwood, sandalwood, cinnamon, cloves (Morita, 1992; Morris, 1984). During the period 833-850 CE, Japanese aristocrats stopped importing incense from China, and began the manufacture (and blending) of incenses. Prince Kaya formulated the famous *Six Scents* (described in the *Kunshū Ruishō* c. 12th century); all the *Six Scents* are compounded of the same six elements at different ratios: aloes, cloves, seashells, amber, sandalwood and musk. A characteristic seventh ingredient defined the final scent, for example, if frankincense was added the scent created was the *Black* incense (Gatten, 1977). Agarwood gained popularity in the late 16th century CE, and connoisseurs appointed by Shoguns Ahsikaga Yoshimasa defined the fragrances of six recognised types of *jinkoh* fragrances, some elements for the description and possible geographical origin of these six fragrances are still used today (Morita, 1992). The six jinkoh at this time was rare and costly and mainly traded for gift to distinguished individuals (Bedini, 1994). To this day agarwood is held as sacred in the Kareki Shrine in the north-western shore of Awajishima (Whelan, 2014).

2.3.6 Aromatics during European age of exploration

Beginning in the 14th century European nations started to influence the international trade of spices. The Portuguese, followed by the Spanish and then by the end of the 16th century, the Dutch and English traded spices from their source in India and east Asia to lucrative markets in Europe. While much is written about the European trade of spices as tea, cinnamon, cloves, pepper and nutmeg (Freedman, 2008; Turner, 2004), there is very little consideration to European involvement in the trade of incense products like agarwood. Chaudhuri (1978) described that while pepper and calicoes were the bulk of imports of the English East India Company in the 17th century, there were other commodities, among them gum resins (aloes, myrrh, olibanum), listed in the Company's import record. For the VOC (Dutch East India company), the trend was similar to the English company, pepper was the main commodity in the 16th and middle of the 17th century (Glamann, 1958), later in the last decades of the 17th and 18th centuries a shift in commodities imports/preference was evidenced in the VOC records and new products were traded (i.e. coffee, tea, textiles) and other lost their economic significance (i.e. pepper) (Glamann, 1958). In terms of fragrance products, incense blends of the West were different from the East, in the West the ingredients commonly used for incenses were frankincense with myrrh, galbanum and onycha (Schafer, 1963). Later in the 19th century CE Australian and other merchants began to exploit sandalwood in the Pacific to balance their trade deficit with the Chinese associated with consumption of tea (Shineberg, 1967).

2.3.7 Evidence of trade of aromatics and agarwood

Spices and aromatics were important commodities in ancient trade (Hull, 2008) and their trade is considered one the first expression of globalization. Long-distances and intricate trade routes or corridors (i.e. incense/spices routes, Silk Road, Red Sea Route) were travelled to obtain the expensive goods and then reach their markets in the Mediterranean. The Spice Road is one of the most influential trade routes in ancient commerce and an example of the beginning of world trade (Haq, 1968). Products that were high in value and light of weight were possible to trade via the overland route, where a single merchant could bear the load (King, 2007). The mention of aromatics and agarwood in particular, in classical literature (i.e. Greek, Romans, and Arabic writings) and other ancient texts (i.e. *Arthaśhāstra*) establish that the good was known, traded, valued and widely used. In the Justinian's Digest of the Roman law (*The Digest or Pandects of Justinian*, 1932), a list of 54 articles were subject of import duty at Alexandria, in this list spices; including aloes (agarwood) are mentioned. In the *Arthaśhāstra* agarwood and other aromatics were also subject to tax, the duty value at 1/10th or 1/15th of the price of the products. Agarwood (and other fragrant woods) was also of great value for the Chinese people. Evidence of trade of incense wood between China and Southeast Asian settlements between mid-tenth century to 14th century (Song-Yuan Period) are confirmed by archaeological data. High concentration of Chinese ceramics (including high quality ceramics) were found at the Satingpra (South Thailand) sites and it is believed were interchanged for forest products, especially high-value incense plants (Stargardt, 2001).

2.4 Agarwood in contemporary times

2.4.1 Incense

Agarwood remains an important part of spiritual and secular life for several cultures. In Muslim communities, it is used during religious rituals, exclusive social events, to honour special guests, and as a body, garment and house fragrance (Antonopoulou *et al.*, 2010; Jung, 2011). Hansen (2000) explained agarwood use among Yemeni people and found that it is considered a sign of status, wealth and refinement. Hansen (2001) also described that chips of wood were placed among tobacco to sweeten the smoke, and women used it to perfume their clothes, hair and skin by putting small pieces of agarwood in a hand-held charcoal censer (*mabkharah*). Agarwood is also burned to celebrate important events in daily life, for instance in Tunisia, agarwood is burned at the 3rd, 7th and 40th day after the birth of a child (Hansen, 2000). Niebuhr related that Arabs used fragrant wood, possibly aloewood to give a pleasant taste to tobacco: 'A custom peculiar to Arabia, is, that persons of opulence and fashion carry always about them a box filled with odoriferous wood'. They put a bit of this wood into any person's pipe, to whom they wish to express particular respect; and it communicates to the tobacco a fragrant smell and a very agreeable taste (1792:224).

Across many Arabian cultures the incense ritual often signifies purification at the conclusion of a guest visit or other social event (Palgrave, 1865). Agarwood is often used as the final offering before guest departure. Kanafani-Zahar (1983) highlighted the widespread saying in the United Arab Emirates (UAE) 'there is no visit after aloewood' (Ma ba'dil 'ûd gu'ûd). Also in the UAE when guests are hosted in the homes, both perfumes and incense feature prominently at the end of a fuãlah (small meal encompassing food, coffee and aromatics) (Kanafani-Zahar, 1983). Scenting of fabrics (clothing, bedding and prayer articles) with essential oils and incense smoke is also common practice across Arabia. A variety of aromatics are used for this purpose, but agarwood is most frequently used by both men and women. Agarwood essential oil is particularly associated with darker garments since dark colour can stain lighter fabrics. Clean fabrics are scented with incense smoke by placing on a rack, which is positioned above a censer with burning incense. The scent in the clothes can be prolonged by their storage in a basket specific for this purpose (Kanafani-Zahar, 1983). In Japan incenses are burned in 40% of households every day (Moeran, 2009). Incense rituals (kōdō) are still practised among artists, intellectuals and wealthy individuals (Katz, 1996). Incense is also burned by younger generations during their leisure time and when entertaining friends (Moeran, 2007). Incense blends are categorized according to either of two base ingredients agarwood or sandalwood. An incense recipe can have between 20 to 30 ingredients added to the base ingredients (Moeran, 2009).

Kaiser (2006) described the fragrance profile for high-quality incense smoke agarwood sensed in the first 12 minutes after heating at 160 during the first 2 minutes a 'very sweet-balsamic, woody floral and reminiscent of α and β-vetivone and related components' was perceived. Afterwards (2-4 minutes) the smoke became 'woodier, incense-like with a distinctive spiciness, and shades of vanilla and musk', then the scent is 'deep noble woody, incense, amber shade, and after nine to 12 minutes the scent is 'deep woody with phenolic note of castoreoum, sweet vanilla note'.

2.4.2 Perfume

The characteristic aroma of agarwood is highly complex and based on the accord of numerous constituents. Naef (2011) identified a total of 150 compounds in the resin, mainly sesquiterpenes, chromones, agarol, agarofuranes, agaraspirol and many other volatile aromatics. Aromatics can be classified as categories of odours that reflect the olfactory profile of the fragrance such as spicy, rosy, smoky, earthy, etc. (see Rhind, 2014 and reference therein for complete list). The main categories of fragrance are described as woody, coniferous, resinous, balsamic, spicy, herbaceous, green, agrestic, floral, citrus and fruit (Rhind, 2014). Agarwood and sandalwood are examples of plant species that belong to the combined fragrance group known as *woody, coniferous resinous and balsamic* scent. In China, agarwood is one of the five most important ingredients in Chinese perfumery, incenses and other goods (Yunjun, 2013). In Taiwan, agarwood is one of the components for the manufacture of incense, ranging from the very high quality to daily use incense (Wuchen) product made of low-grade agarwood (Chang *et al.*, 2007).

The ubiquitous use of perfumes in Arab cultures and the reverence for agarwood is highlighted by Kanafani-Zahar (1983), who outlines specific use in the UAE. Aromatics can be categorised as scented oils used alone or in mixtures for scenting the body or hair, or perfumed incense for scenting the body and clothes. It is considered necessary to use perfumes only to a body after it has been cleansed, and they are most regularly used by women (Gibb, 1939). The most common areas for oil application are hair, in and behind the ears, neck and nape, armpits and nostrils. Some oils are associated with specific areas and agarwood can be applied to all these areas excluding the armpits. Agarwood also is a trademark in Arab perfumery, which has developed fine scents named *Mukhallats* (Marian, 2011). These Arabian fragrances are marketed with an emphasis on the provenance of the raw materials, and use a combination of essential oils (i.e. oud or agarwood) and other natural plant-derived ingredients. For example, *Hutan Kalimantan*, (agaraura.com), is a *Mukhallat* that feature the 'woodiest oud oil (agarwood) from East Kalimantan softened with two varieties of oakmoss and deepened with Indonesian vetiver'. In *Princess Hamida Blend* (arabianoud.co.uk), the components of this exclusive *Mukhallat* includes in the top notes oud oil, in the heart notes, mandarin, ylang-ylang, and as base notes, taif rose and frankincense.

2.4.3 Therapeutic use

In India the consumption of agarwood smoke has been attributed to a warming and calming effect promoting a deep meditational state. Also the heartwood is used as a cardiac tonic, carminative and refrigerant effect (Burfield & Kirkham, 2005). Powdered agarwood is purported to have 'gentle' antiseptic properties for treatment of ear and eye infections as well as on open wounds (Burfield & Kirkham, 2005). In traditional Chinese medicine agarwood properties are related to relieve pain, asthma, nausea, an vomiting, while also promoting qi energy (Huang *et al.*, 2013).

The medical properties ascribed to agarwood products are detailed in Table 2.1. In the UAE Kanafani-Zahar (1983) reports that agarwood oil can be used when a person is sick, where it is described as being 'simultaneously heavy and light' and is considered to relieve headaches, nausea, and faintness by opening the pores, and clearing sinuses to 'release excess moisture or air'. Additionally, agarwood oil as well as a mixture of saffron and water can be used on the nose of newly circumcised male. Other use reported, is the marketing of agarwood tea leaves as a healthy product with multiple benefits.

Table 2.1 Therapeutic properties of different agarwood products.

Species	Therapeutic properties	Constituent	Reference
Aquilaria malaccensis	Central nervous system	jinkoh-eremol and agarospirol	Okugawa *et al.* (1996)
Aquilaria malaccensis	Central nervous system	jinkoh-eremol, agarospirol,	Okugawa *et al.* (2000)
Aquilaria	Antidepressant	Diterpenoids	L. Yang *et al.*

Species	Therapeutic properties	Constituent	Reference
sinensis			(2013)
Aquilaria sinensis	Acetylcholinesterase inhibition	2-(2-phenylethyl) chromone derivatives, qinanones A-E (1-5)	D.-L. Yang *et al.* (2013)
Aquilaria sinensis	Ainhibition against α-glucosidase, inhibitory activity against AChE, cytotoxicity against human gastric cell line (SGC-7901) among three types of tested human cancer cell lines (BEL-7402, K562, and SGC-7901)	2-(2-phenylethyl) chromone derivatives	Liao *et al.* (2016), Dahham *et al.* (2015)
Aquilaria sinensis	Anti-inflammatory activity	Sesquiterpenoids	Zhao *et al.* (2016)
Aquilaria sinensis	Anti-microbial activity	Essential oil	Chen *et al.* (2011)
Aquilaria sinensis & *A. crassna*	Laxative properties	Ethanol/ water extract of agarwood leaves (polyphenols)	Ito *et al.* (2012), Hara *et al.* (2008)
Aquilaria sinensis & *A. crassna*	Anti-nociceptive, anti-inflammatory, anti-oxidative activities	Ethanol extract of agarwood leaves	Zhou *et al.* (2008), Sattayasai *et al.* (2012)
Aquilaria malaccensis	Anti-allergic activity	Phorbol ester from seeds	Korinek *et al.* (2016)
Aquilaria sinensis	α-glucosidase inhibitors (diabetes treatment)	Ethyl acetate (EtOAc) soluble fraction of the 70% aqueous ethanol extract from the leaves of *A. sinensis*	Feng *et al.* (2011)

2.4.4 Trade and supply chains

At least 18 countries are involved in the harvesting of agarwood trees and 46 countries are involved in the global trade and consumption of agarwood products (UNEP-WCMC, 2015). The legal international trade of agarwood is authorized through a series of permits that is managed by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and national regulatory bodies (laws and procedures). Agarwood is traded in a diverse range of products from raw produce (i.e. leaves, seeds, specimens), to resin extract and oil (Appendix 1). There is a lack of a common terminology and measurement units among growers/harvesters, traders, consumers and authorities, leading to difficulties in monitoring harvesting and trade (CITES, 2007).

The four largest consumer markets for agarwood products include the UAE, Saudi Arabia, Taiwan and Japan (TRAFFIC, 2010). The trade of agarwood is complex and involves many intermediaries and countries in the process. During the period 2005-2013, the UAE imported agarwood from at least 25 countries. Indonesia, Malaysia, Singapore (re-exporter country), Thailand and Vietnam have been the regular country-suppliers of the UAE market for the period 2005-2013. In the UAE, 39 companies (as of November 2007) are registered as agarwood traders with the CITES management authorities, with the majority operating in Dubai (Antonopoulou *et al.*, 2010). Much of the trade conducted by unregistered individuals is not recorded in official statistics and may represent a significant proportion of the entire trade. There are no clear divisions in the supply chain of agarwood, with vertical integration in many companies allowing them to simultaneously operate as importer, wholesaler, exporter and retailer (loc. cit. 2010).

Taiwan is no member of the CITES-convention, however all trade involving agarwood products implement CITES regulation through its national laws and authorities. Two types of trade channels can be defined in Taiwan market, businesses that trade agarwood for medicinal purposes and businesses that supply agarwood for non-medicinal purposes. Most of the imported agarwood in the period 2005-2013, came from Indonesia, Singapore, Malaysia, Vietnam and Thailand (UNEP-WCMC, 2015). There are 35 importers and three exporters (as of 2005) registered at the national authorities of Custom. Importers negotiate the acquisition of raw material directly with the countries of origin or Singapore. As in UAE, the supply chain of agarwood has a vertical structure; allowing a company to manage and integrate one or more of the processes across the supply chain. Low-grade agarwood dominates the market in Taiwan, probably as a result of the rareness in the offer of high-quality grade agarwood or perhaps reflects a change in the consumption market (TRAFFIC, 2005). In Japan, the trade of agarwood products is regulated by the Ministry of Economy, Trade and

Industry, who acts as CITES Management Authority of Japan. During the period 2005-2013 at least 14 countries figured as supplier countries, with Hong Kong, Singapore, Indonesia, Vietnam and Taiwan being the regular suppliers. It is believed that only a few importers operate in Japan (less than 10 companies) that would supply most of the businesses dealing with agarwood products (Compton & Ishihara, 2004).

2.4.5 Production

Southeast Asian countries are the main providers of agarwood, with Indonesia, Singapore (re-exporter country), Malaysia, Thailand and Vietnam representing up to 90.4% of the volume exported. In Indonesia, middle traders and exporters must be licensed and registered at the Directorate of Forest Protection office and CITES. At present 45 companies are certified as exporters and work with the agarwood association and local authorities of the Directorate of Forest Protection (Indonesian CITES Scientific Authority & Indonesian CITES Management Authority). Middle traders often hire third parties as local collectors and authorities do not typically monitor these collectors (Indonesian CITES Scientific Authority & Indonesian CITES Management Authority). Chips and powder are the two products that dominate agarwood exports, accounting for 90% of the volume traded between 2005 and 2013 (Figure 2.1) (UNEP-WCMC, 2015). This is because manufacturers prefer to process the raw material, and avoid mixing agarwood of different qualities (Moeran, 2009; Persoon, 2008). Data of exported and imported oil are insignificant (represent less the 1% of the trade for both indices); however, 38% of the volume exported comes from UAE (UNEP-WCMC, 2015).

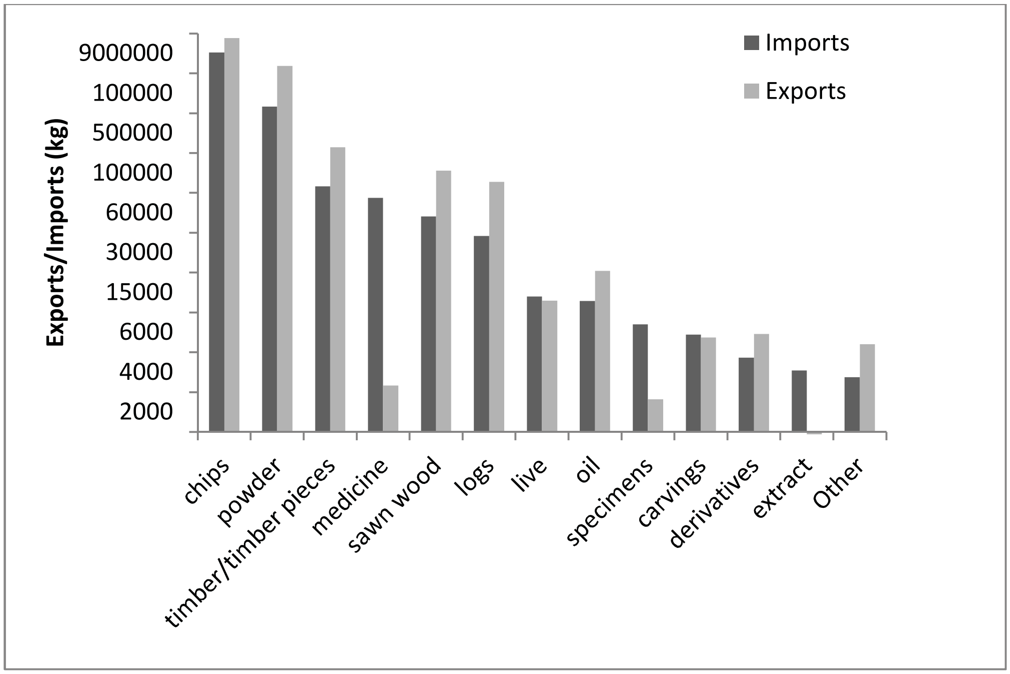

Figure 2.1 Global Exports and Imports of agarwood (in kg) between 2005 and 2013. Others include: dried plants, stems, roots, leaves and seeds. Source: Prepared by author using data from UNEP-WCMC (2015).

2.4.6 Agarwood harvesting

Agarwood is derived from several species within the genera *Aquilaria*, *Gyrinops*, *Aetoxylon* and *Gonystylus* (Thymelaeaceae), with *Aquilaria* representing the principle source (Gunn *et al.*, 2004; Subasinghe & Hettiarachchi, 2015). The species *A. malaccensis*, *A. crassna*, *A. filaria*, *A. sinensis* provide much of the agarwood on the international market and while methods of harvesting vary between countries, it is invariably an extractive process. Two methods of collection have been described 1) felling the infected tree or 2) by cutting the portion of the stem/trunk that contain the resin but leaving the tree standing for further cuts, ensuring incisions do not touch the core of the tree to preserve structural integrity of the tree (Soehartono & Newton, 2001a; Yamada, 1995). Before harvesting, trees are evaluated to determine the presence and quality of agarwood, a process that involves making cuts in the stem in areas with defects or holes (Donovan & Puri, 2004). Agarwood is graded according to its resin content (density), colour and odour (Bhuiyan *et al.*, 2009; Gunn *et al.*, 2004).

2.4.7 Agarwood sustainability

Agarwood production is beset by illegal harvesting, lack of proper management practices, and high trading prices. In most of the natural range of agarwood, illegal trafficking is a major concern for the sustainability of the industry, compromising not only the livelihood of local communities (which harvest the resin from natural areas), also national incomes and international relationships (Jim, 2015; Teck & Awang, 2010). To promote the sustainability of the trade in agarwood, countries involved in the harvest and trade of agarwood have implemented a series of mechanisms and policies that would help to monitor agarwood activity. For example, member countries of the CITES convention agreed to include all producing-agarwood species (not only *Aquilaria* genus) in Appendix II of CITES (CITES, 2015). Species listed in Appendix II are not considered seriously threatened with extinction, but trade of specimens from the species is controlled to ensure their survival. Other *Aquilaria* species are classified as vulnerable according to the 1994 IUCN Red List Criteria, including *A. banaensis*, *A. beccariana*, *A. microcarpa*, and *A. sinensis*.

In most of its range of occurrence, wild *Aquilaria* is a protected species, and its harvesting is allowed only from private land or plantations (ITTO-CITES, 2015). Malaysia and Indonesia, where agarwood is harvested from natural areas, have implemented a voluntary national export quota based on regular inventories of the available resources (Figure 2.2) (ITTO-CITES, 2015). In Indonesia, the national harvesting quota initiative has been running for the last 20 years and is established by the Directorate of the Forest Protection and Nature Conservation department. This harvesting quota is reviewed every year and permits for extraction are issued by the local authorities of the forest protection and nature conservation offices, and then distributed among the middle traders and exporter members of the agarwood association. Transport permits are also issued in accordance to the annual quota and harvest permits provided (Indonesian CITES Scientific Authority & Indonesian CITES Management Authority).

In Malaysia, domestic laws are the framework to regulate agarwood activity. National authorities (State Forestry Departments in Peninsular Malaysia, Sabah and Sarawak, and the Sarawak Forestry Corporation in Sarawak) issue licences and removal pass for collectors (Chua, 2008). A CITES permit is mandatory to export agarwood products/derivatives issued by the Malaysian Timber Industry Board (MTIB, the CITES Management Authority representative) in Peninsular Malaysia and Sabah and by Sarawak Forestry Corporation in Sarawak. As agarwood is classified as minor forest product, the laws are less rigorous regarding the licence required for harvesting agarwood, leading in some limitations in the regulation (and monitoring) of the activity and recording of legal trade at national level (Teck & Awang, 2010). There have been discrepancies in the quantity reported as exported and what is been approved in the licences (data for 1995-2005), where the figures for exports exceeded in almost a factor of two of what was approved. The reasons behind these differences are related to illegal trade and exports of agarwood without the respective permit (Teck & Awang, 2010).

Other initiatives promoted the establishment of plantation programmes through incentive schemes and registration process (Figure 2.2) (ITTO-CITES, 2015). As have been acknowledged by CITES-countries that agarwood-producing species can be grown under controlled conditions and artificially propagated (Decision 15.95 [Rev. CoP16]). Some of the producer countries have indicated the difficulty in identifying agarwood traded products, which include mostly dry samples, to species level. As a result, discussions have been going on in the need to reinvest in methodologies/technologies such as molecular analysis to assist in the identification of agarwood at species level. If DNA in agarwood products (wood) could be isolated, an easy how-to-guide can be developed to assist traders and governments in the assessment of agarwood products (CITES-PC, 2002).

2.4.8 Agarwood plantation/cultivation

Cultivation of *Aquilaria* trees for the production of agarwood (chips and oil) is now a common practice in producer countries and other regions as an option to alleviate the pressure on the remaining natural stands. For example, in Bangladesh, China, Indonesia, India, Malaysia, Vietnam large areas have been planted with agarwood-producing species. Indonesia has planted more than 3 million trees of *A. malaccensis*, *A. microcarpa*, and *Gyrinops versteegii*; China has reported around 5285 ha mainly of *A. sinensis* between 2006 and 2010 (Yin *et al.*, 2016). In Malaysia ca. 984 ha of *A. malaccensis* have been established over the last two past decades (Figure 2.2). Private investor or entrepreneurs have also established agarwood-producing plantations. In Australia 49,000 trees mainly of *A. crassna* were established in 2009 by Wescorp Agarwood (WA)[1]. It is expected by 2020, when WA has reached its capacity of production, to export around 100 million Australian dollars annually. In Sri-Lanka Asia Plantation Capital (Pte) Ltd. has invested in the establishment of agroforestry systems with *Aquilaria* spp[2], also other private initiatives are investing in planting *Aquilaria* trees (i.e Sadaharita Plantations) (De Zoysa & Inoue, 2014).

Aquilaria plantations are managed similar to other tree-crop plantations. For example, in Bangladesh agar trees (mainly *A. agallocha*) were planted in small blocks on degraded land or denuded hills or around farmers' homestead. The management of the plantation included, site preparation before planting, weed control, mulching, watering in the first year of plantation,

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[1] Wescorp Agarwood, 2016, http://www.wescorp.com.au/agarwood.htm

[2] Asia Plantation Capital PTe Ltd, 2017. https://www.asiaplantationcapital.com/plantations/sri-lanka

pruning, pest control (leaf sucker) and fertilization (organic and conventional). Spacing recommended between plants is of 2 x 2 m to 4 x 4 m in square pattern. In this region, farmers artificially induce the formation of agarwood using iron pegging technique on trees that have 6 or 7 years of age. And after 3-5 years of wounding, trees are ready to be harvested (Rahman *et al.*, 2015). Growth rate reported for diameter and height for *Aquilaria* trees grown in plantation was 1.4 cm year^{-1} and 1 m year^{-1}, respectively (Lok & Zuhaidi, 2011). Similar practices to manage agarwood-producing plantations have been reported in other regions of the natural occurrence of the species (i.e. Malaysia, China) (Lok & Zuhaidi, 2013; Xiao-xia *et al.*, 2010). Methods of inoculation for inducing agarwood formation vary among regions, but techniques that yield high-quality agarwood are preferred (Liu *et al.*, 2013).

In *Aquilaria* plantation of particular importance is to evaluate traits related to growth performance. This is because a minimum diameter is required to induce the formation of high-quality agarwood (Suharti *et al.*, 2011; Turjaman & Hidayat, 2017; Van Thanh *et al.*, 2015). Studies about provenances variation in growth traits of *Aquilaria* spp. have suggested that plant height and collar diameter are characters of interest when selecting trees for breeding purposes (Noor Mohamed *et al.*, 2015). Polyploidy has also been evaluated in *Aquilaria*. Siti Suhaila *et al.* (2015), induced polyploidy in *A*. *malaccensis* to measure how the chemical profile of stems, root and leaves were affected. The results showed that the tetraploid had higher content of sequisterpenes (and other volatiles) in comparison to diploid, especially in stems. These first results indicated that high-quality agarwood can be obtained using tetraploid.

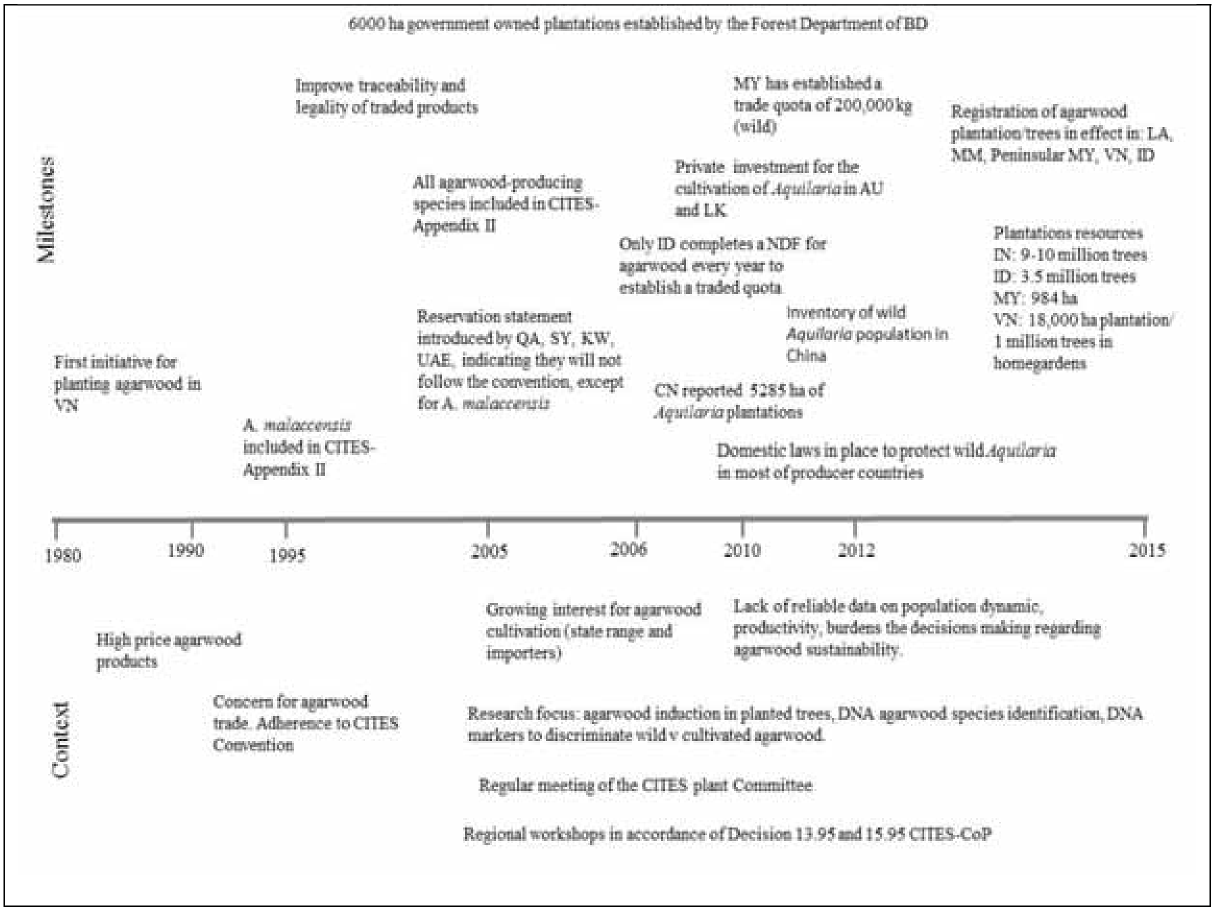

Figure 2.2 Timeline of agarwood actions to improve trade and sustainability of the activity. Source: Author’s compilation from ITTO-CITES (2015), Yin *et al.* (2016). BD: Bangladesh, QA: Qatar, SY: Syrian Arab Republic, KW: Kuwait, UAE: United Arab Emirates, CN: China. AU: Australia, LK: Sri Lanka, Vn: Vietnam, ID: Indonesia, IN: India, LA: Laos, MY: Malaysia, MM: Myanmar, NDF: non-detriment findings. CoP: conference of parties

2.5 Conclusions

Recorded use of agarwood dates back at least c 1400 BCE and throughout its history it has been highly esteemed in both its spiritual and secular uses and synonymous of luxury, exclusivity and intimacy. The use of agarwood together with myrrh to anoint the body of Jesus Christ following crucifixion supports its deeply spiritual and religious connection. The reference of agarwood in the Kāmasūtra supports its unparalleled importance to physical and sensual intimacy. Its long historical use has been associated with cultures where aromatics are deeply ingrained in cultural experience, such as the Middle East, India, China and Japan. Among the many available plant-based aromatics, agarwood has always been considered to be among the most important for its use as the basis for incense and other fragrant products. In current times agarwood products play a prominent place in cultural and folkloric affairs and traditional medicine. The traditional natural sources of agarwood in India and Southeast Asia have been continuously exploited for centuries, and are now becoming rare and threatened. In response, all agarwood producing-species of *Aquilaria* genus have been listed on Appendix II of CITES, for the purpose of preventing the over-exploitation and increasing the likelihood of the survival of natural *Aquilaria* trees. These listings together with respective national conservation policies, laws and strategies have been implemented as a framework to improve the sustainability of the agarwood trade. While these initiatives are encouraging, illegal trafficking of agarwood products remains an issue for maintaining positive relationships between countries, and sustainable agarwood harvest and trade.

Chapter 3. Morphological diversity of *Aquilaria* species (Thymelaeaceae) using elliptical Fourier descriptors of leaf outlines

3.1 Abstract

Leaf shape analysis of three *Aquilaria* species was undertaken using Elliptical Fourier descriptors (EFD) within a research planting in tropical Australia. This approach was taken to develop a method for discriminating the species in the absence of reproductive traits or genetic analysis and as a morphological scale of leaf variation. Leaf outlines were analysed to distinguish variability between species. Principal components analysis and canonical discriminant analysis were applied to group the species based on 25 Fourier descriptors. Four components were required to explain 78% of the variance and the first and second canonical functions discriminated three groups of species therefore EFD was a useful approach for analysing leaf outline variability and distinguish species in the genus *Aquilaria*. This approach provides a rapid method that could assist in the species identification in *Aquilaria* where reproductive traits are absent.

3.2 Introduction

Aquilaria species occur naturally in eastern India, south China, Southeast Asia and Papua New Guinea with 20 known species (Ding Hou, 1965; Rae, 1991; The Plant List, 2013). *Aquilaria* species are trees or shrubs and can be found in areas ranging from sea level up to 1000 m above sea level and in different soil conditions. *Aquilaria* species produce a fragrant resin with high market demand in Arab countries, China, Japan, Taiwan and Singapore. Historically this demand has been satisfied through the unfettered harvesting of the species' natural populations. As such many species in the genus have been listed as threatened or endangered (IUCN, 2013). Reforestation with *Aquilaria* is now being undertaken in producer countries and other tropical areas to meet increasing demand and alleviate harvesting pressures within natural populations. These programmes have relied on different germplasm sources to establish the plantations and plantings may comprise multiple species. Accurate identification of established trees is important to help determine species composition in planted populations. This knowledge can then be incorporated into the management of the plantations based on the biological tolerances and market preferences for certain species.

Leaves, flowers and seeds can be evaluated to identify and classify cultivars or distinguish morphological variations between species (Bruschi *et al.*, 2003; Campos *et al.*, 2005; Jones *et al.*, 2013). In a plantation, identification of juvenile specimens will depend upon vegetative characters and development of simple morphological tools. Elliptical Fourier analysis (EFA) is commonly used in plants to study the shape of different organs (Iwata *et al.*, 1998; McLellan & Endler, 1998; Williams *et al.*, 2012; Yoshioka *et al.*, 2004). EFA is a mathematical interpretation of outlines which are decomposed into a Fourier series of sine and cosine curves (White *et al.*, 1988). EFA involves the orthogonal decomposition of a shape into a series of additive harmonic ellipses, with each Fourier harmonic referred to a different aspect of outline shape of the object of interest (Ferson *et al.*, 1985; Kuhl & Giardina, 1982). The Fourier coefficients, two per harmonic, describe the size (amplitude) and angular offset associated to the starting point (Haines & Crampton, 2000). These series of Fourier harmonics, called elliptical Fourier descriptors, are then typically subjected to morphometric statistical analysis to reduce dimensionality and discriminate groups based on their shape. Elliptical Fourier descriptors have been shown to discriminate among species and geographical location based on leaf shapes. EFA is useful when a specimen lacks homologous points (landmarks) such as some plant leaves. EFA of leaf shape has aided the identification of the three sympatric white oak species (*Quercus frainetto* Ten., Q. *petraea* [Matt.] Liebl. and Q. *pubescens* Willd.) in Italy (Viscosi & Fortini, 2011), description of taxonomic groups within *Monstera* spp. (Araceae) (Andrade *et al.*, 2008) and identification of four species of southern African *Strychnos* L. section *Densiflorae* Duvign. (Adebowale *et al.*, 2012).

Apart the general description of leaf morphology presented in taxonomic reviews, there is no previous research of leaf shape in *Aquilaria* genus. *Aquilaria* species identification relies mostly on reproductive characters associated with flowers, fruits and seeds (Kiet *et al.*, 2005; Peterson, 1997; Rae, 1991) (See Methodology for botanical description of the species). For example, the keys to *Aquilaria* species growing in Vietnam developed by Kiet *et al.* (2005) are based on fruiting or flowering material. While reproductive characters are useful discriminating traits, they are not available in juvenile trees and in mature specimens their availability is seasonally dependent. In contrast leaves are easy to collect and always available in healthy trees, so defining leaf shape characters that discriminate among species can aid identification in the absence of reproductive traits.

In this study, EFA technique was used to describe variation in leaf outlines for three putative *Aquilaria* species growing within a research plantation. This morphometric technique was used because leaves in *Aquilaria* species generally have a lack of homologous and consistent landmarks; only two landmarks could be drawn: petiole-blade junction and blade length. This issue limited the use of morphometrics based on landmark configuration (Viscosi & Cardini, 2011; Zelditch *et al.*, 2004). The aim of this study was to quantify differences in leaf shape between three putative species to determine if it can be used to discriminate among them. The germplasm used to grow *Aquilaria* came from different localities of Southeast Asia.

3.3 Methodology

Three species of the genus *Aquilaria* growing in a research plantation close to Innisfail, Queensland, Australia (17.52°S, 145.02°E) were identified using vegetative and reproductive characters. Botanical description of the three *Aquilaria* species is provided in Table 3.1 based on botanical characters outlined in Kiet *et al.* (2005), Peterson (1997), Rae (1991). In the plantation, *A. baillonii* is defined by its robust trunk and expanded paraboloid crown-shape. Leaves are oval to ovate in shape with mucronate apices and pinnate leaf venation. The stigma is regularly lobed; fruits ovoid (egg shaped) with widest axis at the base. Seeds are dark brown (11.76 mm long) with a long funicle aril dark orange ca. 15 mm. *A. crassna* trunk is straight and canopy is spheroid in shape and dense and often exhibits pronounced branching at lower height along trunk and branches plagiotropic. Leaves are dark green, smaller, elliptical with acuminate apices. Stigma is capitate; fruits are rounded (spherical) with calyx visibly loose. Seeds are dark brown with a long funicle aril brownish. *A. subintegra* has a distinctive tapering trunk with visible buttressing. The canopy is a fattened cone in shape with very light branching. Leaves are oblong in shape. Flowers are smaller than *A. crassna*, *A. baillonii*, with a distinctive sweet-bouquet aroma. Fruits are elliptical. Seeds are dark brown and the long funicle aril dark reddish.

Table 3.1 A comparison of three putative *Aquilaria* species extrapolated from the literature.

Descriptor/Species	*A. crassna*	*A. baillonii*	*A. subintegra*
Habit	Tree 15-30 m tall	Tree 10 m tall or shrub 2-4 m tall	Shrubs or small tree
Bark	Grey or whitish; smooth or rugose	Bark colour/surface ND	Bark colour/surface ND
Leaves	Elliptic to lanceolate; 8-9.5 (12.5) cm by 3.6-6.6 cm Base attenuate to obtuse Apex acuminate Petiole 4-5 mm, lightly hairy	Alternate, ovate or ovate-oblong; 13-15 (-17) cm by 5.3-6.5 cm Base obtuse to rounded; Apex slightly acuminate. Petiole 5-7 mm.	Acuminate, (14) 19-27.5 cm by (5) 7-10.5 cm, Base cuneate to obtuse; Petiole pubescent or glabrous, 5-10 mm long
Inflorescences	Inflorescence axillary, in 1-2 umbels, axis 0.3 -1 cm	Inflorescence supra axillary; axis not more than 1 mm without bract	Inflorescence axillary, 8-20 flowered, 1-3 cm long
Flowers	Flowers yellow; Flowers size ND Pedicel 0.6-1 cm Hypanthium campanulate 3.5-4.5 mm Sepals 5; ovate, spread or reflexed. Petaloid appendages ovate, 1 mm Stamens 10, anthers oblong, 1 mm, glabrous	Flowers colour ND Flowers up to 1 cm at anthesis; -Pedicel 3-4 mm -Hypanthium cylindrical, 7-9 mm; base slightly curved, glabrous -Sepals hairy inside; appendages petaloid 10, short, hairy. -Stamens 10, anthers sessile.	Flowers white, Flowers size ND Pedicel puberulous, 6-13 mm long Calyx tube 5-12 mm long; Calyx lobes 3-5 by 1.2-2.5 mm Petal united at the base, 1-1.5 mm long -Stamens sessile, anthers 1-2 mm long
Ovary	Ovoid, sessile, 2.5-4.5 mm long Style 0.7-1 mm; Stigma capitate, blackish	Obovoid	Sessile, 2-3 mm long; Style 0.5-1 mm; Stigma globose, 1 mm
Fruit	Capsules 4 x 3 cm; velvety, yellowish	Fruit shape and size ND	Elliptic
Seed	Seeds 1 (-2), blackish; shiny	Seeds number and colour ND	Seeds narrowly elliptic; Funicle glabrous attached along one side of the elongated part

Source: (Kiet *et al.*, 2005; Peterson, 1997; Rae, 1991), ND: no data available.

3.3.1 Leaf collection

A total of 320 leaves were sampled from three putative species. For each species 10 to 11 trees were sampled. All trees selected for this study were grown under full sunlight with no supplemental water (water supplied by rainfall only), and were subject to standard agronomic practices (fertilization, weed control, pruning, and pest control). Leaves were harvested from trees of 4.75 years (56.7 months) of age. Tree growth rate (expressed in diameter at 150 mm above ground level) estimated at the site of study was 3.39 cm year^{-1}(± 0.72). On each tree 10 fully expanded mature leaves were harvested from a single branch sun-exposed located north of the lower crown (up to 3m). Only leaves free from insect or mechanical damage were included in the samples. Leaves that were not fully developed were not collected and therefore not included in the sample. Each collected leaf was scanned with the upper (adaxial) surface using an EPSON V370 with a resolution of 300 dpi.

3.3.2 Elliptical Fourier descriptors and statistical analysis

Leaf images were processed with two different image processing and analysis software (SHAPE Hiroyoshi Iwata, http://lbm.ab.a.u-tokyo.ac.jp/~iwata/shape/ and ImageJ 1.49n - Wayne Rasband National Institute of Health, USA). Before images were loaded in ImageJ and SHAPE, they were adjusted with image editing software (Paint.net) to ensure continuous colour along the margin of the leaf. A total of 25 harmonics were used per leaf to estimate the EFDs on both software. Before loading images in SHAPE, files were converted to BITMAP images. Once in SHAPE, images were transformed in binary images (black and white) to estimate a chain-coded contour per object and then calculate normalized EFDs values per leaf. A principal component analysis of the EFDs coefficients was performed using the variance–covariance matrix of the coefficients and the software returned the PCA scores for each object. The variations in shape that can be accounted for by each PCA were obtained and graphed using PrinPrint tool/application from SHAPE. In ImageJ images were converted to a black and white image, and then leaves were delineated using the ROI (region of interest) manager to indicate the objects to be computed. The elliptic Fourier analysis was performed using the plugin Elliptic Fourier Descriptor (EFD) (Boudier & Tuppe, 2009). EFD coefficients were exported to a spreadsheet and averaged per individual to overcome variability within individuals. Averaged EFDs from ImageJ were used to perform a canonical discriminant analysis (lda function contained in R software) to find linear combinations of the original variables that could give best separation between species.

3.4 Results

3.4.1 Leaf outlines

The PCA analysis of the EFDs demonstrated that for *Aquilaria* species the use of Elliptical Fourier analysis helped to differentiate species by based on leaf outlines/shape. Four components were required to explain 78% of the variance (Table 3.2). The combined variation contained within the first and second principal components accounted for 58% of total variation in leaf outline/shape. The effect of shape by each PC is illustrated in Figure 3.1. PC1 describes the widening along the length of the leaf and the length to wide aspect of the leaf and captured the range of leaf shape found in the three species; PC2 is capturing the variation in leaf shape from lanceolate to oblanceolate. PC3 and PC4 explain the variation in the apices and reflect the outline of the leaf. PC5 is related to the general shape of the leaf (Figure 3.1). The canonical analysis indicated that groups of species can be separated using leaf outlines, 73% of separation is achieved with the first canonical function and the remaining 27% is explained by the second canonical function. The first and second discriminant functions plotted in Figure 3.2 show that these two functions help in the differentiation of the three *Aquilaria* species. *A. baillonii* is clearly separated from *A. subintegra* and *A. crassna* by the second discriminant function.

Table 3.2 Importance of the first five principal components explaining the variability of the data.

Principal Components	Eigenvalue	Proportion per PC	Cumulative proportion PC (%)
PC1	3.20E-03	44.60	44.60
PC2	9.53E-04	13.28	57.89
PC3	7.90E-04	11.02	68.91
PC4	6.64E-04	9.26	78.17
PC5	4.13E-04	5.75	83.93

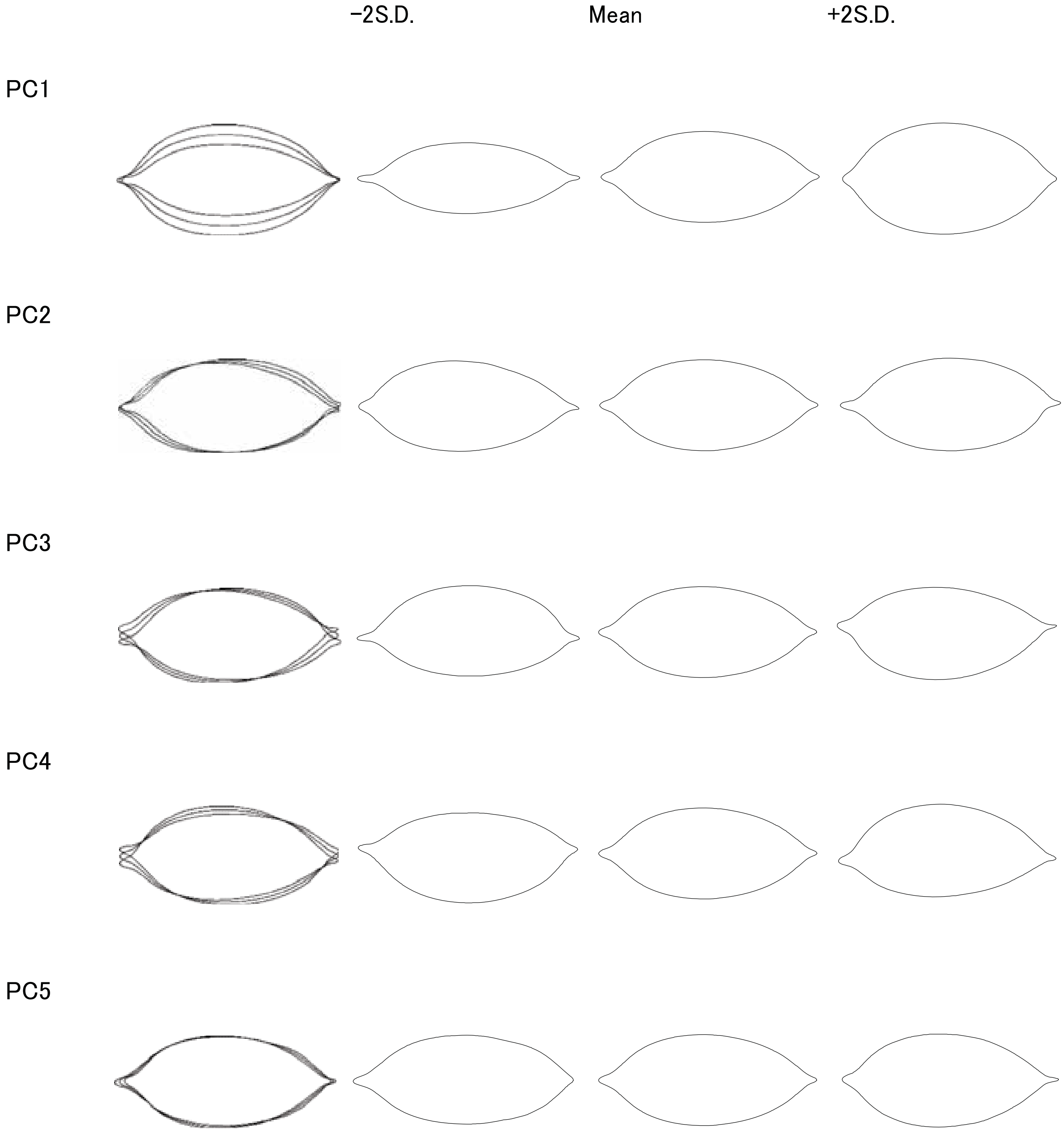

Figure 3.1 Leaf shape variation accounted for by the five first principal components returned from SHAPE.

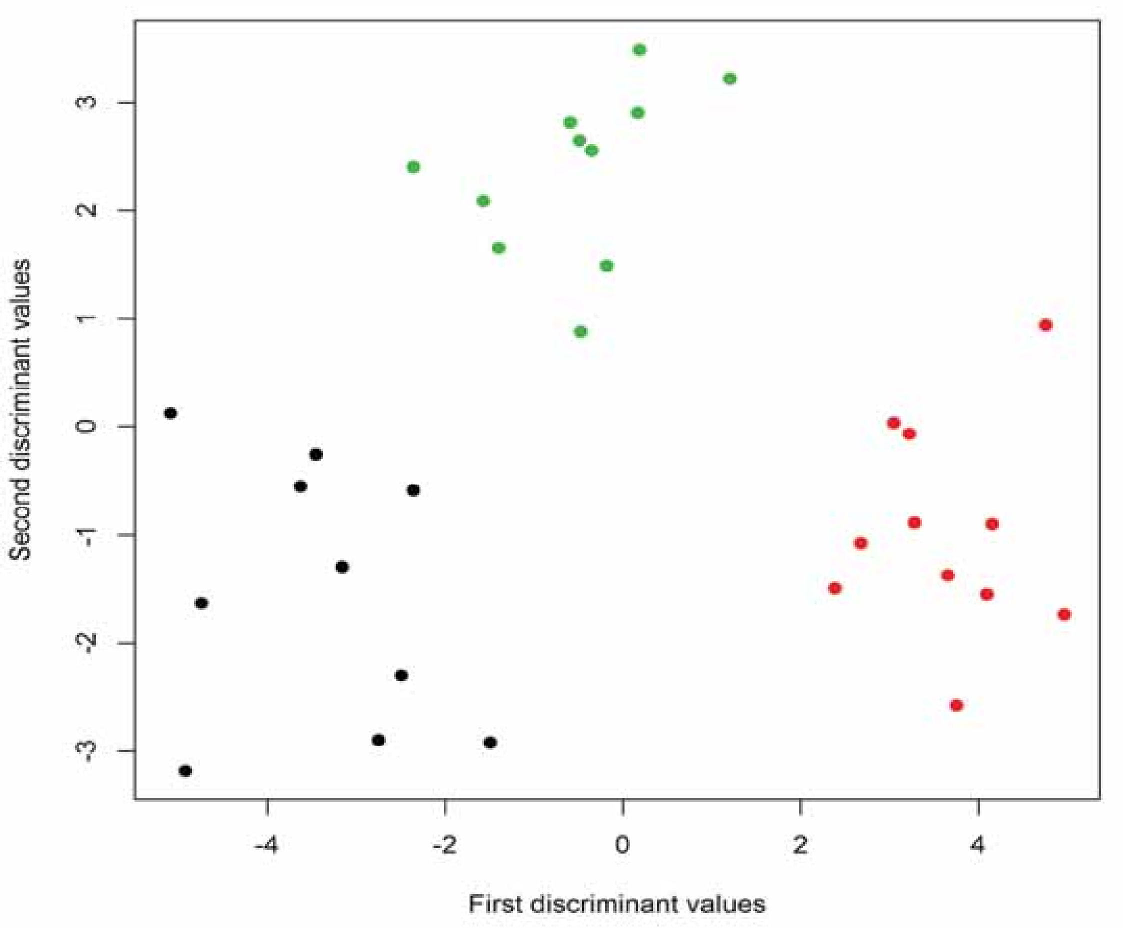

Figure 3.2 Canonical discriminant analysis of the elliptical Fourier descriptors and three *Aquilaria* species (red circle= *A. crassna*, green circle= *A. subintegra*, black circle= *A. baillonii)*.

3.5 Discussion

Morphological studies are a reliable method used for characterisation and generation of information about systematic and ecology (McLellan & Endler, 1998). Several methods have been developed to quantitatively characterize the shape of objects (Rohlf, 1990; Rohlf, 1998). For example, landmark methods (location of points to describe an object) have been used in the study of population biology and biosystematics (Fink & Zelditch, 1995; Rohlf *et al.*, 1996; Viscosi *et al.*, 2009). Another morphological approach is the analysis of the entire outline of an object using Fourier analysis. This method can be applied to simple shapes such as ovals to more complex object such as irregular leaf outlines (Adebowale *et al.*, 2012; McLellan & Endler, 1998; Viscosi & Fortini, 2011). This study demonstrated that mathematical interpretation of leaf outlines using elliptical Fourier descriptors can be used as a tool to differentiate individuals at species level in the genus *Aquilaria* in young plantations. However, the three putative species were first identified based on other characters mainly flower and form characteristics. Validation studies that include a more extensive range of material (leaves) are required to determine whether this approach can be used to differentiate other species of the genus occurring in both natural environments and plantations in the area of natural distribution. The use and selection of a particular morphometric method for analysing leaf shape is important as each available method considers different aspects of the leaf shape leading to biased results (Chitwood & Sinha, 2016). In this study, elliptical Fourier descriptors is a simple technique that can support identification of *Aquilaria* species, when reproductive and other diagnostic characters are absent and studies at molecular level are out of the reach of the breeder.

The analysis of outlines to describe shapes and reconstruct images is advantageous when landmark-based analysis is not suitable to perform. McLellan and Endler (1998) suggest that elliptical Fourier analysis provides accurate reconstruction of the outline of complex object and can explain the overall complexity of the shape. Other studies have reported the use of leaf shape description to group species (Andrade *et al.*, 2008; Jensen *et al.*, 2002; Viscosi & Fortini, 2011). Jensen *et al.* (2002) analysing leaves outline of two *Acer* species and their hybrid were able to distinguish two core groups (species) but it was not possible to differentiate their hybrid. Viscosi and Fortini (2011) found that elliptical Fourier analysis was a useful technique for analysing the leaf shape of three oak species and a valuable tool that could discriminate species in the subgenus *Quercus*.

3.6 Conclusions

Elliptical Fourier descriptors of the leaf outline of *Aquilaria* species provided a rapid method to study shape/outline variability and identify species in the genus *Aquilaria*. The leaf outline methodology is a fast and convenient technique that may support national authorities and growers of *Aquilaria* in the identification of individuals in the genus *Aquilaria*.

Chapter 4. Relationship between leaf functional traits and productivity in *Aquilaria crassna* (Thymelaeaceae) plantations: a tool to aid in the selection of high-yielding trees

4.1 Abstract

Physiological traits are frequently used as an indicator of tree productivity. *Aquilaria* species growing in a research planting were studied to investigate relationships between leaf-productivity traits and tree growth. Twenty-eight trees were selected to measure isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) and monitor six leaf attributes. Trees were sampled randomly within each of four diametric classes (at 150 mm above ground level) ensuring the variability in growth of the whole population was sampled. Model averaging technique based on the Akaike's information criterion was computed to identify whether leaf traits could assist in diameter prediction. Regression analysis was performed to test for relationships between carbon isotope values and diameter and leaf traits. Approximately one new leaf per week was produced by a shoot. The rate of leaf expansion was estimated as 1.45 mm day^{-1}. The range of $\delta^{13}C$ values in leaves of *Aquilaria* species was from -25.5 ‰ to -31 ‰ with an average of -28.4 ‰ (± 1.5 ‰ SD). A moderate negative correlation (R^2 = -0.357) between diameter and $\delta^{13}C$ in leaf dry matter, indicated that individuals with high intercellular CO_2 concentrations (low $\delta^{13}C$) and associated low water-use efficiency sustained rapid growth. Analysis of the 95% confidence of best-ranked regression models indicated that the predictors that could best explain growth in *Aquilaria* species were $\delta^{13}C$, $\delta^{15}N$, petiole length, number of new leaves produced per week and specific leaf area. The model constructed with these variables explained 55% (R^2=0.55) of the variability in stem diameter. This demonstrates that leaf traits can assist in the early selection of high-productivity trees in *Aquilaria* species.

4.2 Introduction

Physiological traits are commonly used as an indicator of crop productivity. The examination of traits with a potential relationship with growth performance can lead to the identification of characters of use for early selection within tree breeding programmes. In forestry where stem diameter increment is desirable, some leaf characteristics have been used to identify highly productive genotypes (Bunn *et al.*, 2004; Marron *et al.*, 2007). Leaf traits with a potential to predict physiological performance and growth include content of carbon and nitrogen, specific leaf area, leaf area, petiole length, leaf density and carbon isotope ratios (Dillen *et al.*, 2009; Marron *et al.*, 2005; Martínez-Garza *et al.*, 2005).

Leaf stable carbon isotope ratios ($\delta^{13}C$) can provide detailed information about physiological processes in plants. Values of carbon isotope ratios represent a relationship between discrimination against $^{13}CO_2$ during gas exchange and the ratio of intercellular and atmospheric CO_2 concentrations (ci/ca) (Dawson *et al.*, 2002; Farquhar *et al.*, 1982). This value can provide a retrospective measure of the long-term environmental and physiological effects that influence CO_2 diffusion and CO_2 consumption (Farquhar *et al.*, 1989; Sparks & Ehleringer, 1997). Variations in leaf $\delta^{13}C$ values have been attributed to environmental conditions and genetically controlled physiological parameters (Cernusak *et al.*, 2013; Dawson *et al.*, 2002). For example, leaf $\delta^{13}C$ content is influenced by irradiance (Martinelli *et al.*, 1998; Waring & Silvester, 1994), soil moisture (Ehleringer & Cooper, 1988), nitrogen availability (Guehl *et al.*, 1995; Livingston *et al.*, 1998), and CO_2 concentrations (Bettarini *et al.*, 1995). Several studies indicated that genotype could influence carbon isotopes values (Flanagan & Johnsen, 1995; Johnsen *et al.*, 1999).

Carbon isotope ratios can be used as an indirect and integrative measure of plant water use efficiency (Farquhar & Richards, 1984), photosynthetic capacity (Virgona & Farquhar, 1996), stomatal conductance, leaf nitrogen content (Sparks & Ehleringer, 1997), and relative growth (Ehleringer, 1993). In a common Sonoran desert shrub, Ehleringer (1993) found that individuals with high leaf carbon isotope discrimination values (corresponding to more negative values of $\delta^{13}C$ in leaf biomass) had greater growth rate when neighbouring competitors were removed. This result is consistent with those reported by Condon *et al.* (1987) and Masle and Farquhar (1988) for wheat seedlings. However, Poorter and Farquhar (1994) when studying carbon isotope discrimination, transpiration and intercellular CO_2 concentration across 24 wild species with different relative growth rate, found no relationship between ^{13}C discrimination and growth parameters, except with the fractional root biomass allocation and specific root length. In *Pinus radiata* the $\delta^{13}C$ in tip needles was found to have a positive linear correlation with nitrogen concentration (Livingston *et al.*, 1998). Fardusi *et al.* (2016) analysed the pattern of the relationship between $\delta^{13}C$ and productivity across 34 non-tropical woody plants, and found a positive global intra-specific relationship between $\delta^{13}C$ and growth, stronger for biomass than height and non-significant for diameter. The results of these studies demonstrate that relationships between $\delta^{13}C$ and growth parameters can vary between species and environments.

Leaf morphological attributes have been demonstrated to be a useful tool for predicting growth. For instance, Dillen *et al.* (2009) found that individual leaf area of the largest leaf along the main stem in a *Populus* population was one of the best growth predictors irrespective of the site and family. Moreover, leaf production rate was strongly correlated with growth in one of the families evaluated.

Possen *et al.* (2014), studying the differences between genotypes of *Betula pendula* using 13 physiological and morphological traits and their relation to growth, established that SLA and the fresh mass to dry mass ratio correlated well with total biomass. These values were consistent across years of evaluation for genotypes of high yield biomass. Marron *et al.* (2005) found that leaf area and SLA were better predictors of productivity than leaf number increment and leaf area increment rate in *Populus deltoides* x *P. nigra* clones. Bunn *et al.* (2004) showed that the most productive genotypes of *Populus* had rapid rates of leaf expansion and slow rates of leaf production.

Aquilaria species produce a highly prized, fragrant resin known as agarwood. This resin has a long history of use since ancient times (Miller, 1969). Agarwood is now widely traded and used for perfume, incense, medicine and religious and secular purposes (Barden *et al.*, 2000; Hansen, 2000; Jung, 2011). This valuable non-timber forest product is formed in the wood after a process of injury and pathogen attack (Donovan & Puri, 2004; Persoon, 2007). *Aquilaria* species comprise shade tolerant trees that grow in the understorey of mature tropical forests of Southeast Asia (north-eastern India to southern China, Cambodia, Laos, Vietnam, Thailand, Malaysia, Indonesia, Philippines and Papua New Guinea) occurring across a wide range of altitude from a few meter above sea level to up to 1000 m (Donovan & Puri, 2004; Faridah-Hanum *et al.*, 2009). They can be found on various soil types including sandy, rocky and/or calcareous, and in topographic positions ranging from floodplains to steep slopes (Akter *et al.*, 2013; Faridah-Hanum *et al.*, 2009). The high demand for agarwood has resulted in overharvesting of the natural population and *Aquilaria* species have been listed in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2015) and as an endangered or vulnerable species in the IUCN list (IUCN, 2013). In recent years a boom in cultivation of *Aquilaria* species (i.e. *A. crassna*, *A. sinensis*, *A. malaccensis*, and *A. agallocha*) has taken place in the area of natural occurrence of the species to supply the demand for agarwood and alleviate the pressure on natural populations (Akter *et al.*, 2013; Hoang Ha & Nghi, 2011; Jha, 2014; Lok *et al.*, 1999; Lok & Zuhaidi, 2011; Nakashima *et al.*, 2005; Persoon, 2008; Rahman *et al.*, 2015; Saikia & Khan, 2014b; Suharti *et al.*, 2011).

In assessing productivity within *Aquilaria* plantations particular attention is given to stem diameter as a pre-requisite to induce the formation of agarwood in the wood (decay prevention process) (Blanchette *et al.*, 2005; Liu *et al.*, 2013). Identification of traits that are associated with plant growth and high-yielding trees is considered important to develop early selection criteria. Identification of traits that are associated with plant growth and high-yielding trees is considered important to develop selection criteria. In this study, cultivated *Aquilaria* species grown in a single environment were monitored to quantify the relationship between isotope ratios, leaf-productivity traits and tree growth. The aim of this study was to identify physiological and/or morphological traits (or combinations) that are correlated with tree growth characters stem diameter and/or plant height in *Aquilaria crassna*. We tested the hypothesis that tree productivity can be predicted by functional leaf traits, with the view that these traits can then aid in the early selection of high-yielding trees.

4.3 Methodology

4.3.1 Study site

The study area is located near Innisfail in north Queensland, Australia (17.52°S, 146.02°E) which has a humid tropical climate with an annual rainfall of 3565 mm and mean daily temperature between 20°C and 28 °C (Bureau of Meteorology, 2015). The soils are classified as Ferrosols (ASRI, 2015) which are high in free iron oxide (ASRI, 2015). Mean daily irradiance measured in a site near to the plantation was 33 mol m^{-2} day^{-1}. A research planting of 704 trees of *Aquilaria* spp. was established in January 2010, using wild-collected seeds of several *Aquilaria* species from south-east Asia, on a site previously used for sugar cane production. Trees were grown under full sunlight, with no supplemental water (water supplied by rainfall only) and were subject to standard agronomic practices (fertilization, weed control, pruning, and pest control).

4.3.2 Tree selection

Twenty-eight trees of *A. crassna*, representing a natural range of diameter increments from lower to upper range of the planted population were measured for carbon and nitrogen isotope ratios and other leaf traits. All trees selected for this study were based on measurements of height and diameter at 4.75 years (56.7 months) after planting. The annual increment in diameter at 150 mm above ground level and height across all trees measured in this study was 3.39 cm (± 0.72) and 128.02 cm (± 33.41), respectively. Four diameter classes were defined (1= 10-12.9 cm, 2 = 13-15.9 cm, 3= 16-18.9 cm, 4, = 19-23 cm) and seven trees per diameter class selected randomly for this study. The mean diameter of classes 1 to 4 was 11.83, 14.59, 17.86 and 20.01cm respectively, which correspond to annual diameter increments of 2.49, 3.07, 3.76 and 4.21 cm. These *A. crassna* trees formed the basis of the stable carbon and nitrogen isotope and leaf trait measures.

4.3.3 Stable carbon and nitrogen isotope measurements

Fully expanded, mature leaf samples from each tree were taken from sun-exposed branches located at the four cardinal compass directions from the top third of the crown (Flanagan & Johnsen, 1995). These were mixed and dried at 65°C, before being finely ground using a ring mill. A subsample of 2 mg of the leaf powder was loaded into tin capsules and combusted in an elemental analyser (ECS 4010 Costech Analytical Technologies, Inc, CA, USA) coupled to an isotope ratio mass spectrometer to determine the relative abundance of ^{13}C and ^{15}N. Stable carbon isotope ratios were expressed as δ^{13}C (‰) relative to the Pee Dee Belemnite (PDB) standard. The values for δ^{13}C were calculated as: δ^{13}C (‰) = (Rp/Rs -1)* 1000, where Rp and Rs are the ^{13}C/^{12}C ratios for plant tissue and PDB standard, respectively. Abundance of nitrogen isotope was expressed as δ^{15}N (‰) relative to the ^{15}N composition of atmospheric N_2. The equation for δ^{15N} (‰) was: δ^{15}N (Rp/ Rs -1)* 1000, where Rp and Rs are the ^{15}N/^{14}N ratios for plant tissue and atmospheric N_2, respectively. Carbon and nitrogen contents were estimated from the samples used to determine the abundance of ^{13}C and ^{15}N.

4.3.4 Leaf traits

Six leaf morphology and development traits were recorded between December 2014 and January 2015 every three or four days over a period of six weeks (total of eleven points of measurement), which included the following: (1) Leaf development on three shoots per tree monitored twice per week. Shoots were observed from dormant bud to ‘complete leaf expansion’ (point of comparison, near mature leaf in the branch, leaf expansion stage does not necessarily mean a mature leaf) to distinguish and classify the developmental stages of the leaf. Descriptions of each stage are outlined in the results. (2) Rate of leaf lamina expansion in length (Lexp, mm day^{-1}) was measured in three shoots per tree, twice per week with digital calipers. (3) Number of new leaves produced in a shoot was monitored twice per week in three shoots per tree. (4) Petiole length (PeLen, mm) and width (PeWid, mm) were measured on a single visibly largest leaf in the canopy using a digital caliper. (5) Leaf area (LA, cm^2) was measured using a leaf area meter (CID 203 handheld laser leaf area meter-CID Bioscience Inc, Camas, WA, USA) on a single visibly largest leaf in the canopy. (6) Specific leaf area (SLA, cm^2 g^{-1}) of the single visibly largest leaf in the canopy was calculated as the individual leaf area divided by the individual leaf dry mass (dried at 70° C for 48 hours).

4.3.5 Data analysis

Number of new leaves produced on a shoot was averaged as the number of new leaves per week (NL.week) to facilitate representation of the data collected. ANOVA and Tukey tests were conducted to estimate statistical differences between diametric classes and leaf morphological traits (NL.week and Lexp). Linear associations between abundance of carbon isotopes with each of diameter and height and with associated leaf attributes (nitrogen content, C: N ratio and $\delta^{15}N$) were determined using linear regression. An explorative analysis was carried out seeking to identify leaf traits that could predict stem diameter variation in *Aquilaria*. Stem diameter was preferred over height and volume because a certain diameter is required to induce the formation of agarwood in cultivated plants and also height and diameter were highly correlated. Model averaging technique based on the Akaike's information criterion (AIC) was computed to fit a model that could predict actual stem diameter. The MuMIn package (Barton, 2015) and dredge and model.avg functions from R were used to compute the set of best ranked models. The second order AIC (AIC_c) values were used because of the relatively small sample sizes (Symmonds & Moussalli, 2011). A list of best models was specified using confidence intervals at 95%. The predictor variables were standardized using standardize function. The following variables were included in the analysis: petiole width (PeWid, mm), petiole length (PeLen, mm), leaf area (LA, cm^2), specific leaf area (SLA, cm^2 g^{-1}), % carbon (PC), % nitrogen (PN), Carbon: Nitrogen ratio, Leaf expansion (Lexp, mm), number of new leaves per week (NL.week), stable carbon isotope ratio ($\delta^{13}C$, ‰) and stable nitrogen isotope ratio ($\delta^{15}N$, ‰). Carbon isotope regression analysis with D and H and other leaf traits and the average modelling technique analyses were done with 27 trees, as one observation was treated as an outlier and removed from the data set. While ANOVA and Tukey tests performed between diametric classes and NL.week and L.exp were done with the entire sample (28 trees). All the statistical analyses were undertaken using R (R Core Team, 2015).

4.4 Results

4.4.1 Leaf development

Three stages in leaf development were determined in *Aquilaria* species. The first stage is leaf emergence; where the lamina is visible but completely rolled. Second stage commences as the leaf begins unfolding and concludes when it is completely unfolded. Unfolding is observed at the base and apices of the blade. Third stage is leaf expansion, where the entire lamina is unfolded, but still actively expanding in both length and width (Plate 4.1). The duration of leaf stages 1 and 2 are each three days and stage 3 will progress for a further 11.5 days until the leaf has achieved its full mature size. The number of leaves growing in a single shoot varied between 1 to 10 leaves, with an average 4 leaves. Approximately one new leaf was produced each week on a given shoot (Figure 4.1). Rate of leaf expansion in length was in 1.45 mm day^{-1}. There were no significant differences between diametric classes in leaf expansion rate or number of new leaves per week (*p* >0.05).

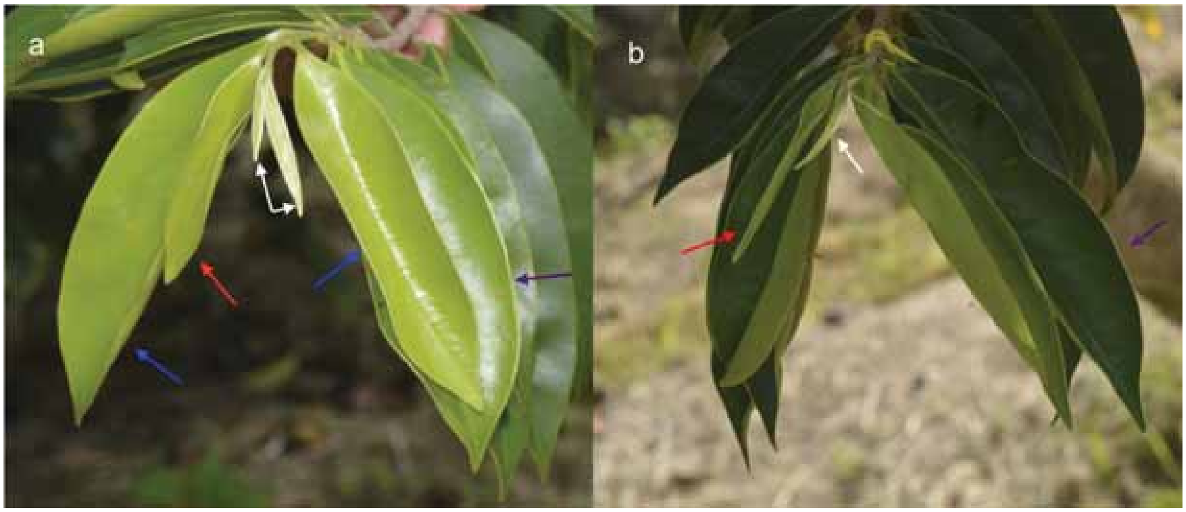

Plate 4.1 Stages of leaf development monitored across 28 individuals of *Aquilaria crassna*. White arrows: leaf emergence; red arrows: leaf unfolding; blue arrows: leaf expansion; purple arrows: leaf full expanded.

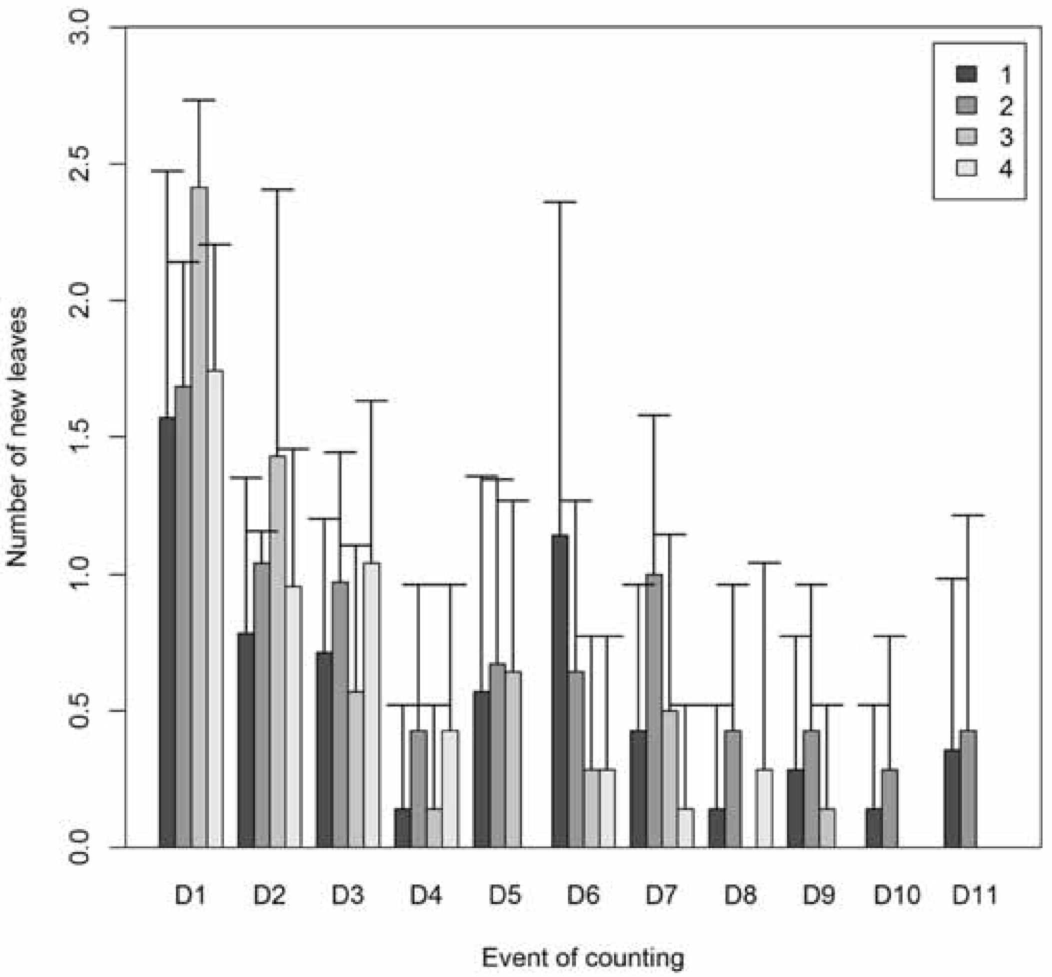

Figure 4.1 Mean leaf production across three shoots in *Aquilaria crassna* in four stem diameter classes (1= 11.83 cm, 2 = 14.59 cm, 3= 17.86 cm, 4, = 20.01 cm) for 11 observations (D1 to D11, D: day) over a period of six weeks in December 2014 and January 2015. N trees: 7 per each class. Vertical lines represent standard deviation. No showed bars indicate absence of new leaves.

4.4.2 Foliar carbon and nitrogen isotope ratios

Range of δ^{13}C values in leaves of *A. crassna* was from -31 ‰ to -25.5 ‰ with an average of -28.4 ‰ (± 1.5 ‰ SD). More depleted values of leaf δ^{13}C were recorded in trees with both larger diameter and height (Figure 4.2).

Fitting a linear regression model between stem diameter and $\delta^{13}C$ showed that 35% of the variability (R^2) in diameter can be explained by leaf ^{13}C in leaf ($p < 0.001$, Figure 4.2). Leaf $\delta^{13}C$ and nitrogen contents were positively correlated (R^2 0.45, $p < 0.001$) (Figure 4.3). The average of leaf C: N ratio was 25.5 (± 3.95) with values ranging from 19.02 to 35.17. A significant relationship (R^2 0.58, $p < 0.0001$) was found between $\delta^{13}C$ and C: N ratio. Large values of C:N ratio were associated with more negative values of $\delta^{13}C$. Values of $\delta^{15}N$ ranged from -0.75 ‰ to a maximum of 4.26 ‰. Values greater than 0 ‰ are indicative of material enriched in ^{15}N compared to atmospheric N_2. There was no significant correlation between $\delta^{15}N$ and N content ($R^2 = 0.06$, $p = 0.22$) (Figure 4.3).

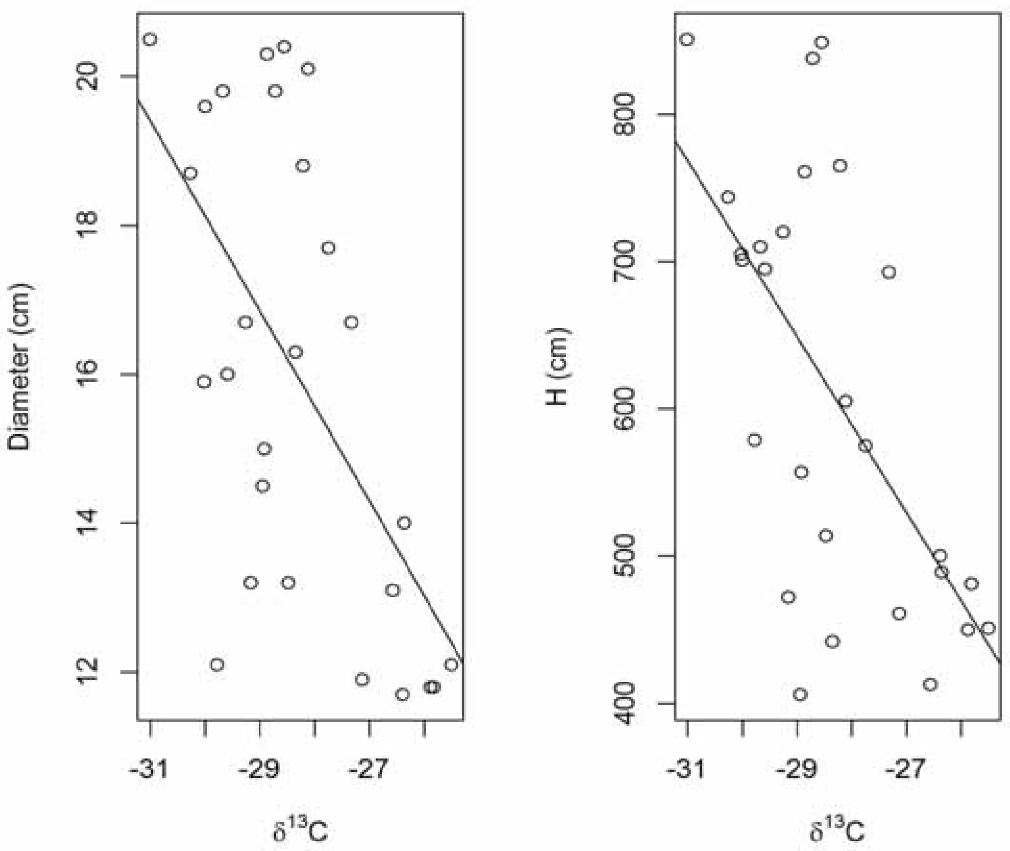

Figure 4.2 Relationship between diameter (cm), height (cm) and $\delta^{13}C$ across 27 individuals of *Aquilaria crassna*. The regression equation for diameter is $y = -20.1445 + -1.2758x$, $R^2 = 0.357$, $p = 0.000999$. The regression equation for height $y = -1083.72 + -59.75x$, $R^2 = 0.3861$, $p = 0.000542$.

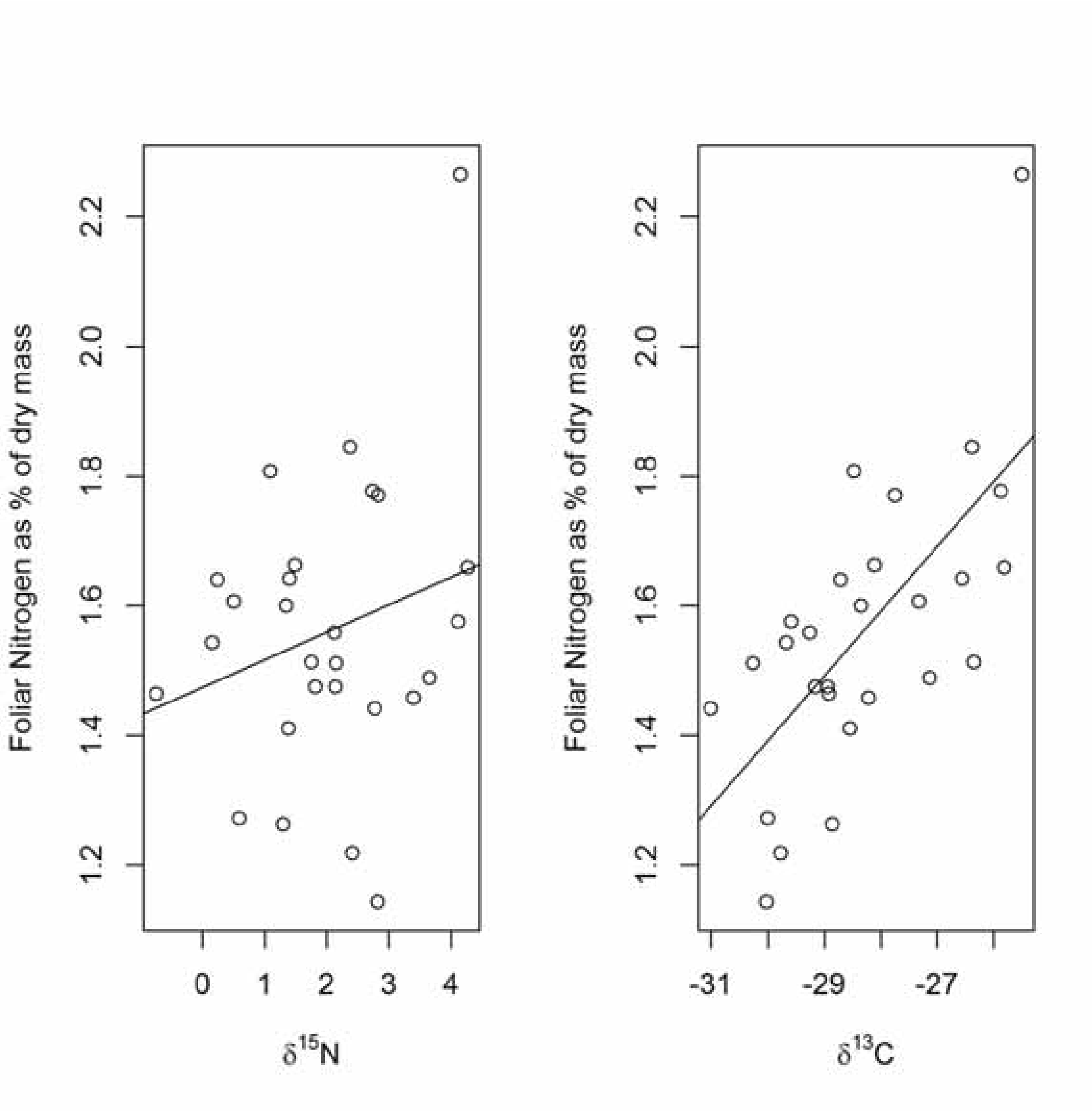

Figure 4.3 Relationship between foliar nitrogen (% of dry mass), $\delta^{15}N$ ($R^2 = 0.0593$, $p = 0.221$) and $\delta^{13}C$ ($R^2 = 0.4479$, $p = 0.0001348$) across 27 individuals of *Aquilaria crassna*.

4.4.3 Relationship between stem diameter and leaf traits

Aside from $\delta^{13}C$, correlations between leaf traits and either tree height or stem diameter recorded in this study were generally weak (Figure 4.4). Analysis of the 95% confidence of best-ranked regression models revealed that, $\delta^{13}C$, $\delta^{15}N$, petiole length (PeLen), number of new leaves produced per week (NL.week) and specific leaf area (SLA) were the best predictors of growth (expressed in diameter) in *Aquilaria crassna* (Table 4.1). When diameter was modelled as a function of PeLen, $\delta^{13}C$, $\delta^{15}N$, SLA and NL.week, it was found that $\delta^{13}C$, and $\delta^{15}N$ contributed significantly to the model (Table 4.2), and it seems that PeLen could contribute to the model as it was slightly over the 0.05 value. The model with all components could explain 55% (R^2 0.55, Table 4.2) of the variability in diameter in *A. crassna*.

Table 4.1 Relative importance values (Akaike weights) of the predictors using model averaging (MuMIn package in R). $\delta^{13}C$; $\delta^{15}N$; PeLen= petiole length; LA= leaf area; SLA= specific leaf area; NL.week= production of new leaf week^{-1}; PC= %C; C.N= ratio C/N; PN= %N; Lexp= leaf expansion; PeWid= petiole width.

Variables	**Importance**	**N containing models**
$\delta^{13}C$	0.82	395
$\delta^{15}N$	0.53	350
PeLen	0.43	278
NL.week	0.31	286
SLA	0.27	221
LA	0.24	263
Lexp	0.17	195
C.N	0.17	208
PN	0.16	206
PC	0.16	207
PeWid	0.15	185

N containing models refers to the number of times the variable is picked in the possible combinations of models.

Table 4.2 Regression coefficients for linear model of diameter as a function of $\delta^{13}C$, $\delta^{15}N$, petiole length (PeLen), specific leaf area (SLA), number of new leaf per week (NL.week).

Variable	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-4.97135	11.84026	-0.42	0.6788
$\delta^{13}C$	-0.8491	0.39234	-2.164	0.0421 *
$\delta^{15}N$	-0.94026	0.42957	-2.189	0.0400 *
PeLen	-0.83644	0.41405	-2.02	0.0563 .
SLA	0.0571	0.03676	1.553	0.1353
NL.week	-1.81633	1.96462	-0.925	0.3657

0.01= (*); 0.05= (.); 0.1 ( ). Residual standard error: 2.392 on 21 df. Multiple R^2: 0.5522, Adjusted R^2: 0.4455. F-statistic: 5.178 on 5 and 21 DF. The multiple linear regression equation for diameter is $y = -4.97135 + -0.8491\delta^{13}C + -0.94026\delta^{15}N + -0.83644PeLen + 0.0571SLA + -1.81633NL.week$, *p*-value: 0.002993.

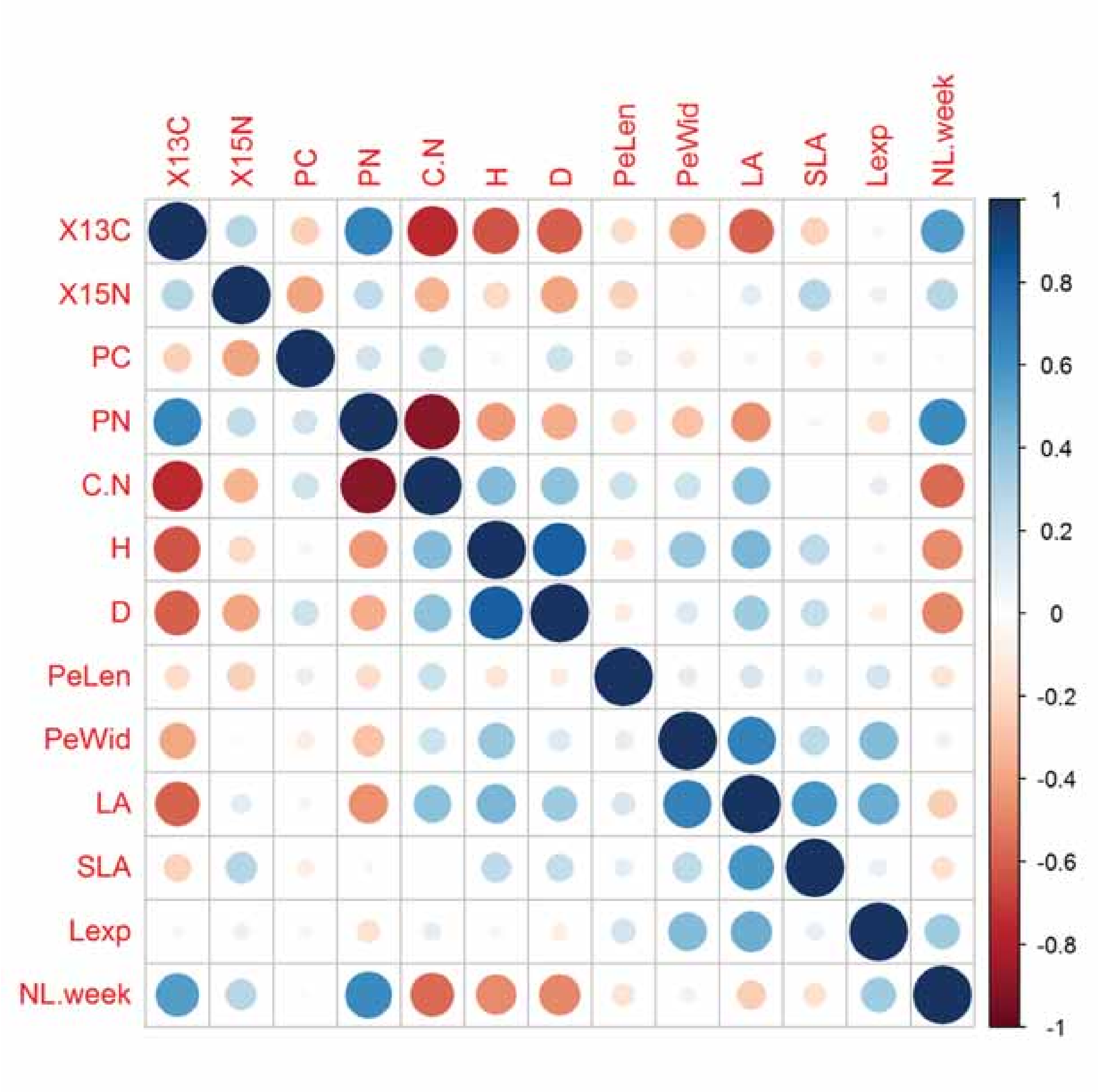

Figure 4.4 Correlation matrix of leaf attributes, height, diameter in *Aquilaria crassna* (true13C = δ^{13}C, true15N = δ^{15}N, PC=% of carbon, PN=% of nitrogen, PeLen = petiole length (mm), PeWid =petiole width (mm), LA = Leaf area (cm^2), SLA =specific leaf area (SLA, cm^2 g^{-1}), Lexp = expansion rate mm day^{-1}, NL.week=number of new leaves per week). Red circles: negative correlation. Blue circles: positive correlation. Intensity of colour and size of circles are indicative of the intensity of the correlation.

4.5 Discussion

Values of δ^{13}C in leaves found in this research were less depleted in ^{13}C than other data reported for tropical trees growing in natural areas. Kapos *et al.* (1993) studying the foliar variation of δ^{13}C for four tropical species growing at different forest condition and canopy position, found that two canopy species reached $\delta^{13}C$ values between -33.5 and -29.4 ‰, and for two understory species (shaded conditions), between -36.6 and -32.2‰. Less negative $\delta^{13}C$ values were associated to species growing near the edge of the forest. Martinelli *et al.* (1998) reported an average foliar $\delta^{13}C$ value of -32.1 ‰ (±1.5 ‰ SD) for trees growing in a tropical forest and they attributed more negative values with a higher ratio of stomatal conductance to photosynthesis. Ometto *et al.* (2006) estimated similar average values as found by Martinelli *et al.* (1998) for tropical trees, and they proposed that the differences in $\delta^{13}C$ values were associated to height and forest structure. However, foliar $\delta^{13}C$ values of planted *A. crassna* were more comparable to plantation species. $\delta^{13}C$ values reported for a five year old pine trees plantation (*Pinus halepensis*) in semi-arid Spain, were similar (-22.6 to -21.6 ‰) to the values estimated for *A. crassna* trees (Querejeta *et al.*, 2008).

In this study of *A. crassna*, tree size (total height and stem diameter) was negatively correlated with δ^{13} C. Other researchers have found links between stem increment and foliar $\delta^{13}C$ (Brown *et al.*, 2011; Ducrey *et al.*, 2008); however, the relationship has generally been positive, such that faster stem growth was associated with less negative foliar $\delta^{13}C$. Because foliar $\delta^{13}C$ correlates positively with intrinsic water use efficiency (WUEi) (Farquhar *et al.*, 1982), these previous findings indicate that faster growth was associated with increased WUEi. The WUEi is defined as photosynthesis divided by stomatal conductance, so it is clear that it can be influenced by either parameter. In *Picea glauca* Sun *et al.* (1996) found a positive correlation between foliar $\delta^{13}C$ and biomass production, and proposed the likely causal mechanism was variation in photosynthetic capacity. In a genetic study of growth in *Picea mariana* (Johnsen *et al.*, 1999) foliar $\delta^{13}C$ again showed a positive correlation with height growth and these authors suggested that these results combined with earlier gas-exchange work (Johnsen & Major, 1995) indicated that variation in $\delta^{13}C$ was due to differences photosynthetic capacity rather than stomatal conductance. On the other hand, Cao *et al.* (2012) found evidence across six *Populus* species for high growth rates being related to both high WUEi and low WUEi, depending of the geographic origin of the species. In *A. crassna*, the negative correlation between foliar $\delta^{13}C$ and plant growth (diameter and height), suggests an optimal carbon gain when stomatal conductance is high, allowing carbon dioxide to diffuse more freely into the leaf, and thereby raising the intercellular CO_2 concentration. It suggests that this is a strategy that can be favoured in environments where water is not limiting, such as in the wet tropics. In this case, high stomatal conductance would lead to higher photosynthesis rates with little risk of a penalty for profligate water use in the form of water stress and stomatal closure. In other words, because water is not limiting in this environment, there should be no adverse cost to having low WUEi.

A positive correlation between foliar $\delta^{13}C$ and nitrogen content was found in *A. crassna* plantations. Leverkus *et al.* (2015) found the same trend when evaluating the survival, foliar nutrient status, and foliar isotopic composition ($\delta^{13}C$ and $\delta^{18}O$) of two sympatric oak species planted at two different altitudes. Both species had a positive correlation between leaf N content and $\delta^{13}C$. Ma *et al.* (2010) also found a positive relationship between $\delta^{13}C$ and N content of the youngest fully expanded leaves of *Malus* rootstock. Previous studies have highlighted the positive effect of foliar N content on photosynthetic capacity (Gough *et al.*, 2004; Reich *et al.*, 1994; Sparks & Ehleringer, 1997). This positive relationship between $\delta^{13}C$ and nitrogen implies that leaves with higher photosynthetic capacities also had lower intercellular CO_2 concentrations (c_i) (Sparks & Ehleringer, 1997). However at high leaf N concentrations, for example, N content > 16 mg g^{-1} (data reported by Livingston *et al.* (1998) for *Pinus radiata*), there is no further increase in photosynthetic capacity with increasing N content. In this study, the positive relationship between leaf $\delta^{13}C$ and N content did not translate into higher growth rates at higher leaf N contents. It seems that *A. crassna* trees growing in a high-rainfall tropical environment can benefit more from having high c_i than from having high leaf N, because realized photosynthesis rates depend both on N allocation to photosynthetic capacity and on the average operating c_i, with either one leading to increased realized photosynthesis.

Natural abundance of $\delta^{15}N$ in plants is a useful tool for evaluating the N status in various ecosystems (Emmet *et al.*, 1998; Pardo *et al.*, 2007; Wang *et al.*, 2014; Watzka *et al.*, 2006). In *Aquilaria* plantations the range of values for leaf $\delta^{15}N$ (-0.75 ‰ to 4.26 ‰) observed was similar to the range of those observed for 32 tropical species along a rainfall gradient (1800 mm to 3500 mm year^{-1}) in lowland tropical forest in Panama (leaf $\delta^{15}N$:−1 ‰ to 5 ‰) (Santiago *et al.*, 2004). Foliar $\delta^{15}N$ is determined by the isotopic composition of the primary nitrogen source (NO_3^-, NH_4^+, N_2) and by the efflux to influx ratios across the root surface in the case of NO_3^- and NH_4^+ (Cernusak *et al.*, 2009; Denton *et al.*, 2001; Werner & Schmidt, 2002). Fertilization can result in variation in $\delta^{15}N$, often showing a positive correlation with intensity of fertilizer application (Watzka *et al.*, 2006). In this study, *A. crassna* grew in sites previously impacted by fertilizer application to sugar cane production, the preceding land use. However, no relationship was observed between $\delta^{15}N$ and leaf nitrogen content, and the correlation between leaf $\delta^{15}N$ and growth was negative. This could indicate a stronger preference for NO_3^- nitrogen in the faster growing trees, in line with their ability to take advantage of the site conditions associated with the abandoned sugar cane plantation.

Anatomical and morphological leaf traits are commonly used to assist in the selection of high-productive individuals. In this study, the leaf traits other than $\delta^{13}C$ and $\delta^{15}N$ correlated weakly with diameter or height (Figure 4.4). However, foliar δ^{15}N, foliar δ^{13} C, petiole length, specific leaf area and number of new leaves per shoot per week explained 55% of the variability in diameter. Only foliar δ^{13} C and δ^{15}N and petiole length contributed significantly to the model, but the latter is weakly significant at 5% level (0.0563, Table 4.2). In other studies, leaf area and petiole length have been good predictors of growth (Bunn *et al.*, 2004; Dillen *et al.*, 2009; Marron *et al.*, 2007). Specific leaf area has been consistently reported as correlated with plant performance (Poorter & de Jong, 1999; Poorter & Bongers, 2006; Possen *et al.*, 2014). As demonstrated by Reich *et al.* (1998) SLA can influence A_{max}- N relationship. Yu (2001), however, did not find a correlation between leaf traits and growth (height and diameter) in four hybrid *Populus* clones. This is the first attempt to link morphological traits and stable isotopes with growth performance in *Aquilaria* species. This study reveals that a combination of morphological leaf traits with stable isotopes could support the selection of productive individuals, and contribute to the development of the species as a plantation crop. However, trials (i.e. clonal trial) of the superior material are required to demonstrate the genetic efficiency of the selected traits to justify their inclusion as a criterion in tree improvement programmes.

4.6 Conclusions

Foliar δ^{13}C of *A. crassna* was less depleted in comparison to other tropical trees growing in natural areas, but similar to that reported for trees growing in plantations. Diameter was correlated with δ^{13} C and better performance was linked with depleted values in ^{13}C. Positive relationship was found between δ^{13}C and N content. However, the negative relationship between δ^{13}C and stem diameter growth suggests that high water-use efficiency associated with high foliar N did not confer a growth advantage. On the contrary, individuals that had high intercellular CO_2 concentrations (low δ^{13}C) and therefore low water-use efficiency grew more rapidly. This pattern differs from many studies carried out in non-tropical regions (Fardusi *et al.*, 2016), and may be the result of the very high water availability at the study site. Foliar δ^{15}N in *A. crassna* was similar to other values reported for tropical plants. In *A. crassna* morphological leaf traits (new leaves per week, petiole length, specific leaf area) and stable carbon and nitrogen isotopes ratios explained 55% of the variability in growth (basal diameter), indicating the potential of leaf traits in assisting the selection of productive individuals.

Chapter 5. Relationship of nutrient content, CO_2 rate and SPAD-502 readings in *Aquilaria crassna* (Thymelaeaceae)

5.1 Abstract

Cultivation of *Aquilaria crassna* for production of its resin-infused fragrant wood will depend on knowledge of its adaptability to environments commonly associated with reforestation. In this study, I evaluate the photosynthesis-irradiance (PE) relationship in *A. crassna* to determine the species adaptability to reforestation sites with different light environments. Here I also evaluate the relationship between leaf chlorophyll content using SPAD-502 with each of light saturated photosynthesis and leaf nutrient content to understand the optimal conditions for plant growth. A significant positive polynomial relationship (R^2=0.805) was found between CO_2 assimilation and photon flux density (PFD). CO_2 assimilation increased linearly with PFD peaking at PFD of 1000 μmol m^{-2} s^{-1} (A_{max}), followed by a gradual decline and increased variability at higher PFD values. *A. crassna* is well adapted to the maintenance of photosynthesis under highly variable light environments. In this environment, CO_2 assimilation is optimised at average solar radiation levels during the middle of the day, and maintained under both low (heavy cloud) and high (full sun) light situations. The effect of relative leaf chlorophyll content on the rate of CO_2 assimilation was determined at a constant PFD of 1000 μmol $m^{-2}s^{-1}$, with a significant positive relationship (R^2=0.855) found between the two. CO_2 assimilation increased to a maximum of 10.8 μmol m^{-2} s^{-1} in leaves with a high internal chlorophyll index (60-70). A significant positive relationship (R^2=0.98) between chlorophyll content and % leaf nitrogen was also found for this species. In this study SPAD-502 readings can give useful indications about leaf nitrogen content in *A. crassna*, and hence, about site quality. This study also confirms that *A. crassna* can grow under heterogeneous light conditions and that management of plant nutrition can be used to optimise CO_2 uptake in *A. crassna* plantings as revealed by the SPAD values and CO_2 relationship.

5.2 Introduction

Agarwood is a resin-infused fragrant wood harvested from several species of *Aquilaria* and *Gyrinops* (Thymelaeaceae) and traded internationally for incense, perfume, traditional medicines and religious uses (Barden *et al.*, 2000; Yunjun, 2013). Agarwood has been traded since ancient times (Miller, 1969), but more recent exploitative harvesting of agarwood has led to widespread depletion of their natural stands (Donovan & Puri, 2004; Zhang *et al.*, 2008). To meet the high demand for agarwood and provide an alternative to the wild-harvested supply, cultivation of species such as *A. malaccenssis* (Lok *et al.*, 1999; Lok & Zuhaidi, 1996; Rahman *et al.*, 2015; Uddin *et al.*, 2008), *A. sinensis* (Liu *et al.*, 2013; Xiao-xia *et al.*, 2010) and *A. crassna* (Nakashima *et al.*, 2005; Persoon, 2008; Van Thanh *et al.*, 2015) has been increasing in recent times. For these species to make a successful transition to planted forestry it is important to develop an understanding of their adaptability to cultivation and develop reliable methods of production (Akter *et al.*, 2013; Thiru *et al.*, 2014; Uddin *et al.*, 2008).

Aquilaria species are generally shade-tolerant, understory trees, which grow in mature evergreen or semi-evergreen mixed hardwood hill forests (Donovan & Puri, 2004). The physical environment of its native habitat typically contrasts with open-cultivated environments with little or no existing canopy, particularly with respect to solar radiation, temperature, water and nutrient availability. Selection of trees for reforestation requires an understanding of their abiotic and biotic stress tolerances (Lawson & Michler, 2014) and matching their ecological suitability with modified planting environments (Jones, 2013). The intensity of irradiance is an important factor influencing survival and growth in regenerating tropical tree species (Favaretto *et al.*, 2011; Gyimah & Nakao, 2007). For *A. malaccensis* saplings growing in a low light (~20 and 170 µmol m^{-2} s^{-1}) forest understory, Soehartono (2002) found that mean height increment was positively related to light availability. These authors found similar results in a nursery experiment with a greater range of midday light intensities (29-347 µmol m^{-2} s^{-1}). In contrast, saplings of *A. crassna* growing under plantation conditions had significantly greater height growth under shade (425 µmol m^{-2} s^{-1}) compared with full sun (1130 µmol m^{-2} s^{-1}). It is therefore important to determine the physiological adaptability of *Aquilaria* species to variable and high irradiance environments associated with cultivation and restoration. In this study is estimated CO_2 assimilation of *A. crassna*, across a range of light intensities and the influence of relative leaf nutrients on chlorophyll content and photosynthesis to provide an insight of its adaptability to cultivated environments that would influence the silviculture and nutrition management in this emerging forestry species.

The relationship between solar irradiance and photosynthesis varies between plant species (Kitajima, 1994; Vera *et al.*, 1999) and is influenced by the various succession and life-history strategies (Montgomery & Chazdon, 2002; Nogueira *et al.*, 2004; Valladares *et al.*, 1997). To understand the factors influencing leaf photosynthetic capacity in any species, it is important to determine the nature of the photosynthesis-irradiance (PE) relationship, particularly the levels of irradiance at which the rate of photosynthesis is maximised (light-saturated photosynthetic rate - A_{max}). A_{max} is influenced by leaf nitrogen (Bown *et al.*, 2010; Evans, 1989; Reich *et al.*, 1994; Ripullone *et al.*, 2003; Rosati *et al.*, 1999), chlorophyll fluorescence (Baker, 2008; Edwards &

Baker, 1993; Furbank & Walker, 1986) and chlorophyll content (Kura-Hotta *et al.*, 1987; Takai *et al.*, 2010). The positive association between leaf nitrogen and rate of photosynthesis is found across a wide range of trees including 14 species from the Zimbabwean woodlands (Tuohy *et al.*, 1991), eight Australian savanna tree species (Eamus *et al.*, 1999) and a range of temperate north American tree species (Nicodemus *et al.*, 2008; Salifu *et al.*, 2008). This relationship is brought about by the nitrogen dependent proteins of the Calvin cycle (particularly rubisco) and thylakoids, which represent the majority of leaf nitrogen in C_3 plants and support photosynthesis (Berveiller *et al.*, 2010; Evans, 1989). This relationship can ultimately influence plant performance whereby plant/leaf nitrogen and leaf chlorophyll have a positive association with growth in species such as *Pinus taeda* (plant height, stem diameter, needle and stem dry weights) (Tsay *et al.*, 1982), *Populus deltoides, Platanus occidentalis, Quercus falcata* var. *pagodafolia* (aboveground net primary productivity) (Henderson & Jose, 2005) and *Jatropha curcas* (fruit production) (Yong *et al.*, 2010).

Growth in plants is often determined by the rate at which their leaves can assimilate carbon dioxide through photosynthesis. Photosynthesis in turn is influenced by the level of leaf chlorophyll (Lichtenthaler *et al.*, 2007; Sarijeva *et al.*, 2007) and nitrogen (Kattge *et al.*, 2009). Given the association between nitrogen content and yield across a number of tree species, management of nitrogen fertilization is an important factor in maximizing yields. Nutrition management in many horticultural crops depends on regular tissue sampling to guide the application of fertiliser (Beverly, 1994; Blackmer & Schepers, 1995). This is important since the application of fertilizers based on actual plant requirements rather than a scheduled regime has both environmental and economic advantages (Schepers *et al.*, 1992). While plant tissue analysis is very useful tool in this regard, a more rapid method of assessment can improve the responsiveness of nutrition management interventions. In this investigation, a hand-held chlorophyll meter was used to estimate chlorophyll content in *A. crassna* leaves.

The SPAD-502 meter (Konica-Minolta, INC, Osaka, Japan) offers a simple, rapid and non-destructive, way of determining the chlorophyll content or greenness of a leaf, which is expressed as a chlorophyll index. This index is calculated by measuring the transmittance of two light wavelengths (red (650nm) and infrared (940nm)) through the leaf (Cassol *et al.*, 2008). The SPAD index is recorded in dimensionless units and is proportional to the chlorophyll content (Loh *et al.*, 2002). The use of chlorophyll meter is widely accepted and frequently applied in a range of field (Cassol *et al.*, 2008; Kumagai *et al.*, 2009; Netto *et al.*, 2005; Schepers *et al.*, 1992), ornamental (Wang *et al.*, 2004) and forestry crops (Bauerle *et al.*, 2004; van den Berg & Perkins, 2004). The chlorophyll index determined using a SPAD-502 meter, has been correlated with actual chlorophyll and nitrogen content in various tree crops (Boussadia *et al.*, 2011; Loh *et al.*, 2002; Sibley *et al.*, 1996). Such correlations however may not be assumed for all crops, where Fu et al. (2013) found no association between leaf greenness, determined using a SPAD-502, and nitrogen concentration in the fruit crop species *Litchi chinensis*.

The aims of this study were to determine the nature of the relationships between (a) photosynthesis and irradiance, (b) light saturated photosynthesis and leaf chlorophyll content and (c) leaf chlorophyll and nutrient content of *A. crassna* leaves. Understanding the nature of these relationships can potentially influence matching the species with proposed reforestation sites, and provide an insight into how the resources and light environment affect growth and productivity in *A. crassna* plantings.

5.3 Methodology

5.3.1 Chlorophyll-internal leaf nutrient

A. crassna leaves were collected in May 2014 from an agarwood plantation site in Innisfail in tropical North Queensland, Australia (17.52°S, 146.02°E). The leaves ranged from deep green to pale yellow in colour (Plate 5.1). Once collected the leaves were washed in water and placed in bags for transportation to the laboratory. These leaf samples were kept in the fridge for one hour prior to analysis. The leaves were then categorised into SPAD Index increments of 10 and placed in separate containers. The index increments ranged from <15, 15-24.9, 25-34.9, 35-44.9, 45-54.9, 55-64.9, 65-75. Once categorised, 30 leaves were randomly selected from each group and an arithmetic mean was obtained. Samples were placed in a drying oven set at 80°C for 72 hours. These dried samples were sealed in envelopes and sent to Nutrient Advantage Laboratory for internal nutrient analysis. The leaves were evaluated for concentrations of 16 nutrients including: primary macro nutrients, nitrogen (ammonium (mg/kg), nitrate (mg/kg) and % nitrogen (kjeldahl)) phosphorus (%), potassium (%); secondary macro nutrients, calcium (%), magnesium (%), sulphur (%); and trace minerals, boron (mg/kg), chloride (%), cobalt (mg/kg), copper (mg/kg), iron (mg/kg), manganese (mg/kg), molybdenum (mg/kg), selenium (mg/kg), sodium (%), zinc (mg/kg).

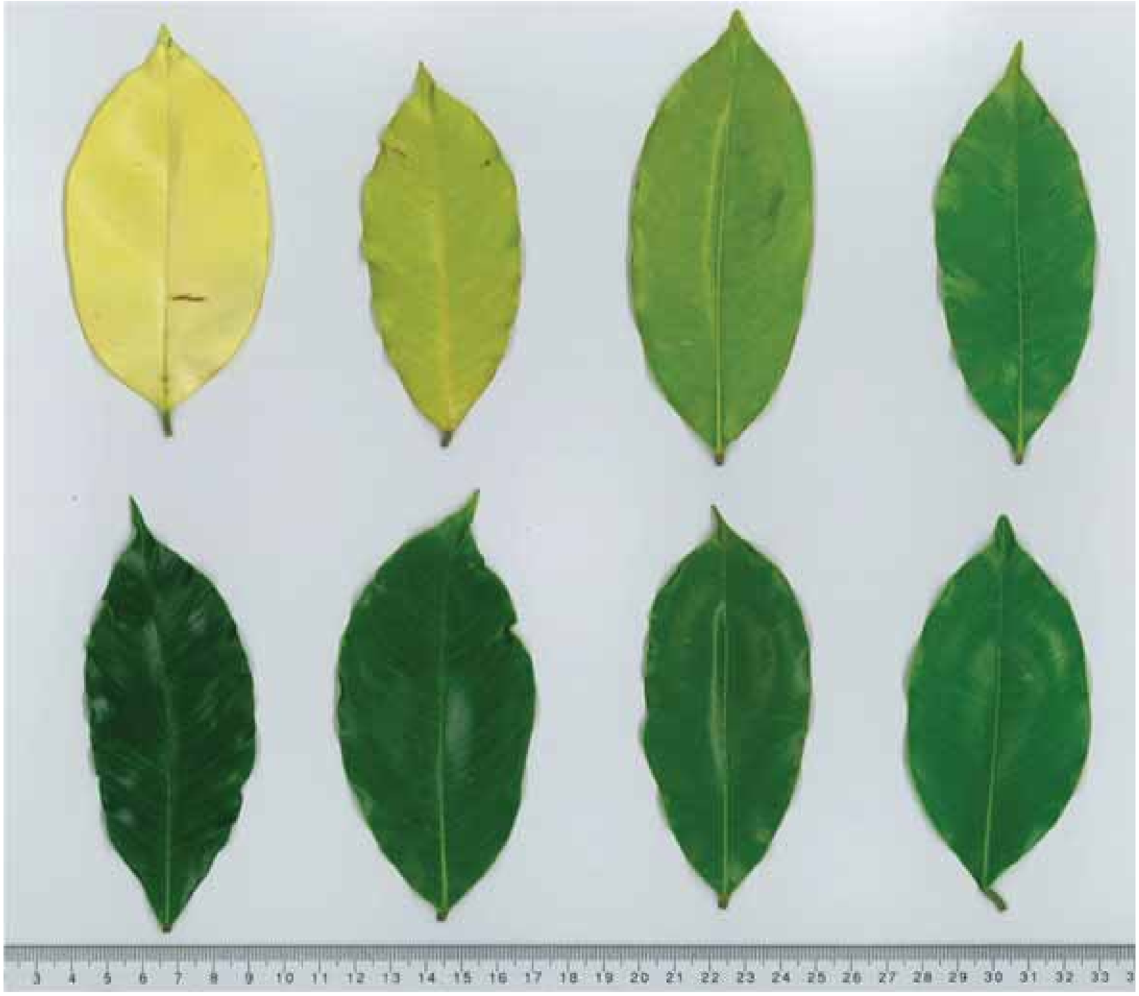

Plate 5.1 Leaves of *Aquilaria crassna* corresponding to chlorophyll index values (SPAD 502-Plus, Konica Minolta, INC, Osaka, Japan) of 0, 10, 20 30 (top, left to right), 40, 50, 60 & 70 (bottom, right to left).

5.3.2 Photosynthesis-irradiance

The relationship between photosynthesis and irradiance was determined using six genotypes of *A. crassna* that were between 18 and 24 months old. Leaves tested had a mean chlorophyll index of between 35 and 50 (SPAD-502 Plus, Konica-Minolta, INC, Osaka, Japan). The CO_2 assimilation rate was measured across twelve photon flux densities –PFD- (0, 50, 100, 200, 400, 600, 800, 1000, 1200, 1500, 1800 & 2500 μmol $m^{-2}s^{-1}$) using a portable photosynthesis system (Li-Cor 6400, Lincoln, USA). Measurements of CO_2 assimilation were logged at 20-second intervals for eight minutes. Between PFD treatments a period of five minutes was permitted before new measurements were logged. This period was necessary to ensure leaves reached steady state values defined as the coefficients of variation (CV) of CO_2 and water vapour within the chamber of less than 0.25%

(Caruso *et al.*, 2005). Two genotypes were measured during the morning (09:00-12:00) and afternoon (13:00-16:00) to determine the influence of the time of day on CO_2 assimilation. All measurements were conducted at a temperature of 25±3°C. Maximum ambient photosynthetically active radiation was measured at the growing site using a PAR-sensor (Skye Instruments Ltd, UK) and logged every 10 minutes (Unidata - prologger) during February (altitude of the midday sun is ~90° on 1st February) and in May (altitude of the midday sun is ~57° on 1st May).

5.3.3 Photosynthesis-chlorophyll

The relationship between photosynthesis and relative leaf chlorophyll was determined across 52 individual 28-month old trees. The relative level of leaf-chlorophyll was determined in 25 leaves of each tree using a hand-held chlorophyll meter (SPAD-502 Plus, Konica-Minolta, INC, Osaka, Japan). The mean chlorophyll content for each tree was calculated and a leaf with a mean of ±5 chlorophyll units (as determined by six replicate leaf measures) was selected to measure photosynthesis. The light-saturated leaf CO_2 assimilation rate was measured on individual leaves at a consistent PFD of 1000 μmol $m^{-2}s^{-1}$. All measurements were conducted at a temperature of 25±3°C.

The leaves of *A. crassna* demonstrate a typical colour response (increase in green pigmentation) in relation to the level of leaf chlorophyll (Plate 5.1). In cultivation under full-sun conditions it is common to locate leaves ranging from 20 to 60 units. Leaves with values between zero and ten represent an extreme chlorophyll deficiency and those with value around 70 represent extreme chlorophyll luxury. During this study while it was possible to locate individual leaves with values under ten and over 70 it was not possible to locate any individual trees with such mean values. Visually healthy trees can have a mean chlorophyll index of between 30 and 60 and therefore it is of interest to determine the difference in photosynthesis between these less extreme levels.

5.3.4 Data analysis

Data analysis was performed using Minitab 17 software. A polynomial linear regression was fitted to describe the relationship between CO_2 assimilation rate and PFD. Relations between nitrogen, copper concentration and chlorophyll index were tested with linear regression.

5.4 Results

5.4.1 CO_2 assimilation and irradiance

In *A. crassna* a significant ($P<0.05$) relationship ($R^2=0.805$) was found between CO_2 assimilation rate and PFD. A cubic regression model (Figure 5.1) most accurately accounted for this relationship. CO_2 assimilation increased linearly with PFD peaking at between 800 and 1200 µmol m^{-2} s^{-1} PFD, followed by a gradual decline and increased variability at higher PFD values. Light saturated photosynthetic capacity (A_{max}) was considered to occur at a PFD of 1000 µmol m^{-2} s^{-1} with a CO_2 assimilation of 7.15 µmol m^{-2} s^{-1}. Mean CO_2 assimilation was 2.1 µmol m^{-2} s^{-1} at a PFD of 50 µmol m^{-2} s^{-1}, therefore the light compensation point (where net photosynthesis is zero, LCP) is considered to occur at below this level of radiation.

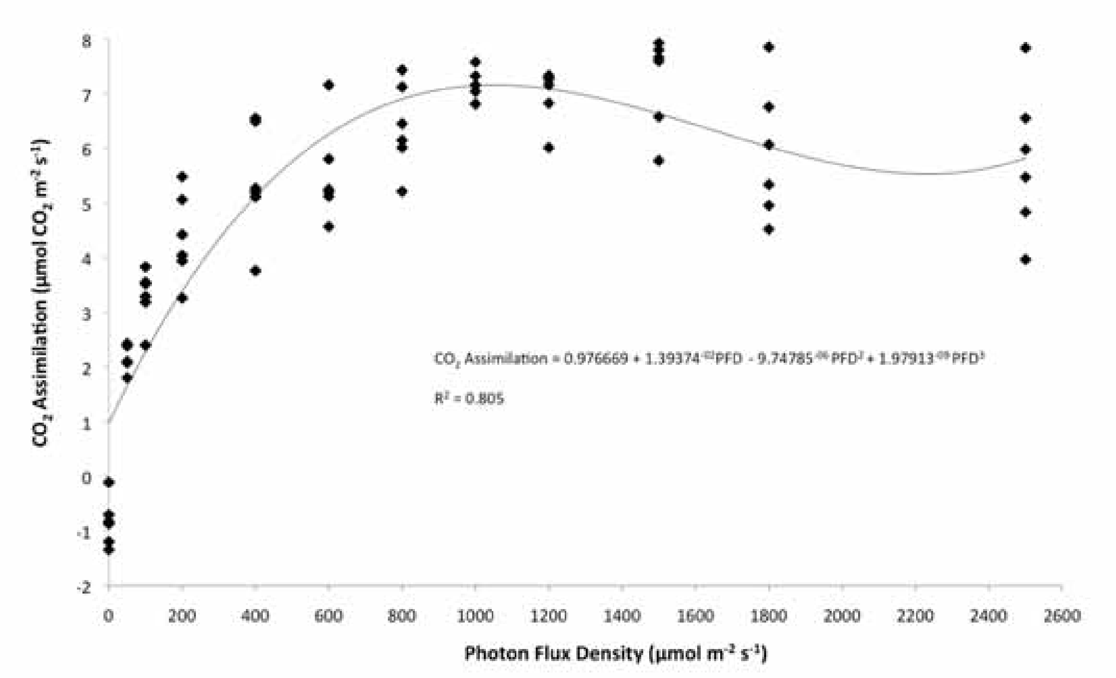

Figure 5.1 Relationship between CO_2 assimilation and Photon Flux Density (PFD) in the leaves of six genotypes of *Aquilaria crassna* with a mean chlorophyll index of 40.

The mean and mean maximum ambient PAR measured at between 10:00 and 14:00 hrs was significantly greater in February (1298 and 2334 µmol m^{-2} s^{-1} respectively) compared with May (804 and 1703 $µmol^{-1}$ m^{-2} s^{-1}). No statistical difference in mean minimum ambient PAR (i.e. under heavy cloud) was found between the February and May measures (Figure 5.2). The mean daily irradiance for February and May was 33 and 19 mol m^{-2} day^{-1} respectively. CO_2 assimilation rate under heavy cloud (PFD 250-200 µmol m^{-2} s^{-1}) is 4.1 µmol m^{-2} s^{-1}, which is 42% lower than maximum CO_2 assimilation (7.15 µmol m^{-2} s^{-1}) at PFD of 1000 µmol m^{-2} s^{-1}.

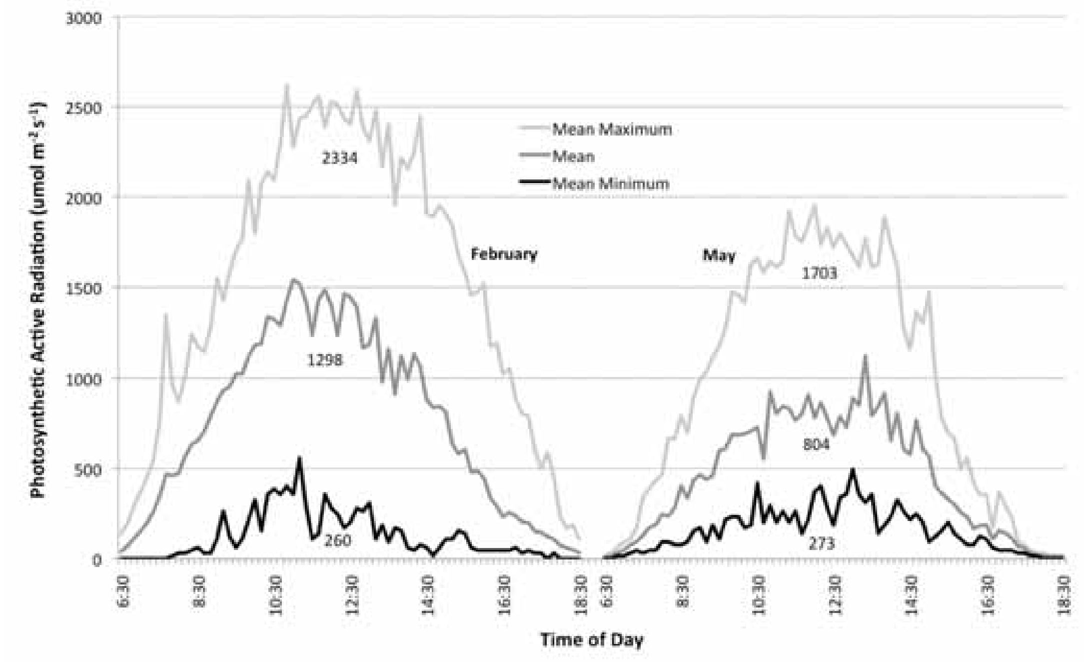

Figure 5.2 The mean, maximum (full-sun) and minimum (cloudy) Photosynthetically Active Radiation (PAR µmol m^{-2} s^{-1}) at the study site throughout the day (06:30-18:30hrs) during the months of February (left line series) and May (right line series). Values under each line series represent its mean PAR between the hours of 10:00 and 14:00.

5.5 Chlorophyll index and internal leaf nutrients

Of the 16 nutrients evaluated for relationships with mean leaf chlorophyll index, leaf nitrogen (% Kjeldahl), calcium (%) and copper (mg kg^{-1}) were found to have an association (Figure 5.3). These nutrients were found to have a positive relationship with leaf chlorophyll with R^2 values of 0.91, 0.98 and 0.94 for N, Ca and Cu respectively. The relative increase in leaf nutrient between the lowest (15) and highest chlorophyll (70) index values was 2.9 times for N, 2.1 times for Ca and 2.5 times for Cu.

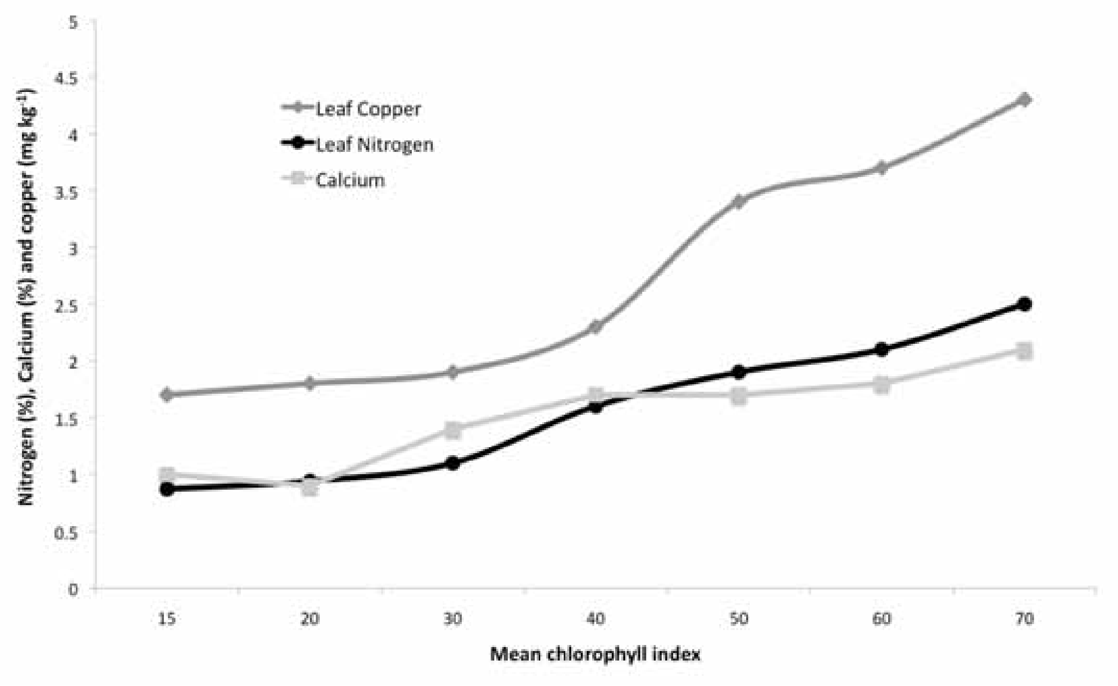

Figure 5.3 Relationship between leaf nitrogen (% Kjeldahl) and copper (mg kg^{-1}) concentration each with mean leaf chlorophyll index (SPAD-502 Plus, Konica-Minolta, INC, Osaka, Japan) in 28-month old *Aquilaria crassna*.

5.5.1 CO_2 assimilation and chlorophyll index

A significant ($P<0.05$) positive relationship ($R^2=0.855$) between mean tree chlorophyll index and CO_2 assimilation rate was found for *A. crassna*, which was best described by a quadratic model (Figure 5.4). Across the range of mean tree chlorophyll index the rate of CO_2 assimilation ranged from approximately 2 to 11 µmol m^{-2} s^{-1}. This represents a four-fold increase in photosynthesis from the lowest to highest mean tree chlorophyll index.

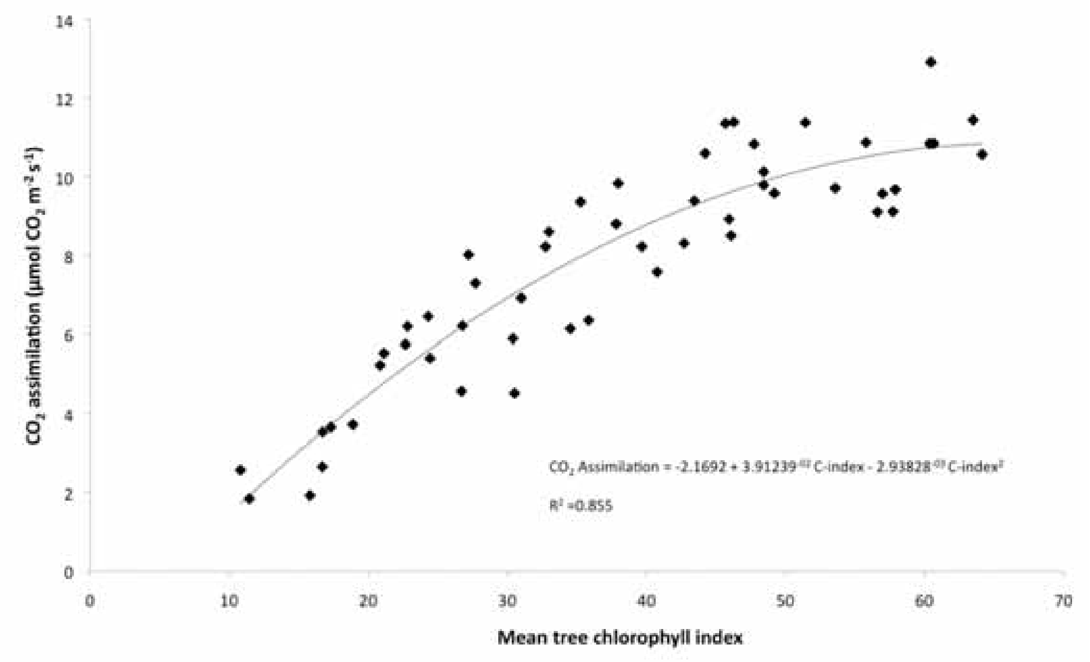

Figure 5.4 Relationship between CO_2 assimilation and mean tree chlorophyll index (C-index) (SPAD-502 Plus, Konica-Minolta, INC, Osaka, Japan) across 52 individual 28-month-old *Aquilaria crassna* trees.

5.6 Discussion

5.6.1 Photosynthesis-irradiance

A_{max} in *A. crassna* occurs at photon flux densities (PFD) between 800 and 1200 µmol^{-1} m^{-2} s^{-1}. Full sunlight at the site between 10:00-14:00hrs in February (2334 µmol^{-1} m^{-2} s^{-1}) and May (1703 µmol^{-1} m^{-2} s^{-1}) is well in excess of the A_{max} range and therefore CO_2 assimilation would be limited under these conditions. A_{max} is however equivalent to the mean radiation occurring during the day (10:00-14:00hrs) in both February (1298 µmol^{-1} m^{-2} s^{-1}) and May (804 µmol^{-1} m^{-2} s^{-1}) (Figure 5.2). Under low light conditions at times of heavy cloud, PAR is approximately 25% and CO_2 assimilation is 42% of that at A_{max} in *A. crassna*. This demonstrates that the species is well adapted to maintenance of photosynthesis under both low and high light situations, and optimises photosynthesis at average solar radiation levels during the middle of the day in the tropical lowlands of north Queensland. The benefits of supplementary shade for *A. crassna* under cultivation (Page & Awarau, 2012), may only be realised during long periods of sunny weather or during the sensitive establishment phase. Poorter (1999) evaluated the responses of 15 rainforest tree species growing under different levels of shade and found that species growing at 25-50% of ambient light had the highest growth rate;

although they did not quantify ambient radiation. Similar results for 16 timber species were reported, where the maximum growth rates were obtained with irradiance ranging between 10-44% of the 22.74 mol m^{-2} day^{-1} ambient light (Agyeman *et al.*, 1999). Species with different life-history strategies can vary in their photosynthetic response to increasing radiation. For instance Riddoch et al. (1991) reported that CO_2 assimilation increased beyond 300 μmol^{-1} m^{-2} s^{-1} PPFD in seedlings of the pioneer species *Nauclea diderrichii* but often declined in the seedlings of the late-successional species *Entandrophragma angolense*. Same patterns were observed by Eschenbach *et al.* (1998), when studying selected tree species of different successional life-strategies and vertical structure of the lowland dipterocarp rainforest. All species responded to an increase to irradiance by increasing net photosynthesis rate. Most of the species reached saturating irradiance between, 230 and 534 µmol m^{-2} s^{-1}, except for the fast-growing pioneer species *Macaranga hypoleuca* and *Acacia mangium* where light saturation of net photosynthesis was reached at 1050 and 1023 µmol m^{-2} s^{-1}, respectively. The light demanding *M. hypoleuca* attained maximum A_{max} (14.2 µmol CO_2 m^{-2} s^{-1}), high LCP (19 µmol m^{-2} s^{-1}) and high saturating irradiance (about 1000 µmol m^{-2} s^{-1}) demonstrating the adaptation of this species to an open sunny environment. While the understory *Shorea xanthophylla*, had low Amax (5.93 µmol CO_2 m^{-2} s^{-1}), low LCP (8 µmol m^{-2} s^{-1}) and low saturating irradiance (483 µmol m^{-2} s^{-1}), indicating the species adaptation to shaded conditions. The photosynthesis-light conditions relationship in *A. crassna* suggest is adaptable to reforestation in areas without existing forest canopies, with maximum photosynthetic productivity occurring under moderately cloudy conditions where incident irradiance is approximately half that of full sun.

This study has demonstrated that photosynthesis in *A. crassna* becomes limited under heavy cloud (PFD 250-300 μmol^{-1} CO_2 m^{-2} s^{-1}). Graham et al. (2003) has also reported this for other rainforest trees where heavy cloud cover negatively affected CO_2 uptake because of the reduction in photosynthetic photon flux. They found that light rather than water or temperature, or leaf nitrogen affected negatively the CO_2 uptake in this conditions. Despite the lower rates of photosynthesis under low light, *A. crassna* maintains a capacity for CO_2 assimilation under very low irradiance. *A. malaccensis* has also been found to survive and grow at very low light availabilities (Soehartono *et al.*, 2002). Given the presence of diverse multistrata agroforestry systems (Michon *et al.*, 2007) that exist in some agarwood producing countries, the adaptability of leaf photosynthetic capacity to highly variable irradiance make *Aquilaria* species suitable candidates for production under such systems. Vincent (2001) found that tree species (*Shorea japonica, Cinnamomum porrectum, Lansium domesticum*) growing in a damar agroforest are adapted to a wide range of light intensities as evidenced in their maximum assimilation rate (A_{max}) response to openness. However, A_{max}

pattern varied according to the life-strategy of the species, with the understory specialist (*L. domesticum*) achieving A_{max} around 15% of light, whereas for the upper-and mid-strata species A_{max} was reached at higher light levels (30% of canopy openness). Kobe (1999) found that radial growth of four tropical seedling tree species (*Trophis racemosa*, *Castilla elastica*, *Pourouma aspera*, *Cecropia obtusifolia*) increased with light availability, and concluded that light levels was the factor that could explain differences in growth performance in the species studied.

A significant positive correlation (R^2 0.855) was found between CO_2 assimilation and PFD. The mean CO_2 assimilation rate ranged from 2 to 7 μmol^{-1} CO_2 m^{-2} s^{-1} in leaves with a mean leaf chlorophyll index of between 35 and 50. Maximum photosynthetic capacity of *A. crassna* was measured at 10.8 μmol^{-1} CO_2 m^{-2} s^{-1} for leaves with high levels of total leaf nitrogen (2.1-2.5%). Shade tolerant rainforest species *Agathis microstachya* and *A. robusta* produced a CO_2 assimilation rate of 4.8-9.8 µmol m^{-2} (Langenheim *et al.*, 1984). Similar results were obtained for rainforest species *Flindersia brayleyana* (7.7 µmol m^{-2} s^{-1}), *Argyodendron peralatum* (9.0 µmol m^{-2} s^{-1}), (Thompson *et al.*, 1992) and *Alseis blackiana* (8.3 µmol m^{-2} s^{-1}) (Pasquini & Santiago, 2012). In contrast, sun demanding tree species such as *Toona australis* (Thompson *et al.*, 1992) and *Entandrophragma angolense* (Riddoch *et al.*, 1991) generated higher CO_2 assimilation values of 12.3 and 16.9 µmol m^{-2} s^{-1}.

In *A. crassna,* LCP, expressed as the light intensity when net photosynthesis is zero, occurs at a PFD of lower than 50 µmol m^{-2} s^{-1} (Figure 5.1). Lower LCP values are found in shade tolerant species compared with both shade intolerant and light intermediate species (Craine & Reich, 2005). This suggests that LCPs can be indicative of species adaptability to low light environments. Bazzaz (1982) studied the light compensation points of 14 species with different life strategies grown under contrasting light conditions (sun and shade), and found that the LCPs of 12 of the 14 species grown in full sun had higher values than their counterparts grown in shade (1% of full sun condition), with values ranging from 4.9 µmol m^{-2} s^{-1} to 61 µmol m^{-2} s^{-1}. Kwesiga (1986) estimated the LCP for four timber species at two PFD regimes (40 µmol m^{-2} s^{-1} and 1000 µmol m^{-2} s^{-1}). Three of the studied species were known as light demanders and one as shade tolerant species in its seedling stage. The LCPs figures for three of the species (included the shade tolerant species) were similar at either low or high PFD (7 to 15 µmol m^{-2} s^{-1}). *Terminalia ivorensis* had the same value at the two PFD conditions and presented the highest LCP values (25 µmol m^{-2} s^{-1}). In Kwesiga et al. study the shade tolerant species did not have the lowest LCP.

5.6.2 Chlorophyll, leaf nutrients and photosynthesis

Positive association between leaf chlorophyll index and leaf nitrogen and other nutrients has been found across several tree species (Bauerle *et al.*, 2004; van den Berg & Perkins, 2004; Yong *et al.*, 2010). In this study a greater concentration of N, Ca and Cu were found in leaves of *A. crassna* with higher SPAD values. In respect to their effect on photosynthesis the influence of N is well documented (Geiger *et al.*, 1999; Niinemets, 1997). Calcium has many varied physiological functions in plant leaves (McLaughlin & Wimmer, 1999) including cell structure (Bolwell, 1993; His *et al.*, 1997), plant signalling (Cheng *et al.*, 2002; Gilliham *et al.*, 2011), stress tolerance (McLaughlin & Wimmer, 1999) and herbivore defence (Howe & Jander, 2008). Given the involvement of Ca in such a wide range of plant physiological functions, the association between chlorophyll index and Ca recorded in *A. crassna* in this study may not necessarily be due to variation of Ca in the chloroplast. Further research would therefore be required to determine the physiological basis for the association. Copper is an important element of the chloroplast protein plastocyanin that is involved in electron transport between photosystems II and I. Moreover, plastocyanin is the most abundant copper-based protein in plants (Katoh, 2003; Peers & Price, 2006; Yamasaki *et al.*, 2008). It is therefore likely that the chlorophyll index measured in this study is related to actual content of Cu in the chloroplasts. The positive relationship may not necessarily be related to a copper deficiency in the leaves, but to an associated reduction in chlorophyll content related to availability of nitrogen. This study demonstrates that the SPAD-502 may be useful field tool for determining broad levels of both leaf nitrogen and copper.

The study demonstrated a clear positive association between mean leaf chlorophyll index and CO_2 assimilation rate in *A. crassna* (Figure 5.4). CO_2 assimilation increased from leaves with an index value of 15 and reached its maximum at a value of 70. Chlorophyll index values for this species under cultivation have been reported to range between 35 and 55 (Page & Awarau, 2012). The rate of CO_2 assimilation increases 32% from 7.9 to 10.5 μmol m^{-2} s^{-1} in trees with a mean chlorophyll index of 35 and 55 respectively. Genetic variation in the CO_2 assimilation capacity can lead to greater net carbon gain, but this may not necessarily lead to greater yields (Driever *et al.*, 2014; Rosado-Souza *et al.*, 2015; Stitt & Schulze, 1994) as yield is influenced by efficiency of light interception and biomass partitioning (Driever & Kromdijk, 2013). Within individual genotypes and cultivars however, greater CO_2 assimilation can potentially support increased metabolism and growth. Tree volume in *Pinus taeda* was positively correlated with leaf-level light-saturated photosynthesis (Aspinwall *et al.*, 2011). In the biomass tree crop *Morus* spp. light-saturated rate of photosynthesis and leaf nitrogen content showed strong positive correlation with biomass productivity. It is therefore likely that management of nitrogen fertilisation to optimise CO_2 assimilation in *A. crassna* can lead to greater productivity. The use of a chlorophyll meter to improve the responsiveness of nutrition management of *A. crassna* plantings can potentially lead to greater fertiliser efficiency, reducing both cost of production and environmental effect.

5.7 Conclusion

This study demonstrates that photosynthesis in *A. crassna* is well suited to the light conditions of humid tropical lowland environments with maximum photosynthesis found at average midday solar radiation levels. *A. crassna* also has a high capacity for maintaining photosynthesis across a range of light intensities from heavy shade to full sun. This pattern agrees with other studies carried out in tropical forest tree species occurring in a wide range of canopy openness (Kobe, 1999; Sterck *et al.*, 2006; Vincent, 2001). This demonstrated photosynthetic adaptability means that *A. crassna* can be accommodated within a range of approaches for reforestation including native forest enrichment, incorporation into complex agroforests and agricultural and grassland reforestation. In this study SPAD readings have a positive relationship with leaf nitrogen, this pattern can be an indicator of site productivity and, be used to maintain suitable levels of leaf chlorophyll to optimise photosynthetic capacity and ultimately plant vigour. However, SPAD values need to be verified with analytical chlorophyll content to determine the relationship between SPAD and leaf chlorophyll content.

Chapter 6. Flower development, stigma receptivity and breeding system of *Aquilaria crassna* (Thymelaeaceae)

6.1 Abstract

While *Aquilaria* species have had a long history of use, there is only limited knowledge of their reproductive biology. In this study, it is described flowering and fruiting phenology as well as the main breeding system operating in *A. crassna*, as a basis for more detailed studies of its reproductive biology. Detailed understanding of flowering and fruiting patterns in this species is also required for developing techniques to assist in the process of controlled pollination. Reproduction in *A. crassna* occurs on an annual basis with the peak occurring in the dry season. The combined duration of the flowering stages was 12.3 days and fruiting stages 59.5 days. In this study, no clear association was found between the timing of flower opening and fruit set. The occurrence of the reproductive stages showed similar pattern of seasonality to natural forest, but differences in the duration of each stage was recorded. Flower anthesis typically occurred at night and flowers remained open for up to 3.5 days. The stigma is receptive when the flower is open (2.3 to 3.6 days). Hand-pollination experiments demonstrate that *A. crassna* have certain degree of self-compatibility and that there is no pollen limitation for fruit production within a monoculture planting. Results indicated that hybrids between *A. crasnna* and *A. baillonii* are possible when these are in close proximity.

6.2 Introduction

The genus *Aquilaria* produces a valuable fragrant resin, known as agarwood, widely used for the production of incense, perfume (essential oils) and for medical purposes. Several species of the genus are reported in the literature as a source of the resin. The resin develops and accumulates in the stem and branches of the tree after a process of injury (Donovan & Puri, 2004; Rasool & Mohamed, 2016). Only a few trees in natural areas (2-3% of the total population) can yield the highly sought resin (Ng *et al.*, 1997). This has led to the overexploitation of the genus in the countries of origin (Persoon, 2008; Soehartono & Newton, 2000). Efforts have been made to understand the process of resin formation with the view to developing methods for resin induction to improve the viability of cultivated agarwood (Liu *et al.*, 2013; Mohamed *et al.*, 2014a; Persoon, 2008).

While the development of resin induction techniques is central to agarwood production in plantations, other traits will also influence cultivation success. These may include growth rate, form, environmental and edaphic adaptability, propensity to respond to artificial induction, resin production and quality. Understanding the variation in these traits provides the basis for selection and is of particular significance in crop improvement. Of equal importance to crop improvement is the development of methods for capturing these traits in current and subsequent generations through vegetative and sexual reproduction. Understanding the breeding system will therefore be a key element for the domestication and improvement of *Aquilaria*.

The study of reproductive phenology is relevant for breeding activities within a domestication programme. The flowering pattern of an individual plant refers to the duration, timing and frequency of flowering, and the number and position of flowers in the inflorescence (Augspurger, 1983; Bawa *et al.*, 2003). Flowering patterns determine the mechanism of reproduction of the species, and these flowering patterns are influenced by environmental and genetic factors (Bawa *et al.*, 2003; Chapman *et al.*, 1999; Elzinga *et al.*, 2007). Despite the long-established use of *Aquilaria* species, only limited understanding exists of their biology to bring about effective domestication. Previous research into the reproductive phenology of *Aquilaria* indicates that flowers are hermaphroditic and borne on terminal branches. The duration of all reproduction stages (budding, flowering and fruiting in days) among six species of *Aquilaria* (*A. beccariana*, *A. crassna*, *A. filaria*, *A. hirta*, *A. malaccensis* and *A. microcarpa*) varied widely among species, reportedly 44.6 days for *A. filaria*, 49.4 days for *A. crassna*, 51.4 days for *A. microcarpa*, 59.3 days for *A. beccariana,* 77.7 days for *A. hirta and* 82.4 days for *A. malaccensis* (Soehartono & Newton, 2001b).

Breeding systems of seed plants range from obligate outcrossing, mixed mating (simultaneous outcrossing and selfing) to predominant selfing (autogamy) (Barrett *et al.*, 1996). Perennial woody plants are often outcrossing due to the presence of incompatibility systems and inbreeding depression (Morgan *et al.*, 1997; Petit & Hampe, 2006). The results of fruit set in bagged flowers (to limit open pollination) of three species of *Aquilaria* (*A. filaria*, *A. malaccensis* and *A. microcarpa*) demonstrated no capacity for autogamous pollination (Soehartono & Newton, 2001b) While information about specific aspects of incompatibility in the genus is missing, several studies of pollen vectors, which influence gene transfer among individuals, suggest insect pollination to be predominant. Principal pollinators reported for *A. crassna*, *A. filaria*, *A. malaccensis* and *A. microcarpa* are nocturnal insects belonging to Pyralidae and Lymantridae family, also diurnal visitors have been reported belonging to Euminidae and Specidae family (Soehartono & Newton, 2001b; Tasen *et al.*, 2009). Other insects recorded as visitors of *Aquilaria* flowers are bees belonging to Apidae and Halictidae families (Hymenoptera), and butterflies of the Pieridae family (Lepidoptera) (Tasen *et al.*, 2009). Tasen et al. and Soehartono & Newton also reported that natural pollinators tended to visit the flowers mainly at night and early morning. Less work has been conducted on seed dispersal agents, with Soehartono and Newton (2001b) reporting limited seed dispersal in natural forests with the majority of seed produced dispersed within only a few meters of the tree.

This study was undertaken to improve our understanding of reproductive biology in *A. crassna*. The aim of this study was to (i) identify and describe the distinct morphological stages in the development of flowers (ii) record the timing and duration of each flower stage in *A. crassna*, (iii) determine the onset and duration of stigma receptivity and (iv) determine levels of self- and cross-compatibility within *A. crassna*, and cross-compatibility of *A. crassna* with *A. baillonii* and *A. subintegra*.

6.3 Methodology

6.3.1 Reproductive phenology

Two discrete experiments were conducted to describe and quantify reproductive phenology in *A. crassna*. The first was conducted between October and December 2012 and the second between October and January 2016.

6.3.1.1 Phenology 2012

The flowers and fruit in seven inflorescences from three trees of *A. crassna* were observed and images taken between October and December 2012. All inflorescences were photographed across three timing intervals 1) daily over a period of 30 days, 2) every two days the next 12 days and 3) every four days the next 45 days. A simple development scale for the characterization of flowering and fruiting pattern was determined based on visual changes identified in the sequence of photos. The duration (in days) of each stage was recorded from the period of anthesis to flower fall or fruit maturity (seed production) for all flowers in each inflorescence. The day of anthesis for the first flower in a given inflorescence was considered Day 1 for the development of the inflorescence and in-frutescence. Flowering and fruiting stages were defined from these observations on the basis of morphological changes in these structures.

6.3.1.2 Phenology 2016

The sequence of flower and inflorescence development was assessed in single inflorescences on three different trees of *A. crassna* growing in an open environment. Photographs were recorded twice daily (0700 and 1900 hrs) over the reproductive season (October-January) in 2015/16. The duration (in hours and days) of each floral stage was recorded for each flower from the period of pre-anthesis to flower or fruit shed or seed production.

6.3.2 Stigma receptivity

To study stigma receptivity a modified procedure from Dafni and Maués (1998) was used to identify esterase activity using a Peroxtesmo esterase indicator paper liquid (one paper+1 ml water). One indicator paper of Peroxtesmo-Ko- peroxidase (15×15 mm) was soaked in 1 ml of distilled water and a droplet of the solution was applied directly onto the stigma. From each species, 15 flowers at 3 different developmental stages (opening, fully open and closing/senescing flowers, Plate 6.1) were examined to determine receptivity (Heslop-Harrison & Shivanna, 1977). A dissecting microscope (x 40) was used to detect any reaction to the solution. The appearance of a blue colour indicates receptivity. Peroxidase activity was recorded as presence or absence of colouration on the stigmatic surface.

Plate 6.1 Three different stages of flower development in *Aquilaria crassna* at which stigma receptivity was examined. (a) Flowers at anthesis. (b) Flowers open. (c) Flowers closing/senescing.

6.3.3 Breeding system

The mating system of *A. crassna* was evaluated through a series of controlled hand pollinations as described by Bittencourt Jr and Semir (2004) and Oliveira *et al.* (1992). These were conducted in individuals growing in a plantation located near Innisfail, north Queensland, Australia (17.52°S, 146.02°E) during two consecutive years (2014 & 2015). Fourteen individuals were evaluated in

2014 and 20 individuals in 2015 (Table 6.1). In the 2014 event, whole inflorescences were enclosed in bud stage using pollen proof Durabweb® bags (150 x 200mm, 12 individuals) or fine mesh bags (200 x 250 mm, 2 individuals). In 2015 only fine mesh bags were used to isolate the inflorescences and two techniques of hand pollination were tested in this year, hand-pollination in advanced bud stage (estimated to be within 24hrs of opening) and open flowers (enclosed at bud stage to prevent open pollination) (Plate 6.1).

Table 6.1 Pollination experiments, number of trees, inflorescences and flowers evaluated in *A. crassna* populations in two consecutive years (2014 & 2015).

Year	Experiment	# Trees	Total Inflorescences	Total Flowers
2014	Bag control (Spontaneous self-pollination)	3	29	143
2014	Controlled-Self	4	22	47
2014	Intra-Cross	4	16	50
2014	Open Pollination	3	55	331
	Subtotal 2014	**14**	**122**	**571**
2015	Bag Control (Spontaneous self-pollination)	4	41	211
2015	Bud-Controlled Self	4	54	105
2015	OF-Control-Self	2	17	45
2015	Bud-Intra-Cross	6	102	183
2015	OF-Intra-Cross	4	28	48
2015	Bud-Inter-Cross-CxB	4	56	85
2015	Bud-Inter-Cross-CxS	4	56	123
2015	OF-InterC-CxB	2	21	38
2015	OF-InterC-CxS	3	15	21
2015	Open Pollination	20	227	956
	Subtotal 2015	**20†**	**617**	**1815**

†In 2015 different pollination experiments were performed on the same tree. Pollination experiments in 2015 conducted at two stages of flower development open flowers and buds. OF denotes= open flowers

In both years, flowers were either hand pollinated using pollen from anthers of the same flower or another flower of the same tree (controlled self-pollination), or cross-pollinated with pollen from a distant tree of the same species (intraspecific cross-pollination). In 2015 controlled pollination of *A. crassna* with pollen from other species (*A. baillonii* and *A. subintegra*) was also conducted. The interspecific crosses were in one direction, with *A. crassna* as the female parent as it was the most abundant species in the plantation. Each tree, branch and inflorescence in both years was allocated a unique code according to its species and pollination treatment (open, spontaneous self- (bag control), controlled self-, intraspecific cross- and interspecific cross-pollination). Hand pollination was carefully done covering the whole surface of the stigma with pollen. Candidate trees were selected based on information derived from a previous study (AL-S unpublished) as well as those identified as being productive seed producers. Fruit set was recorded at fruit initiation stage in 2014. In 2015, seed production from the controlled pollination experiments was recorded and seeds collected to check for embryo availability. For natural fruit set, flowers were tagged and monitored until fruit set and the presence or absence of fruit per inflorescence recorded.

6.3.4 Data analysis

Images were labelled and stacked using ImageJ and PaintNet. Floral stages were defined by direct observation of flower morphology and assessment of images. The duration of the flowering stages was summarized using the time-labelled sequence of images. The differences in peroxidase activity across each flower developmental stages were analysed using a generalized linear model with the glm () function of the statistical package R (R Core Team, 2015). Model fit was assessed using overdispersion statistics. A simple model was fitted (colouration ~ flower stage). The relationship between the number of fruits produced and pollination treatments was presented as a percentage of success or failure in fruit production. Due to the nature of the results obtained it was not possible to analyse any interaction between fruit production and pollination treatments [the data contained too many zeros (failures) in the response variable] or year because different individuals were analysed each year (based on available flowers).

6.4 Results

6.4.1 Flower development

Four distinct stages of flower development were identified (opening, open, closing and closed (Plate 6.2). Anthesis in *A. crassna* generally occurred between 5:00 pm and 7:00 am (Plate 6.3), although some flowers open during the daylight hours of the morning. The process of separation of the tepals until flower is completely open may last up to one hour. Anthers change colour from yellow to orange-brownish after 23 h (range 11-48 h) of flower opening (Plate 6.4). Flower remained open for an average of 2.13 days (range 1-3 days) in 2012 and 3.6 days (range 2-4 days) in 2015 (Table 6.2). The open stage is concluded as the flower tepals begin to close (closing stage), and anthers begin to wilt, stigma turns orange leading to a closed stage of flower development. At the closed stage 'non-fruiting' flowers can persist in the inflorescence between 1 and 18 days until flower fall (Table 6.2). And flowers in which fruit initiation had occurred remained closed between 16 to 25 days (the tepals will remain attached to the developing fruit, Plate 6.5) (Table 6.2). Flower fall occurred in average at day seven (range 1-20 days) after flower opening in 2012 and at day five (range 3-8 days) in 2015. Two stages were identified in the fruit formation: fruit initiation and fruit development (until seed set) (Plate 6.5). Fruit initiation was recorded when there is evidence of size increment within the ovaries, stigma scar prominent; tepals expand to cover a quarter of the fruit. Fruit development can be recognised when fruits are rounded in the apex, with the tip extending beyond the 'level' of the tepals; tepal colour changes slightly to brown. The duration from the beginning of fruit initiation until fruit opening ranged from 30 to 66 days (Table 6.2).

Plate 6.2 (a) Buds ready to open (yellow arrow), opening stage. (b) Anthesis (open stage). (c) Floral developmental stages captured in a inflorescence of *Aquilaria crassna* [blue arrow= anthesis; purple arrow= closing stages, anthers wilted (orange-brownish); red arrow= closed stage (tepals enclosing ovary), fruit initiation or flower fall].

Plate 6.3 Flower development in a sequence of 36 hours shoot in an inflorescence of *Aquilaria crassna*.

Plate 6.4 Anthers wilted (change in colour from yellowish to orange-brownish) after a range of 11 to 48hrs after flower opening in *Aquilaria crassna* inflorescence.

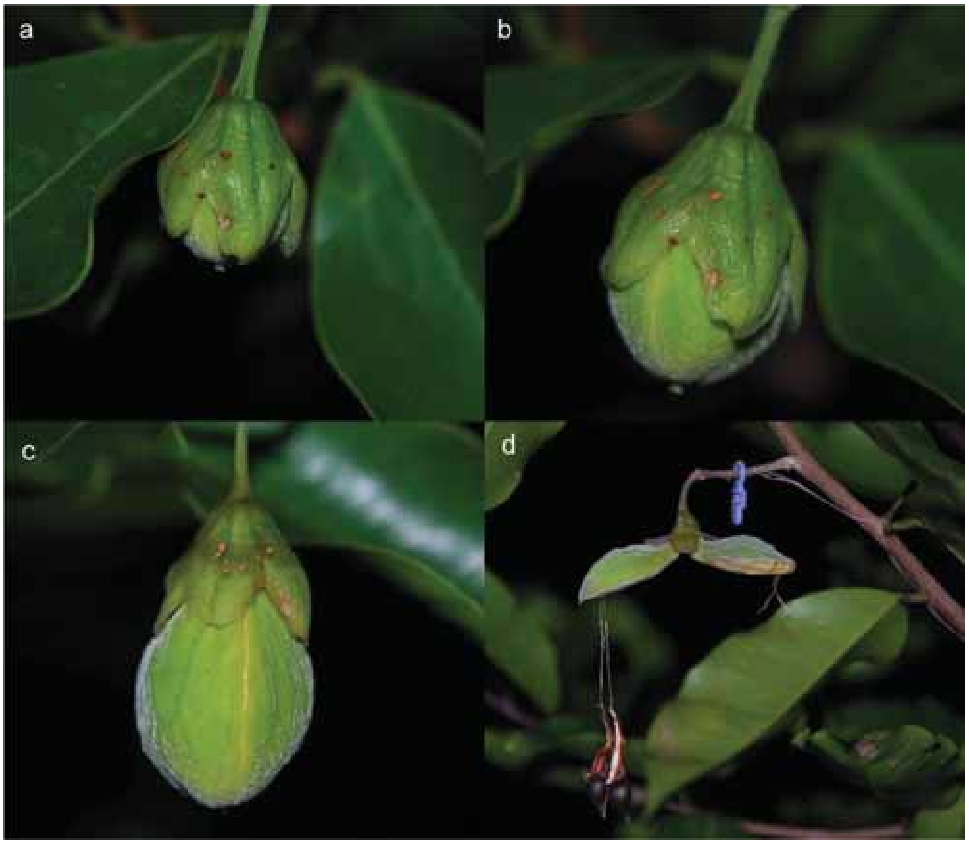

Plate 6.5 Fruit developmental stages identified in *Aquilaria crassna* (a) late stage of fruit initiation where the tip of the swollen ovaries almost reaches the level of the tepals, (b) early- to mid-fruit development stage where the ovaries have continued to expand beyond the level of the tepals (c) late fruit development and (d) fruit open with seed hanging from their arils by delicate threads (duration of hanging varies can last from several hours to days depending on prevailing wind conditions).

Table 6.2 Mean duration (in days) of flowering and fruiting stages in nine inflorescences from seven trees of *Aquilaria crassna* in north Queensland, Australia# in 2012 and 2015.

	2012								2015				
Infl.	**a**	**b**	**c**	**d**	**e**	**g**	**f**	**Mean Year^{-1}**	**h**	**i**	**j**	**k***	**Mean Year^{-1}**
N days flowers opening	1±0 [10]	1±0 [7]	1±0 [8]	1 [1]	1±0[5]	1.25 ±0.5 [4]	na	1.04 ±0.1	0.43 ±0.5[7]	0.12 ±0.35 [8]	0.12 ±0.35 [8]	na	0.23 ±0.18
N days flowers open	2.10 ±0.57 [10]	2.29 ±0.76 [7]	1.70 ±0.48 [10]	2.33 ±0.58 [2]	1.56 ±0.73 [9]	2.80 ±0.45 [6]	na	2.13 ±0.45	3.29 ±0.69 [7]	3.88 ±0.35[8]	3.63 ±0.52 [8]	na	3.6 ±0.3
N days flowers closing	1.55 ±0.88 [9]	1.00 ±0 [5]	1.14 ±0.38[7]	1.00 [1]	1.00 ±0 [3]	2.13 ±0.83 [9]	na	1.30 ±0.46	1 ±0.49 [7]	0.63 ±0.52 [8]	0.75 ±0.71 [8]	na	0.79 ±0.19
Total flower stages	4.65	3.29	3.84	4.33	2.56	6.18	-	4.1 ± 1.25	4.73	4.63	4.5	na	4.62 ± 0.11
N days flowers closed to flower fall	8.57 ±6.65 [7]	10.25 ±6.29[4]	8 ± 4 [4]	2 [1]	4±3 [3]	11.5 ±4.59 [6]	na	7.39 ± 3.67	0.67 ±0.57 [3]	1 ± 0 [3]	2 ± 0.82 [4]	na	1.22 ±0.69
N days flower closed until fruit initiation	25 ±1.41 [2]	-	-	-	-	-		25 ±1.41	-	18 [1]	-	16 [1]	17 ±1.41
N days fruit initiation	4 ±0 [2]	-	-	-	-		8.25 ±2.7 [4]	6.12 ±3		5 [1]		4 [1]	4.5 ± 0.71
N days fruit development	40 ±5.66 [2]	-	-	-	-		58 ±4.32 [4]	49 ±12.72		25 [1]		51 [1]	38 ±18.38
Total days fruit formation until seed sett	44 ±5.66	-	-	-	-		66.25 ±3.8	55.12 ±15.73		30 [1]		55 [1]	42.5 ±17.68

= values are means, Infl.: inflorescence; ± = standard deviation; [ ] = N flowers or fruit; na: data no available; N values correspond to the number of flowers identified in each stage. * Values correspond to fruit development in a hand cross pollination experiment. Some flowers missed one of the stages of flowering development stages.

6.4.2 Stigma receptivity

The stigma was found to exhibit staining (indicating esterase activity) in 20, 100 and 27% of flowers at opening, fully open and closing stages respectively (Table 6.3). At closing stage, the stigmatic surface normally turns orange so it is difficult to distinguish any variation in colour, only in four flowers (27%) was it possible to identify changes in colour. Flower developmental stages had a significant effect on the incidence of stigma staining (Wald$_{test}$ X^2 = 10.7, df = 2, P (> X2) = 0.0048) with a significantly greater incidence recorded in flowers in the open stage (Table 6.4) compared with those in either opening or closing stages. The model testing for the effects of flower developmental stages on stigma receptivity has X^2 = 17.63678 with $p < 0.001$. In the case of having a flower in open stage *v* opening stage, increases the odds of a receptive stigma by a factor of 26 (Table 6.4).

Table 6.3 Esterase activity recorded as a presence or absence of colouration in the stigma surface as indicative of receptivity in three stages of flowers development in cultivated *Aquilaria crassna* (n= 15 flowers per flower stage).

	Opening		**Open**		**Closing**	
Species	Presence	Absence	Presence	Absence	Presence	Absence
A. crassna	3 (20%)	12 (80%)	13 (87%)	2 (13%)	4 (27%)	11 (73%)

Table 6.4 Regression coefficients of a logistic regression model using glm () function testing for the effects of flower developmental stages on stigma receptivity of cultivated *Aquilaria crassna*.

Coefficients	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**	**OR**
(Intercept)	-1.3863	0.6455	-2.148	0.03174*	0.250000
Open stage	3.2581	0.9968	3.269	0.00108 **	26.00000
Closing stage	0.3747	0.8704	0.430	0.66684	1.454545

OR= odd ratios. Null deviance: 61.827 on 44 degrees of freedom. Residual deviance: 44.190 on 42 degrees of freedom. AIC: 50.19. Number of Fisher Scoring iterations: 4.

6.4.3 Breeding systems

Percentage fruit set recorded in this study was greater in open-pollinated flowers than hand cross-pollinated flowers across both years (Table 6.5). The natural fruit set exceeded that of hand cross-pollinated flowers almost by a factor of three in 2014 and by a factor of five in 2015. 0% of flowers set fruit when *A. crassna* was tested for spontaneous self-pollination (bagged but not pollinated) and hand self-pollination in 2014. In 2015, 1.4% of fruit production was recorded in self-pollination experiments and 2.2% for hand self-pollination performed in open flowers (Table 6.5, Plate 6.1). Intraspecific crosses reached between 2.1% (2015) to 6% (2014) of fruit set. Interspecific crosses between *A. crassna* and *A. baillonii* reached 4.7% of fruit set, whereas interspecific crosses between *A. crassna* and *A. subintegra* did not produce any fruit. Values for fruit set by pollination technique (at bud stage or open flower), were similar for intraspecific and interspecific crosses, however in the case of controlled self-pollination only for open flower pollination technique fruit production (2.2%) was recorded (Table 6.5, Figure 6.1). Flowers dried out and fell off after a few days following hand self-pollination or bagging.

Table 6.5 Number of fruit set and seed produced by cultivated *Aquilaria crassna*.

	2014					2015				
Experiment	N trees	N flowers	N fruits	N seeds	% success	N trees	N flowers	N fruits	N seeds	% success
Spontaneous self-pollination **(Bag control)**	3	143	0	na	0%	4	211	3	2	1.4%
controlled self-pollination **(bud pollination)**	4	47	0	na	0%	4	105	0	0	0%
Hand self-pollination **(flower pollination)**	na	Na	na	na	na	2	45	1	*	2.2%
Intraspecific cross pollination **(bud pollination)**	4	50	3	na	6%	6	193	5	5	2.6%
Intraspecific cross pollination (flower pollination)	na	Na	na	na		3	48	1	2	2.1%
Interspecific cross pollination **(bud pollination Ac x Ab)**	na	Na	na	na		4	85	4	5	4.7%
Interspecific cross pollination **(bud pollination Ac x As)**	na	Na	na	na		4	123	0	0	0%
Interspecific cross pollination **(flower pollination Ac x Ab)**	na	Na	na	na		2	38	2	0	5.2%
Interspecific cross pollination **(flower pollination Ac x As)**	na	Na	na	na		3	21	0	0	0%
Open pollination	3	331	48	na	(14.5%)	18	956	446	576	46.7%

na= no data available. *seed missing at the time of counting. Ac= *Aquilaria crassna*, Ab= *A. baillonii*, As= *A. subintegra*

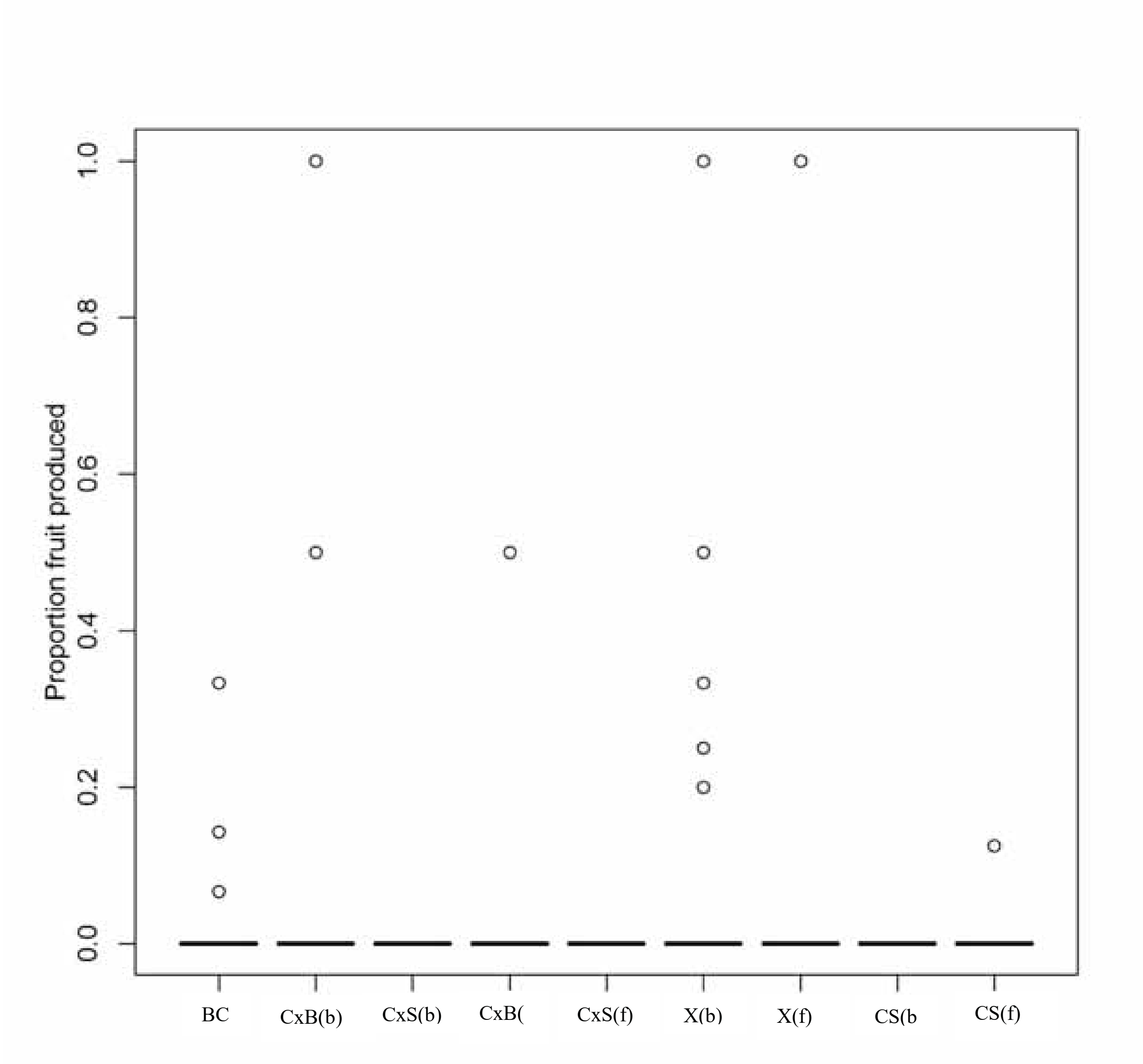

Figure 6.1 Proportion of fruit produced by experiment and both year of data collection (where, BC=bag control/spontaneous self-pollination, CxB= inter-crosses *A. crassna* x *A. baillonii*, CxS= inter-crosses *A. crassna* x *A. subintegra*, X= intra crosses, CS =control self-pollination,). Letters in parentheses denote the flower stage at time of controlled pollination (b)=buds and (f)=flowers

6.5 Discussion

6.5.1 Reproductive phenology

Aquilaria crassna trees in north Queensland flower between October to mid-November (at the end of the dry season and the beginning of the wet season). Flowering in the dry season has been recorded for other *Aquilaria* spp. growing in natural and cultivated environment in Indonesia (Soehartono & Newton, 2001b). In this study, fruit development took place during the wet season

(November-December). Given the limited seed dispersal found for other species of *Aquilaria* (Soehartono & Newton, 2001b), it may be assumed that the primary method of dispersal is wind and gravity. In a sub-montane rain forest in the Philippines it was found that fruit dispersed by the effect of wind or gravity had largest period of fruiting matching with season of heavy rain (typhoon season) (Hamann, 2004). Observations made during this study of different insects (ants, beetles and cockroaches) consuming and fungi growing from (during periods of high humidity) the seed's fleshy aril suggest it may be energy rich and have a possible role in seed dispersal. Seed arils are found to promote dispersal in species/genera such as Acacia (Davidson & Morton, 1984), *Trichilia cuneata* (Foster & McDiarmid, 1983), *Virola surinamensis* (Howe, 1983), and *Cymbopetalum baillonii* (Coates-Estrada & Estrada, 1988). The role of the aril in insect mediated dispersal of *Aquilaria* seed, particularly within a species natural distribution, is something that would be of further research interest.

The inflorescence of *A. crassna* can have up to seven flowers that open gradually and sometimes synchronized (two flowers open at one time) and differently orientated (Plate 6.2, Plate 6.3). These traits combined with longevity of the flower (stay open for 2-4 days) may help in the attraction of and facilitate access for pollinators and reduce the cost of selfing as suggested by other researchers (Goodwillie *et al.*, 2010; Johnston, 1991; Roccotiello *et al.*, 2009). Anthesis in *A. crassna* generally occurs in the evening and early morning. In other members of the Thymelaeaceae family it has been reported that anthesis occurs at dusk (Okamoto *et al.*, 2008). The stigma is receptive during the open stage of the flower development (Table 6.3 Plate 6.1). Pollen shed is largely completed on average within 23 hr after anthesis (Plate 6.4). In this research was observed a higher level of flowers' visitors at night in comparison with low level of visit of insects during the morning. This trend has been observed by other researchers when studying visitors of *Aquilaria* spp. flowers (Soehartono & Newton, 2001b; Tasen *et al.*, 2009). More research is required to quantify foraging activity of flowers' visitors in *A. crassna* growing in Queensland.

The duration of flowering and fruiting documented for *A. crassna* in this study are similar to other species of the genus *Aquilaria* growing in plantations. For example, *A. crassna* flowering stage in our study lasted 2 to 4 more days, respectively, than the reproductive stages reported for *A. crassna* and *A. filaria* growing in Indonesia (Soehartono & Newton, 2001b). Similar patterns in duration (number of days) of flowering were observed in *A. hirta*, *A. malaccensis* and *A. microcarpa.* Fruit development in our study ranges from 30 to 66 days; similar to what has been reported by Soehartono and Newton (2001b) for *A. crassna*, *A. filaria*, *A. microcarpa*, *A. beccariana*, *A. hirt*a, and *A. malaccensis*.

6.5.2 Breeding systems

6.5.2.1 Open pollination

Flowers of *A. crassna* in this study are not pollen- limited as evidenced by the open pollination treatments (14.5% success in 2014 and 46.7% success in 2015). This result is contrary to the breeding system of other plants, where > 60% of plant population is pollen limited (expressed on percent of fruit set) (Burd, 1994; Knight *et al.*, 2005). However, these results are similar to those reported by Harmon-Threatt *et al.* (2009), where they found that the majority of the plants (native and introduced) studied were not pollen-limited. This study also differed greatly to what has been reported for *A. crassna* growing in plantation and natural stands, Tasen *et al.* (2009), showed that the ranges of fruit set in open pollination were of 2 to 3% for natural population and plantation, respectively.

The high success in open pollination experiments in this study may also be associated with resource availability, as the study population is growing in a managed environment. Salinas-Peba and Parra-Tabla (2007) found that *Manilkara zapota* (L.) P. Royen growing in home gardens had greater fruit production in comparison with trees growing in forest, even though flower production was similar at both sites. They attributed this difference to resources being limited for fruit production in the forest. This study also suggests that introduced *A. crassna* has adapted successfully in its introduced range by producing fruit and setting seeds (Table 6.5). Harmon-Threatt *et al.* (2009) mentioned that the successful establishment of introduced plants may be influenced by self-compatible system, as a certain degree of autogamy provides reproductive assurance and also that pollinators are available to provide the service of pollen transfers. In this study, even though there was no systematic record of flowers' visitors, a higher presence of pollinators in *Aquilaria* flowers was observed at night; this observation agrees with other researchers (Soehartono & Newton, 2001b; Tasen *et al.*, 2009) who noted that the most abundant visitors to *Aquilaria* flowers were insects with nocturnal behaviour.

6.5.2.2 Controlled pollination experiments (self-pollination and intra-inter crosses)

High rate of failure in the hand-pollination experiments was recorded in flowers of *A. crassna* in both years, more than 90% of the flowers fell and similar results (failure to produce fruit) were obtained if the hand-pollination was carried out in buds (closed but about to open) or recently open flowers (2015 data, Figure 6.1), suggesting that the technique did not influence the results. One possible explanation for the low rates of fruit set in controlled-pollination treatments are that flowers are sensitive to handling and bagging suffered desiccation after pollination occurred, suggesting that further refinement of controlled pollination techniques would be required before it could be used routinely in plant breeding. Young and Young (1992) suggested possible reasons as to why low reproductive success is achieved in controlled pollination experiments in comparison with natural pollination, for instance, mechanical damage can be effected on the stigma of the flowers during the process of hand-pollination, also the bagging process can negatively influence the results.

Using the index of self-incompatibility (average fruits self-experiments divided by average fruit intra-cross experiments) developed by Ruiz and Arroyo (1978), was found that *A. crassna* is self-compatible (ISI= 0.33), as a value equal or lower to 0.2, the species is categorized as self-incompatible. However, the number of individuals producing fruits following intraspecific cross-pollination (43% of trees produced fruits, data is for the two seasons of evaluation) was greater than the number of trees that produced fruits after self-pollination (24% of trees produced fruits, data is for the two seasons of evaluation). Further research is required to evaluate the viability and vigour of seed produced by self-pollinated trees as in this research it was not possible to evaluate seed germination of controlled pollination experiments. The variation in the level of apparent self-incompatibility between individuals has also been demonstrated for two species of *Santalum*, such as *S. lanceolatum* where only 20% of genotypes had a capacity for producing self-seed (Tamla *et al.*, 2011) and *S. spicatum* with a high level of inbreeding in one family, but a high mean outcrossing rate (95.2%) in all remaining families (Muir *et al.*, 2007). *Ceiba pentandra* populations occurring in a tropical forest fragment had a mixed-mating system, with trees being partially self-compatible and with values for outcrossing of 40% (Quesada *et al.*, 2004).

The results of the controlled-cross pollination experiments in this study are consistent with other studies of *A. crassna* breeding system. Tasen *et al.* (2009) showed that the maximum of fruit production attained in controlled-cross pollination experiment was 3% to 4%. In this study the ranges of fruit success for intra-crosses (6% in 2014 and 2.1 (flower pollination) to 2.6% (bud pollination in 2015) are similar to those reported by Tasen. Inter-crosses (*A. crassna* x *A. baillonii*) were more successful than intra-crosses with estimated fruit production of 5%. Self-pollination (1.4-2.2% for spontaneous self-pollination and hand-self-pollination, respectively) was recorded in *A. crassna* growing in Queensland. Tasen *et al.* (2009) reported as well low rates of fruit set for self-pollination in both plantation and natural forest (0.53%). However, Soehartono and Newton (2001b) showed that spontaneous self-pollination did not occur in this species. This study suggests that a certain degree of autogamy occurs in *A. crassna*.

6.6 Conclusions

Flowering and fruiting patterns of *Aquilaria crassna* can be described using simple development scale based on visible changes experimented by the reproductive structure. Flowering period is concentrated in the dry season and fruiting period in the wet season. The duration of flowering and fruiting stages of *A. crassna* can last up to 9 weeks and were in similar ranges to that the reported in other studies of its conspecific. Flowers of *A. crassna* are remain open > 3 days and anthesis generally occurs between 6:00 pm and 7:00 am with some flower opening during the daylight in the morning. Four developmental floral stages were identified: opening (0.2 to 1 day), open (2.3 to 3.6), closing (0.8 to 1.3 days) and closed-flower fall (1.4 to 7.4) or closed-fruit development (17 to 25 days). Percentage fruit set in controlled pollinated flowers was generally lower (0% to 6%) than for open pollinated flowers (14.5 to 46.7%). The high level of fruit set in open pollinated flowers of *A. crassna* demonstrates that the planted population in Queensland does not appear to be affected by pollen-limitation. Variation in seed set following self-pollination in *A. crassna* with one of seven plants producing 'self' seed may indicate the presence of self-incompatibility in this species that is not necessarily expressed in all individuals. Hybridization between *A. crassna* and *A. baillonii* was recorded in this study but not between *A. crassna* and *A. subintegra*.

Chapter 7. General Conclusions and Summary

7.1 Introduction

Tree cultivation is recognized as a fundamental practice in sustainable forest management and meets a wide range of social, economic and environmental purposes (Constanza *et al.*, 1998; Kanninen, 2010; Paquette & Messier, 2010). The cultivation and domestication of wild species (Leakey & Newton, 1993) such as agarwood can help provide an alternative source for its products, which permits the ongoing protection of conservation areas. The domestication process is of particular importance since it can improve the productivity of the plants and the efficiency of cultivation. Successful tree reforestation programs (i.e *Eucalyptus*, *Pinus*, *Acacia*) are examples of the understanding of key factors to achieve maximum productivity and adaptation to the cultivated conditions. Productivity (expressed in growth rate or dry matter production) in a plantation is a function of management regime (including silvicultural practices) (Gonçalves *et al.*, 2004) and the genotype (Acquaah, 2007).

Domestication follows a series of stages, including the identification and characterization of plant genetic resources, germplasm conservation, selection, propagation, breeding and cultural management (silviculture) (Leakey & Tchoundjeu, 2001). This study has addressed key knowledge gaps in our understanding in four of these areas for agarwood. (1) Characterised morphological variation that defines three commercially important species of *Aquilaria* (*A. baillonii*, *A. crassna* and *A. subintegra*). (2) Determined the relative contribution of leaf development and physiological parameters on the commercially important characters of height and diameter increment. 3) Defined the photosynthetic response to solar irradiation and the influence of nitrogen on photosynthetic output. (4) Characterized important aspects of the reproductive phenology and biology of *A. crassna*.

Several *Aquilaria* species were introduced in tropical Australia to source the highly- sought fragrant resin. The rare resin is produced by species of the genera *Aquilaria*, *Gyrinops Aetoxylon*, and *Gonystylus*. However, *Aquilaria* species are the preferred species as a producer of agarwood, mainly because of the high- quality of the resin and the high prices paid for the premium quality. In other regions (mainly in the areas of natural distribution), *Aquilaria* species have experienced a boom in cultivation (i.e. Lok *et al.*, 1999; Nakashima *et al.*, 2005; Persoon, 2008; Rahman *et al.*, 2011; Soehartono *et al.*, 2002; Suharti *et al.*, 2011; Uddin *et al.*, 2008; Xiao-xia *et al.*, 2010) as an initiative to supply the demand for agarwood. Successful cultivation and domestication of the species is essential to ensure sustainability of the activity and reduce the pressure in the natural stands.

7.2 *Aquilaria* cultivation/domestication

In this study one species of the genus *Aquilaria*, *A. crassna*, is examined in detail. The species chosen is one of the widely-planted species in the area of natural occurrence (Figure 2.2). The commercial value of agarwood and its steady market was another factor to conduct this study. For example, in Indonesia the economic value of agarwood plantations it is expected to be worth of more than 97 million US dollars in 2020 (Turjaman & Hidayat, 2017). In Bangladesh the net present value estimated for 1 ha of agarwood plantation was US $ 62,820 (Rahman *et al.*, 2015). Other tree species that produce non-timber forest products (i.e. resin, latex, oils, and fruits, among others) have been brought to cultivation. Examples include the domestication at industrial level of the valuable rubber tree (*Hevea brasiliensis*) (Priyadarshan *et al.*, 2009; Schultes, 1993); the well-known coffee (Anthony *et al.*, 2002; Philippe *et al.*, 2009) and cacao tree crops (Clement *et al.*, 2003; Motamayor *et al.*, 2002); the recognized litchi (*Litchi sinensis*) for fruit consumption (Sarin *et al.*, 2009); damar gardens (*Shorea spp.*) cultivated in complex agroforest for the production of resin (de Foresta *et al.*, 2004); production of sandalwood (*Santalum* spp.) for its resin (Arun Kumar *et al.*, 2012) and several medicinal plants cultivated in agroforestry systems or as understory crop in forest or plantations in different parts of the world (Rao *et al.*, 2004).

The use of agarwood has a very long history of use across multiple religions and cultures since ancient times. Agarwood's place in history was a part of people's insatiable desire for aromatic products. There is little doubt however that agarwood has been of the most revered of all aromatics. This is evidenced by its widespread use as an item for tributes, offerings and gifts for important people as well as celebrating important events. The fragrance of agarwood is used as a scent of both prestige and sensual attraction for both male and female alike. Its prevalence also in spiritual customs of many prominent religions demonstrates the very high status of and regard for agarwood. People's insatiable appetite for agarwood has resulted in trees being harvested until almost exhaustion of its wild sources. This has been evidenced in other fragrant products with a long history of use, including cinnamon (Gilboa & Namdar, 2015; Meinen, 2002), frankincense and myrrh (Gebrehiwot *et al.*, 2003; Groom, 1981), musk (King, 2007), sandalwood (Arun Kumar *et al.*, 2012; Gillieson *et al.*, 2008; Rashkow, 2014) among other aromatics.

Biological variability in a population is required to begin with the process of selection of the individuals with the desirable variants or traits. Variability in *Aquilaria* species was measured using morphological traits and herbarium keys to assist in the identification of the putative species growing in north Queensland. In this study, the use of leaf-outline analysis using elliptical Fourier descriptor as a technique was sufficient to corroborate that three species: *A. crassna*, *A. subintegra* and *A. baillonii* are present in the state. This technique proves to be an efficient tool in grouping the variability of leaf shape and discriminate species in the genus *Aquilaria*. However, a large sampling protocol that includes leaf samples from trees growing in areas of natural occurrence is required to validate the technique. The lack of homologous points in leaves of *Aquilaria* species limited the use of other techniques (i.e. modern morphometrics) to study leaf variation. Molecular analysis of the *Aquilaria* species will confirm the genetic variation of the species. It is important to have a capacity to quickly determine the species composition of a plantation and/or breeding program. This is important since it would be expected that different species will have different agronomic characters as well as product qualities (agarwood formation) that will influence the commercial viability of its production.

In the *Aquilaria* population, one of the traits of interest is growth (diameter and height increment) as a pre-requisite to induce the formation of agarwood in cultivated stands. To make the selection process more efficient and precise, rigorous techniques are used in addition to the techniques practiced in early selection (skill, judgment and intuition). The analysis of functional leaf traits is a useful tool to evaluate tree functioning (performance) (Bussotti & Pollastrini, 2015; Poorter & Bongers, 2006; Sterck *et al.*, 2006). Leaf functional traits are the expression of an organism's physiological and ecological function (Bussotti & Pollastrini, 2015). According to the results of this study, there is a relationship between growth and leaf functional traits and is illustrated by the model that explain diameter variability (as a growth parameter). Carbon and nitrogen isotopic composition of dry-leaf material, petiole length, specific leaf area and number of new leaves per shoot per week were the parameters that could explain growth performance in *A. crassna*. Stable carbon and nitrogen isotopes and morphological leaf traits are useful to assist with selection of superior individuals. More research is required to elucidate the role of stomatal conductance in *A. crassna* plantations; also, trees need to be evaluated for other traits related to physiological processes and their heritability.

The use of SPAD- 502 Chl meter is considered a suitable indicator for leaf nitrogen content and site productivity in tree species (Bonneville & Fyles, 2006; Bussotti & Pollastrini, 2015; Marenco *et al.*, 2009; Vincent, 2001). In this study leaf chlorophyll content estimated using SPAD-502 Chl meter, was found to have strong correlation with leaf nitrogen content and carbon uptake in *A. crassna*. This is useful information to growers that provides a rapid tool to estimate the current state of leaf nutrient content and provide an insight of site productivity. SPAD values obtained in this research need to be correlated with analytical chlorophyll content to determine the relationship between the SPAD-values and Chl content. Further research to improve the accuracy of the SPAD values include the study of the nature of the relationship between SPAD values and reported morphological leaf-traits (i.e leaf thickness, specific leaf area, leaf water content) that affect SPAD readings. Also this study confirms that *A. crassna* can grow under a wide range of light conditions. This evidence gives the opportunity to growers to accommodate the species under different canopy conditions.

7.3 Breeding system in *A. crasnna*

A. crassna flowering and fruiting patterns are similar to its conspecific (Soehartono & Newton, 2001b; Tasen *et al.*, 2009), flowering season started at the end of the dry period and fruit development occurred during the wet season. Any activity regarding the manipulation of the breeding system of *A. crassna*, *A. baillonii* and *A.* subintegra need to be carried out in October when the species are in full bloom. Introduced or exotic species are sometimes successful in ensuring fitness traits (reproduction, viability). Harmon-Threatt *et al.* (2009) evidenced that introduced plants somehow developed a self-compatible system, ensuring a certain degree of autogamy provides reproductive assurance. In this study, flowers of *A. crassna* had a high rate of fecundity in both years of observation; however, fruit production in 2015 was strikingly superior to that in 2014. This difference can be attributed to genotype variability and/or agronomic practices (i.e. insect pest control that also could affect pollinator populations). Further research is required to determine the factors that could influence the difference in fruit set between seasons. As evidenced in this study, hand-pollination experiments (intra/inter-crossing techniques) experienced a high-rate of failure, indicating that more advanced techniques are required (i.e. indirect anther emasculation,) to manipulate and effectively develop crosses. Emasculation can be performed in the bud stage (buds that are close to open), and pollination of two to three flowers per inflorescence is recommended to improve the survival of the emasculated flowers.

Low rate of fruit success was obtained in the intra-crosses and self-pollination experiments, but it seems that *A. crassna* is self-compatible. In this research, seeds collected from the controlled pollination experiments were germinated and there was no apparent lack of vigour between experiments (data not shown). However, seedlings from the controlled experiments need to be grown and evaluated until the next generation to corroborate viability of fruit and seed. Hybridisation may occur between *A. crassna* and *A. baillonii* as were evidenced in the inter-crosses experiments. More research is required to evaluate the fitness and superiority of the hybrids. With this information *Aquilaria* growers could improve the performance of the tree crop. The use of closely related tree species and their hybrids has influenced the development of many forestry crops. For instance, *Populus* species and hybrids (Bunn *et al.*, 2004; Rood *et al.*, 1986; Vanden Broeck *et al.*, 2005) *Eucalyptus* species and hybrids (Ashton & Sandiford, 1988; Campinhos, 1999; Potts & Dungey, 2004), *Pinus* species and hybrids (Chen *et al.*, 2004; Wachowiak & Prus-Głowacki, 2007).

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Appendices

Appendix 1 Appendix to Chapter 2: Exports of agarwood products (kg) from various *Aquilaria* species for the period 2005-2013 (Source: CITES trade base- UNEP-WCMC).

Taxon	Product	2005	2006	2007	2008	2009	2010	2011	2012	2013	Total
Various *Aquilaria* species (*A. crassna*+*A. filaria*+ *A. malaccensis*, *A. microcarpa*, *A. sinensis*, *Aquilaria* spp.)	chips (kg)	1003,219.8	1020,426.0	552,755.0	685,752.6	980,447.0	928,515.0	1163,951.5	1329,635.2	1306,660.4	8971,361.9
Various *Aquilaria* species (*A. crassna*+ *A. malaccensis* + *A. filaria*+ *A. sinensis*+ *Aquilaria* spp.)	powder (kg)	191,193.5	388,630.3	160,731.0	402,339.3	257,406.0	489,068.0	796,180.2	971,821.8	1068,575.9	4725,945.8
Various *Aquilaria* species (*A. crassna*+ *A. filaria*+ *A. malaccensis*, *A. microcarpa*, *Aquilaria* spp.)	timber & timber pieces (kg)	10,856.7	10,730.9	22,211.9	35,319.0	33,842.8	261,072.0	49,012.9	217,631.3	81,071.2	721,748.9
Various *Aquilaria* species (*A. crassna*+ *A. filaria* +*A. malaccensis*, *Aquilaria* spp.)	sawn wood (kg)		0.0	0.0	807.8	20,731.1	10,000.0	320,858.7	21,710.2	45,943.1	420,051.0
Various *Aquilaria* species (*A. crassna*+ *A. malaccensis*, *A. filaria*)	logs (kg)	10,636.7		1,491.6	1,031.1	155.2	40,142.5	6,678.9	10,537.1	252,916.8	323,589.9
Various *Aquilaria* species (*A. crassna*+ *A. filaria* +*A. malaccensis*, *Aquilaria* spp.)	oil (kg)	15,806.1	608.1	1,164.9	266.5	3,275.9	637.0	2,523.9	4,715.8	8,666.2	37,664.5
Various *Aquilaria* species (*A. crassna*+ *A. malaccensis*, *A. filaria*)	live (kg)			17,516.0	0.0	2,053.0	503.0	495.0	20.0	160.7	20,747.7
Various *Aquilaria* species (*A. crassna*+ *A. filaria*+ *A. malaccensis*+ *A. sinensis*, *Aquilaria* spp.	derivatives (kg)	375.4	84.4	759.1	333.2	1,022.5	57.6	243.5	594.4	6,169.7	9,639.7

Taxon	Product	2005	2006	2007	2008	2009	2010	2011	2012	2013	Total
Various *Aquilaria* species (*A. crassna*+*A. filaria*+ *A. malaccensis*, *Aquilaria* spp.)	carvings (kg)			2.9		427.0	538.4	4,392.5	3,192.6	302.9	8,856.3
Various *Aquilaria* species (*A. crassna*+ *A. malaccensis*, *Aquilaria* spp.)	leaves (kg)			200.0	2,538.0	204.0	10.0	268.5	151.0	101.4	3,472.9
Various *Aquilaria* species (*A. crassna*+ *A. malaccensis*)	roots (kg)	423.5	34.3				202.0		362.9	512.0	3,069.4
Various *Aquilaria* species (*A. crassna*+ *A. malaccensis*, *A. sinensis*)	medicine (kg)					8.6	12.0	106.5	73.7	2,722.7	2,923.5
Various *Aquilaria* species (*A. crassna*+ *A. filaria* +*A. malaccensis*, *Aquilaria* spp.)	specimens (kg)							1,215.9		918.2	2,134.2
Various *Aquilaria* species (*A. crassna*+ *A. malaccensis*, *A. sinensis.*)	stems (kg)		243.0			140.5	165.8	193.2	181.6	54.2	978.3
Various *Aquilaria* species (*A. crassna*+ *A. malaccensis*, *A. sinensis*, *Aquilaria* spp.)	extract (kg)				19.4	0.1	155.9	486.3	47.7	40.0	749.3
A. crassna	seeds (kg)			14.0	21.0						35.0
A. sinensis	dried plants (kg)			27.0							27.0

Values presented in this table are only from those quantities expressed in kg and g originally, as these units of measures were the most common among countries and agarwood products in the CITES trade base. Other units of measures were not taken into account when values were summed

Appendix 2 Appendix to Chapter 5 Polynomial Regression Analysis: CO$_2$ Assimilation versus SPAD Value (Tree)

The regression equation is
CO$_2$ Assimilation = -2.1692068098789137 + 0.39123950363122306 SPAD Value(Tree) - 0.002938281818949126 SPAD Value(Tree)$^{\wedge 2}$

CO$_2$ Assimilation = -2.1692 + 3.91239^{-02}C-index - 2.93828^{-03}C-index^2

S = 1.09575   R-Sq = 85.5%   R-Sq(adj) = 84.9%

Analysis of Variance

Source	**DF**	**SS**	**MS**	**F ratio**	**P**
Regression	2	346.29	173.1	144.2	0.000
Error	49	58.833	1.201		
Total	51	405.12			

Sequential Analysis of Variance

Source	**DF**	**SS**	**F ratio**	**P**
Linear	1	326.5	207.6	0.000
Quadratic	1	19.792	16.48	0.000

Polynomial Regression Analysis: CO$_2$ Assimilation versus PFD

The regression equation is
CO$_2$ Assimilation = 0.9766699728604886 + 0.013937401532000647 PFD - 0.00000974784772868275 PFD$^{\wedge 2}$ + 0.0000000019791347935933487PFD$^{\wedge 3}$

CO$_2$ Assimilation = 0.976669 +1.39374^{-02}PFD -9.74785^{-06} PFD^2+ 1.97913^{-09} PFD^3

S = 1.09349   R-Sq = 80.5%   R-Sq(adj) = 79.6%

Analysis of Variance

Source	**DF**	**SS**	**MS**	**F ratio**	**P**
Regression	3	335.53	111.8	93.54	0.000
Error	68	81.308	1.196		

Total	71	416.83		

Sequential Analysis of Variance

Source	**DF**	**SS**	**F ratio**	**P**
Linear	1	152.66	40.45	0.000
Quadratic	1	148.61	88.73	0.000
Cubic	1	34.256	28.65	0.000