DOCTOR OF PHILOSOPHY

Variation in Thaumatococcus daniellii (Benn.) Benth. and its potential as an intercop with Hevea brasiliensis (Willd. Ex A. de Juss) Mueller-Argoviensis in West Africa

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Variation in *Thaumatococcus daniellii* (Benn.)Benth. and its potential as an intercrop with *Hevea brasiliensis* (Willd. Ex A. de Juss) Mueller-Argoviensis in West Africa

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A thesis submitted in candidature for the degree of Philosophiae Doctor
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ABSTRACT

Thaumatococcus daniellii, the source of the super-sweet protein, thaumatin, is a perennial understory forest herb of West and Central Africa. This thesis investigates its natural variation and potential as an intercrop with rubber (*Hevea brasiliensis)* in West Africa. Field work undertaken in Ghana and Cameroon, resulted in two distinct locations per country being used as sites for the collection of morphological data, and the supply of planting material for experimental sites. Two experimental sites were established: the main experimental site was based at the Missellele Rubber Estate, Cameroon, under plantation rubber; two secondary experimental field trials were established under rubber smallholdings near Akona Junction, Ghana. Natural variation in the plant was studied through the collection of morphological data from four locations in Cameroon and Ghana. Data collected included petiole length, lamina width and breadth, fruit size and mass, and aril number and mass. Arils were extracted and thaumatin was recovered from them to assess if thaumatin content differed amongst populations. Significant differences in morphology were found amongst populations. Thaumatin content, varied significantly amongst populations but the range (1.08-2.00%) was within industry standards for all accessions. A molecular study of variance based on leaf material collected from the four populations detected 364 AFLP markers using three primer sets which were used to investigate phenetic and phylogenetic variance within and among populations. The study revealed that populations geographically close to one another were more closely related genetically, than those with similar phenotypic morphology. There was no evidence to suggest that speciation within *T. daniellii* is occurring despite some reports of this in the literature. Genetic differentiation between populations, 58%, was greater than within regions and within populations. ΦRT values of 0.777 ($p = 0.01$) indicate a strong genetic structure and suggests some isolation amongst populations. Three field trials were established in Cameroon. In the first trial sections of *T. daniellii* rhizomes from each of the four populations studied above were planted under nine year old rubber in a randomized block design to study whether the phenotypic expression of wild populations would be stable maintained across environments, to document phenology and quantify fruit production. The second trial investigated the effect of early growth of *T. daniellii* on rubber production using a second randomised block design. The trial was planted with material from one population each from Ghana and Cameroon, plus material found at the experimental site. The final trial studied the effect of shade on the growth of *T. daniellii*. Two blocks of six plots were established with trees surrounding plots lopped by different amounts. Different methods to measure the shade cast by rubber canopy were compared. Fruit were produced two and a half years after planting with locally adapted material producing significantly more and larger fruit ($p<0.05$) than germplasm obtained from further away from the site. Phenotypic differences measured across locations were not maintained when the material was all grown at the same site, suggesting that the traits under study were more under environmental than genetic control. *T. daniellii* growth was unaffected by rubber tree pruning but intercropping reduced cup lump rubber production by up to 12%. Growth of *T. daniellii* planted from rhizomes and seedlings was investigated in trials established in Ghana under rubber smallholdings. The trials were useful for small holders to gain experience with the plant and an indication of potential fruit production from small holder rubber was obtained. There was no difference in growth and fruit production amongst types of planting material. Overall, the study has demonstrated the practicality of growing *T. daniellii* under rubber and the significant genetic and phenotypic variation found in fruit yield indicate that selection of appropriate material for use at potential cultivation sites may have potential to increase productivity.

Keywords: *Thaumatococcus daniellii, Hevea,* Rubber, AFLP, West Africa, thaumatin, shade response.

DEDICATION

This thesis is dedicated to my family: dla mama y tata, Władysław and Danuta, who are no longer with us, but who would have been immensely proud of me to have got this far and for my brothers, Marek and Raj, my future wife, Sonia, and the new family I am waiting to join.

I also wish to dedicate this work to the memory of Dr Ymke Warren
1970-2010

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Ndom, Dr Anthony Nchanji, Mr Ebenki Smith and other staff of WCS I am in deep gratitude for all your help and assistance. From CDC I would like to particularly thank my dear friends and colleagues, Dr Steven 'DOCTOR' Namijo and Mr Odilous Mbuyeh without whom this work could not have been started, Mr Mebameg Wilson, the current Missellele Estate Manager, Mr Shiti Cletus, and the site overseerers Mr Peter. Also many thanks to friends with whom I have shared good times: Dr Bethan Morgan, James Christie, Sonny Blackwell III, Jo Osborne, Johanna, Sigali, Rudi and Rolf and Heidi and others made my time there a happy experience. I have in my thoughts, Ymke, who, sadly was taken away from us all.

I cannot leave Cameroon without saying an enormous thank you to my most erstwhile assistant, Mr Mark Litute Lyonga – thank you so much for all your help over the years.

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I think that's about it.

WSW.

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ABBREVIATIONS AND ACRONYMS

η^2	Eta squared
ω^2	Omega squared
ΔPCH	Change in a plant characteristic
ΦPR	Between population variation
ΦPT	Within population variation
ΦRT	Regional variation
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
BM	British Museum of Natural History
BR	National Botanic Garden of Belgium
CAR	Central African Republic
CDC	Cameroon Development Corporation
CFC	the Common Fund for Commodities
CLP	Cup lump production
CLR	Cup lump rubber
CNRA	Centre National de Recherche Agronomique, Côte d'Ivoire
cpDNA	chlorplast DNA
CPR	Crop performance ratio
DRC	Democratic Republic of Congo
DNA	Deoxyribose nucleic acid
DSF	Direct site factor
E00	Universal primer that anneals to the EcoR1 adaptor
EcoR1	Rare cutter restriction enzyme
ETO	Etome Village, South West Province, Cameroon
GBL	Gbledi Gbogame Village, Volta Region, Ghana
GD	Genetic distance
GREL	Ghana Rubber Estates Limited
GSF	Global site factor
He	Genetic diversity
ISF	Indirect site factor
IRAD	Institut de Recherche Agricole Pour le Developpement, Cameroon
ISSR	Inter-simple sequence repeat
K	Royal Botanic Gardens, Kew
LAI	Leaf Area Index
LER	Land equivalent ratio

LBV	National Herbarium of Gabon, Libreville
LL	Lamina length
LW	Lamina width
MBE	Mebanga Village, South Province, Cameroon
MI	Missellele
MO	Missouri Botanical Garden
M00	Universal primer that anneals to the Mse1 adaptor
Mse1	Frequent cutter restriction enzyme: (Tru9)
mtDNA	mitochondrial DNA
NJ	Neighbour joining (algorithm)
NSU	Nsuta Village, Western Region, Ghana
OTU	Operational taxonomic unit
P	Muséum National d'Histoire Naturelle, Paris
PAR	Photosynthetically active radiation
PCA	Principal coordinates analysis
PCR	Polymerase chain reaction
Pfr	Far red light absorbing phytochrome receptor
PH	Petiole height
PPFD	Photosynthetic photon flux density
Pr	Red light absorbing phytochrome receptor
RAPD	Random amplified polymorphic DNAs
RFLP	Restriction fragment length polymorphism
R:FR	The ratio of red to far-red light
RFU	Relative fluorescence unit
ROPP	Rubber Outgrowers Plantation Project
rRNA	ribosomal RNA
RY	Relative yield
SLS	sample loading solution - deionised formamide
SM	Simple matching (coefficient)
SP	South Province
SSR	Simple sequence repeats (microsatellites)
SWP	South Western Province, Cameroon
TE	Tapping episode
UPGMA	Unweighted pair group method using arithmetic average
VR	Volta Region, Ghana
WAG	Wageningen University
WR	Western Region, Ghana
XFA	West African Franc

CHAPTER 1

INTRODUCTION

1.1 HISTORICAL CONTEXT AND IMPORTANCE

1.1.1 Historical context

In the late 1960s Inglet and May (1968) were screening African plants for potential sources of alternative natural sweeteners for dietetic uses. They described four plants with unusual sweet tasting properties: *Synsepalum dulcificum* (Schum.) Daniell (now defined as *Richardella dulcifica* (Schum. and Thonn.)), *Sphenocentrum jollyanum* Pierre, *Dioscoreophyllum cumminsii* (Stapf) Diels and *Thaumatococcus daniellii* (Benn.) Benth. (Inglett and May, 1968). On 18th October 1969, following the assertion that sodium cyclamate, an artificial sweetener, was carcinogenic, the United States of America's Secretary of State of Health, Education and Welfare decided to restrict all diet foods containing sodium cyclomate (Adansi, 1970; Shutt, 1986). Interest grew in natural sweeteners, miraculin and monellin from R. *dulcifica* and *D. cumminsii* respectively, not just for their sweet tasting properties but also in their ability to modify taste or the perception of it (Adansi, 1970; Isawumi, 1981).

By 1972 thaumatin proteins had been isolated by van der Wel and Loeve (1972) and found to be the sweetest natural substance eliciting sweet taste responses at concentrations as low as 10^{-7} to 10^{-8} M (Farbman and Hellekant, 1989; Daniell *et al.,* 2000) equating to between 5500 to 1300 times sweeter than sucrose at threshold sweetness, w/v (Higginbotham, 1979). Thaumatin proteins were described as chemostimulatory by Cagan (1973) and found to lose their sweet taste on heating (Korver *et al.,* 1973); crystalline structures (de Vos *et al.,* 1985) and changes in the level of sweetness of three identified thaumatin proteins, TI, TII and TO, from arils at different stages of maturity were elicited (Mackenzie *et al.,* 1985) with Lee *et al.* (1988) identifying, the currently accepted, five forms of thaumatin proteins, I, II, a, b, c, (Faus, 2000). In 1996 crystals of thaumatin were grown in the Life and Microgravity Spacelab aboard Space Shuttle Colombia (National Aeronautics and

Space Administration, 1998) In 1976 Tate and Lyle had patented the process of extracting thaumatin, and named the product Talin®. A series of agronomic studies, both in West Africa and the UK were conducted to determine if the plant could be cultivated (Adansi and Holloway, 1977; Most *et al.,* 1978; Onwueme *et al.,* 1979). Entrepreneurs in Ghana were already supplying fruit by the late 1970s (Enti, 1975) and the next decade saw collection of fruit on a large scale from the Ivory Coast (Bonnehin, 1997).

Masuda and Kitabatake (2006) in their extensive review of the production of sweet proteins describe the production of thaumatin by researchers using many methods: bacteria including, *Escherichia coli*, *Bacillus subtilis* and *Streptomyces lividans*; yeasts including, *Saccharomyces cerevisiae*, *Kluyveromyces lactis, Pichia pastoris* and *Schizosaccharomyces pombe*; filamentous fungi including *Aspergillus oryzae, A. nidulans, A. niger* var *awamori,* and *Penicillium roquefortii*; and transgenically in higher plants, *Solanum tuberosum* L. (potato), *Fragaria* × *ananassa* Duchesne, (strawberry), *Solanum lycopersicum* L. (tomato), *Cucumis sativus* L. (cucumber) and *Pyrus* L. (pear). Higginbotham (1979) suggested that supply from the natural resource would probably dominate the supply of thaumatin, as 30 litres of bacterial culture equating to thaumatin content in one aril was not viable economically: Faus (2000) supported this argument 20 years later stating that

> *'microbial production of this protein (sic. thaumatin) would only be economically feasible if the recombinant microorganism could produce 1g of product per litre'*

and at the time of writing none of the attempts expressing recombinant thaumatin had succeeded in reaching this level.

In 2001-2002 Samartex Timber and Plywood Company, Ghana, began purchasing fruit for the production of thaumatin, during the course of which two research projects were conducted, one on potential income generation through small scale farming (Yeboah *et al.,* 2003) and the other on local knowledge regarding sustainable harvesting and production (Waliszewski, 2002).

1.1.2 Global importance

Thaumatin is a world-wide traded commodity with a market value of about US$6000 per kilogram (Charles Boy, pers. comm. 2009) being used principally for taste modification and flavour enhancement (Boy, 2009), but also as a sweetener in many industries and products (Shimitzu *et al.,* 1994; Wirsig, 2002) including: mouthwashes, toothpastes, yoghurts, beverages and confectionary. The collection of the fruit from Ivory Coast and export of arils to the UK was valued at between US$ 160 000 – 400 000 annually, between 1984-1991 (Bonnehin, 1997).

Its safety was evaluated by Higginbotham *et al.* (1983); since then thaumatin has been approved by the Joint FAO/WHO Expert Committee on Food Additives and the European Food Safety Authority (EFSA) with similar approval existing in Switzerland, the United States, Canada, Israel, Mexico, Japan, Hong Kong, Korea, Singapore, Australia, New Zealand and South Africa (International Sweeteners Association, 2009). It is designated, by the European Union as E957, generally regarded as safe for use as a sweetener and as a food additive (European Union, 1994; European Union, 1995).

With the exponential rise in the number of patients suffering from diseases caused by the consumption of sugar (obesity and diabetes) (Kant, 2005) natural sweeteners could be potential replacements for artificial sweeteners. Increased interest from major soft drinks suppliers using natural sweeteners has led to the use of a natural sweetener, Stevia (RebA) / Rebiana, from *Stevia rebaudiana* Hemsl. (Asteraceae) (Jones, 2007) this being modified with thaumatin to giving a more rounded flavour (Naturex, 2010).

New interest is growing in the exploitation of extracts of *T. daniellii* lamina as an alternative natural preservative for bread and a preserved carbohydrate local to southern Nigeria, 'Eba' (Adebayo and Kolawole, 2010), though Okejale *et al.* (2007) found that the leaves did not have any anti-microbial characteristics but did have an essential oil that was thought to impart a distinctive taste to foods wrapped in the leaves. Other areas of potential interest are in the use of waste *T. daniellii* from extraction of the arils, namely, seed and pericarp, for use as an animal feed due to the high protein content of the structures (Elemo *et al.,* 1999).

1.1.3 Local importance

Abbiw (1990) gives a detailed listing of local uses of *T. daniellii* including: wrapping leaves for foods sold in markets (Falconer, 1992; Burkill 1997); mat making, roofing, preservation of kola nuts (Arowosoge and Popoola, 2006), medicinal uses (Burkill 1997; Neuwinger 2000; Arowosoge and Popoola, 2006), masking the acidity of palm wine and to sweeten foods (Inglett and May, 1968; van der Wel and Loeve, 1972; Shutt, 1986). Though Falconer (1992) suggested a thriving market for leaves in the early 1990s in large markets, such as Kumasi, this has been reduced through the use of plastic bags (pers. obs.).

1.2 *THAUAMATOCOCCUS DANIELLII* (BENNET)BENTHAM

1.2.1 Taxonomic placement

Kingdom	Plantae	Plants
Subkingdom	Tracheobionta	Vascular plants
Superdivision	Spermatophyta	Seed plants
Division	Magnoliophyta	Flowering plants
Class	Liliopsida	Monocotyledons
Subclass	Zingiberidae	
Order	Zingiberales	Gingers
Family	Marantaceae	Prayer-plant family
Genus	*Thaumatococcus* Benth.	
Species	*Thaumatococcus daniellii* (Bennett) Bentham	

The first botanical specimen of *T. daniellii* (*Don* s.n. (BM)) was collected by George Don in Sao Tomé in 1822 (Plate 1.1) (Exell 1944). In 1855, Bennet (1855) termed the plant *Phrynium Daniellii* (Benn.) after the collection of the type specimen by his friend and Army Surgeon, Dr William Freeman Daniell, in 1853 (Daniell, 1855). Dr Daniell had become acquainted with the plant in an earlier visit to West Africa, in 1839, to Old Calabar (present day South East Nigeria) and Ebo ('Ibo' – present day Cross River State), stating that the fruit had come from traders near the area around

Bocqua (Niger Delta) (Daniell, 1855). It was given its current description and name, *Thaumatococcus daniellii* (Benn.) Benth. by Bentham and Hooker (1883)

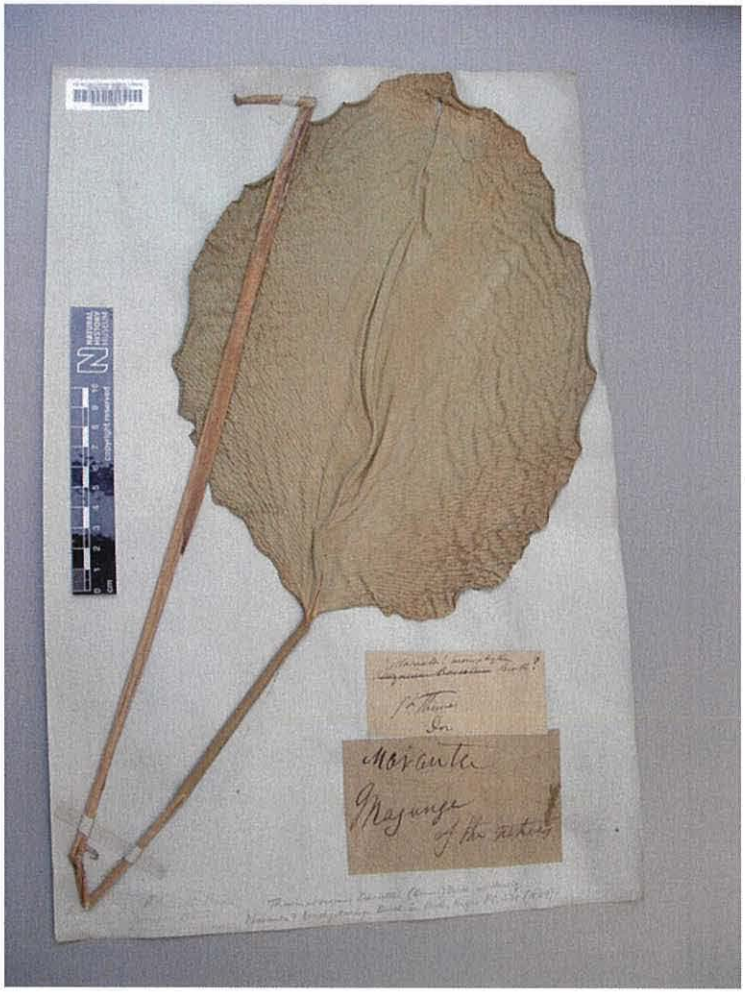

Plate1.1 The first botanical specimen of *Thaumatococcus daniellii*, *Don* s.n. (BM) 1822, courtesy Natural History Museum.

Others have described *T. daniellii*, usually to only a limited extent, in floras, manuals and books (Schumann, 1902; Leonard and Mullenders, 1950; Leonard and Mullenders, 1951; Roberty, 1955; Mangenot, 1957; Schnell, 1957; Raponda-Walker and Sillans 1961; Aubreville, 1964; Koechlin, 1964; Koechlin, 1965; Hepper, 1968; D'Orey, 1981; Letouzey, 1986; Burkill 1997; Andersson, 1998). The most detailed descriptions are those of Jean Koechlin and George Mangenot.

Dhetchuvi (Dhetchuvi, 1993; Dhetchuvi, 1996) and Dhetchuvi and Diafouka (Dhetchuvi and Diafouka, 1993) described the var. *puberulifolius* form based on the observation of minute hairs on the underside of leaves, principally from herbarium specimens, and shorter inflorescence (rachis) length. Ley and Claßen-Bockhoff ((in

press); 2005; 2009) and Ley (Ley, 2008) suggested her material included a second species (sp 1. nov), from Gabon, but at the time of writing this has not been formalized.

1.2.2 Botanical description

Below is a botanical description of *Thaumatococcus daniellii* based using the above references, principally (Mangenot, 1957; Koechlin, 1964; Koechlin, 1965; Hepper, 1968) and personal observations during the past eight years.

1.2.2.1 Lamina

Laminae are ovate-elliptic in shape, rounded at the base, with a short acuminate (pointed) tip (Plate 1.2). Maximum length is about 55 cm and breadth about 35 cm. Texture is papery, though leaves from some provenances 'feel' thicker and more substantial. The midrib is pronounced abaxially, with numerous principle parallel veins and secondary veins diverging from the midrib at about 45°, in a sigmoidal pattern to the edge of the lamina. Principle parallel veins are approximately 1cm from each other with many secondary veins in between. Cross veins are seen between secondary veins. The colour of leaves when unfolding is bright and light green but they turn darker with maturation. The leaves are glabrous on both sides at maturity. Some simple hairs are present on lower surface in some young leaves, generally near the midrib, but can only be seen with the use of a magnifying lens.

Plate1. 2 *Thaumatococcus daniellii* lamina

1.2.2.2 Pulvinus

The pulvinus (sometimes referred to as a callus (Letouzey, 1986) is a dark curved extension of the petiole proximally limited by a distinctive groove – a V-shaped notch (Plate 1.3), some 20 cm from mature lamina and felt as a thickening in the petiole. Movement of the leaf during the day is caused by changing of turgor pressure in the cells of the pulvinus, due to increasing sugar concentration in the apoplast (cell walls) (Raven *et al.,* 2005).

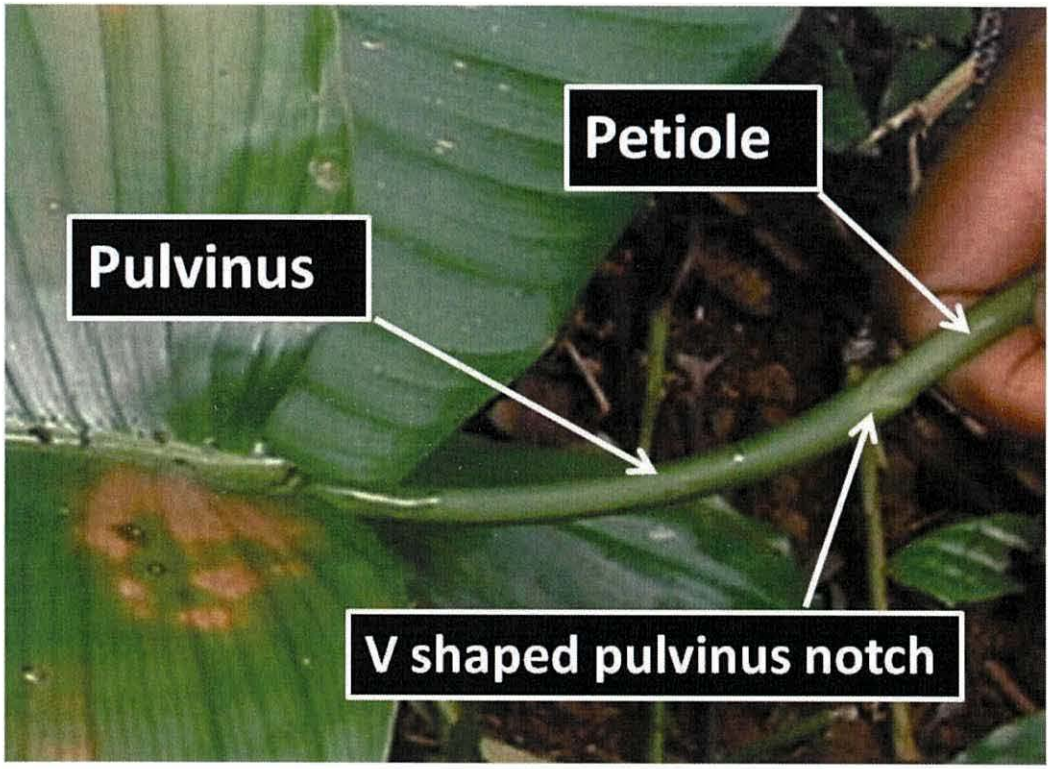

Plate1. 3 Pulvinus and V shape pulvinus 'notch' of *Thaumatococcus daniellii.*

The pattern of venation can be seen. The knuckle of the person holding the petiole can be seen.

1.2.2.3 Petioles

In mature plants these arise predominantly indivually from each node on the rhizome, at intervals of 10-15 cm. Sometimes there is a papery sheath, light in colour, at the base of the petiole. Petiole diameter is approximately 1 cm, and the structure is strong and flexible. Petioles can be as long as of 2.5-3 m to the pulvinus, but mature short petiole plants have been reported (Mebanga, South Province, Cameroon). The Volta Region has very tall plants.

1.2.2.4 Rhizome

Rhizomes are slender, scaled, simple or forked (Plate 1.4), and 1-2.5 cm in diameter. They are light brown/cream in colour, with lighter papery scales. They are usually subterranean at a depth of eight to 20 cm, but many arch out of the ground, rhizomes can grow up to more than five meters.

Plate1.4 Cut rhizomes from the Volta Region, showing scales, bud points and forking.

1.2.2.5 Raceme/Rachis

This arises at ground level, at the base of a petiole (Plate 1.5; Figure 1.1, part c and d). The rachis is the main axis of the inflorescence, to which flower buds, flowers, and fruit are attached. This is a cyme, 5-20 cm long, and may bear flowers, flower buds and fruit at the same time, sometimes in numbers as high as eight. More than one rachis can be associated with a single petiole.

Plate1. 5 Crimson red fruit of *Thaumatococcus daniellii* borne on three raches from the base of a single petiole

1.2.2.6 Flower buds

The flower bud is ellipsoid, varying in colour from light green or almost yellow to purple and green, or green and brown, being 2.5 cm long and 1 cm wide (Plate 1.6).

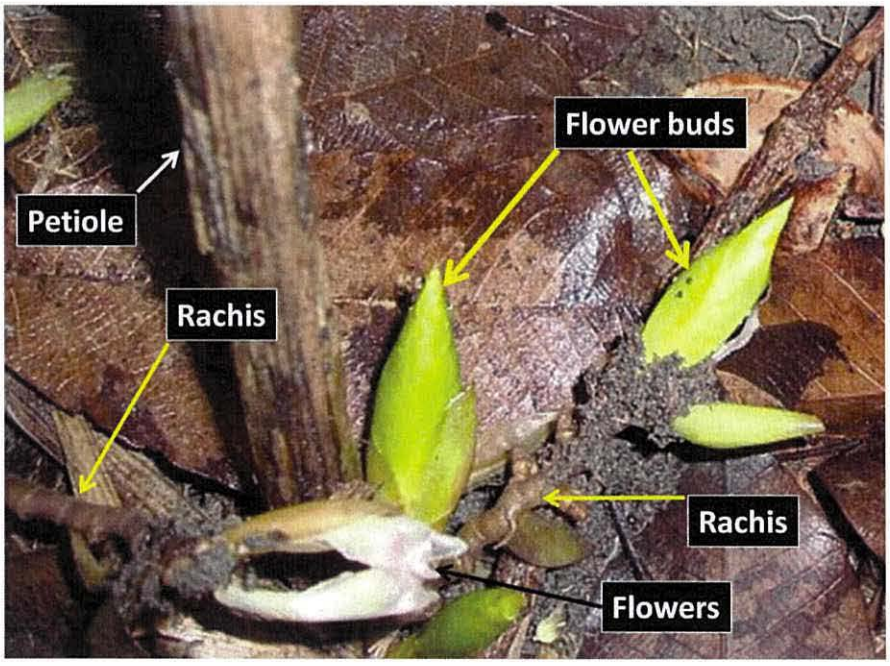

Plate1. 6 Floral structures of *Thaumatococcus daniellii*

1.2.2.7 Floral structure

Flowers are delicate as long as the subtending bract (about 4 cm), with a long corolla tube. Flowers are zygomorphic. (Kress, 1990), white-purple (Plate 1.6) or yellow (the latter in Gabon is possibly a different species). Ley and Claßen-Bockhoff (2005) display the contrasting flower colours (Plate 1.7) Andersson (Andersson, 1998) states that staminodes are absent, in conflict with Letouzey (Letouzey, 1986).

Plate1. 7 Two differently coloured flowers of *Thaumatococcus*.

Yellow flowers *Thaumatococcus* sp.1.nov from Gabon. Purple flowers *T. daniellii* from West Region, Ghana.

1.2.2.8 Fruit, seeds and arils

The fruit are indehiscent; borne on the rachis at or very near ground level (Plate 1.5). The pericarp is leathery, and hard, changing in colour during maturation from white to green to brown to dark brown (almost black) to orange to light red and then to the typical crimson red colour. Seeds, are white when immature, black at maturity. Seeds are surrounded by a thick mucilaginous gel, which is highly hydrophilic. Water entering the fruit may lead to swelling of the mucilaginous gel forcing the fruit to open (Martin Laasen, pers. comm. 2005). Fruits are typically between 2.5-3.5 cm in width, and 3-4 cm in length, though more massive fruit are found in the Volta Region. Three-seeded fruits are pyramidal in shape and this tri-locular form, containing three seeds, predominates, although fruits with only one or two seeds, and different in shape, are not unusual. Seeds are large, about 1 cm in length and of pyramidal or deformed crescent shape, narrower at the base. The seed coat is smooth but rough on drying. An aril is located at the top of the seed (Plate 1.8), cream coloured and about 4-5% of the total fruit mass (fresh weight). Fruiting is thought to occur about 13

weeks after first flowering (Onwueme *et al.,* 1979) and to be dependent on the onset of the more intense rainy season (Boy, 1994; Waliszewski *et al.,* 2005).

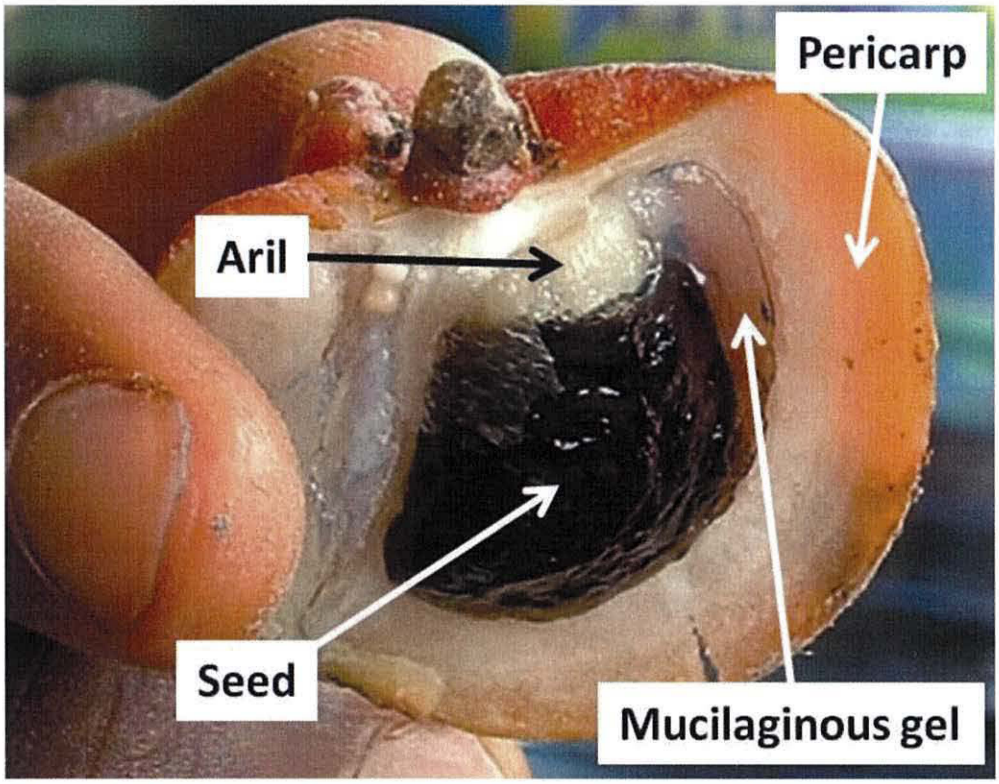

Plate1. 8 Internal structure of *Thaumatococcus daniellii* fruit

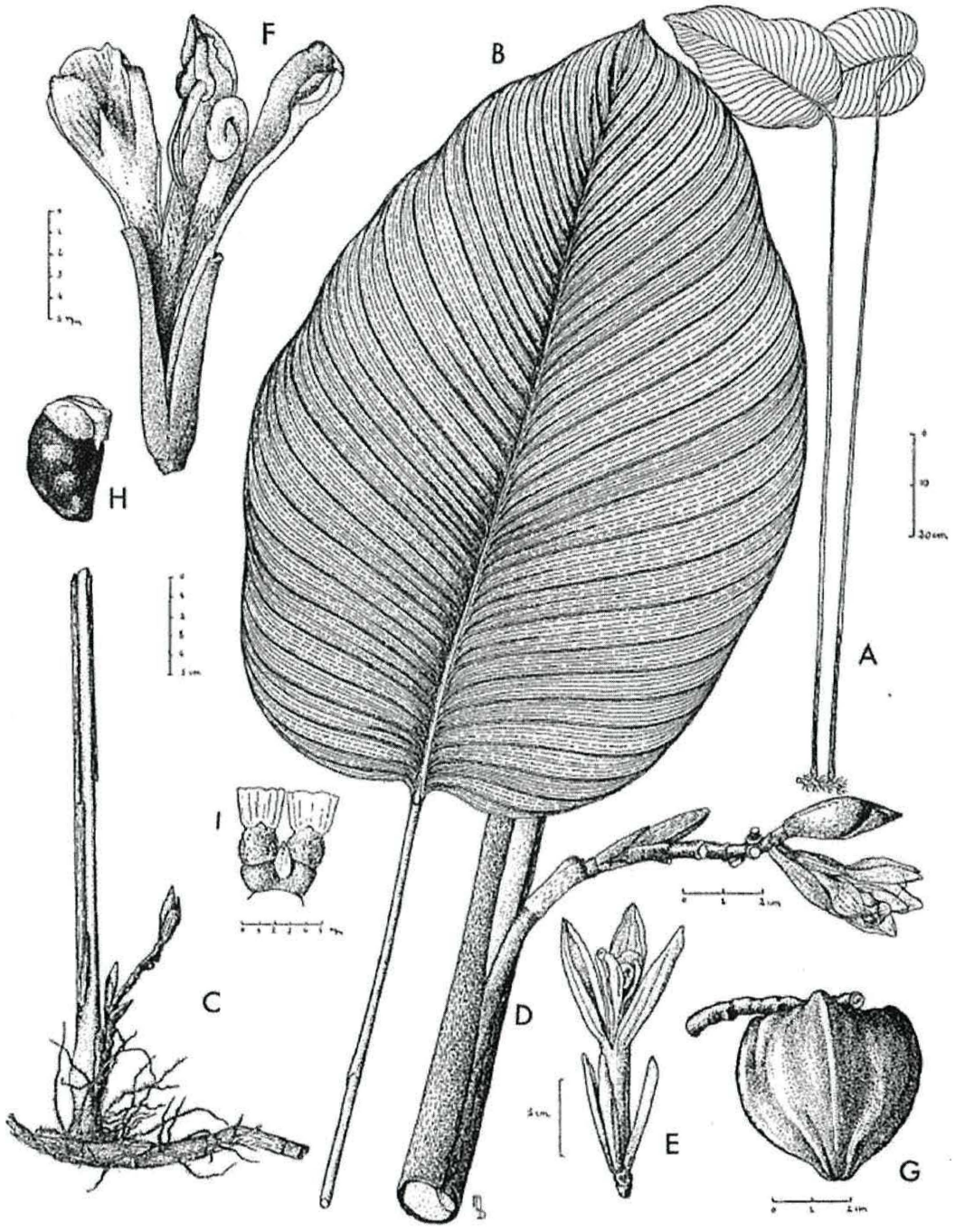

Figure 1.1. *Thauamtococcus daniellii*, after (Mangenot, 1957). Drawing by M. Devillers

A: General aspect of a young plant. B: Lamina, note the groove where the lamina attaches to the petiole, and the groove indicating the distal limit of the pulvinus. C: Rhizome with inflorescence, rachis and sheath and lower part of petiole. D: Rachis emenating from the petiole at the base of the plant, with flowers and buds shown, and attachment scars of previous flowers/buds/fruits. E: Flower. F: Three staminodes and the style. G: Fruit. H: Seed. I: Flower attachment structure.

1.2.3 Range, ecology and habitat

Thaumatococcus daniellii is native to West and Central Africa, and a floristic element of the Guineo-Congolian phytochorion (Waliszewski *et al.,* 2005) mapped by White (White, 1979; White, 1983), but apparently absent from Benin and most of Togo. The range of *T. daniellii* across Africa was mapped first for an unpublished thesis by Dhetchuvi (1996). The first published map of the distribution (Figure 1.2) showing the relationship with mean annual rainfall appeared in 2005 (Waliszewski *et al.,* 2005). The range of the plant suggests it thrives where there is a mean annual rainfall of >1500 mm, well distributed - at least nine months of the year with mean rainfall > 50 mm per month, and a mean annual temperature between 23-26°C (Waliszewski *et al.,* 2005).

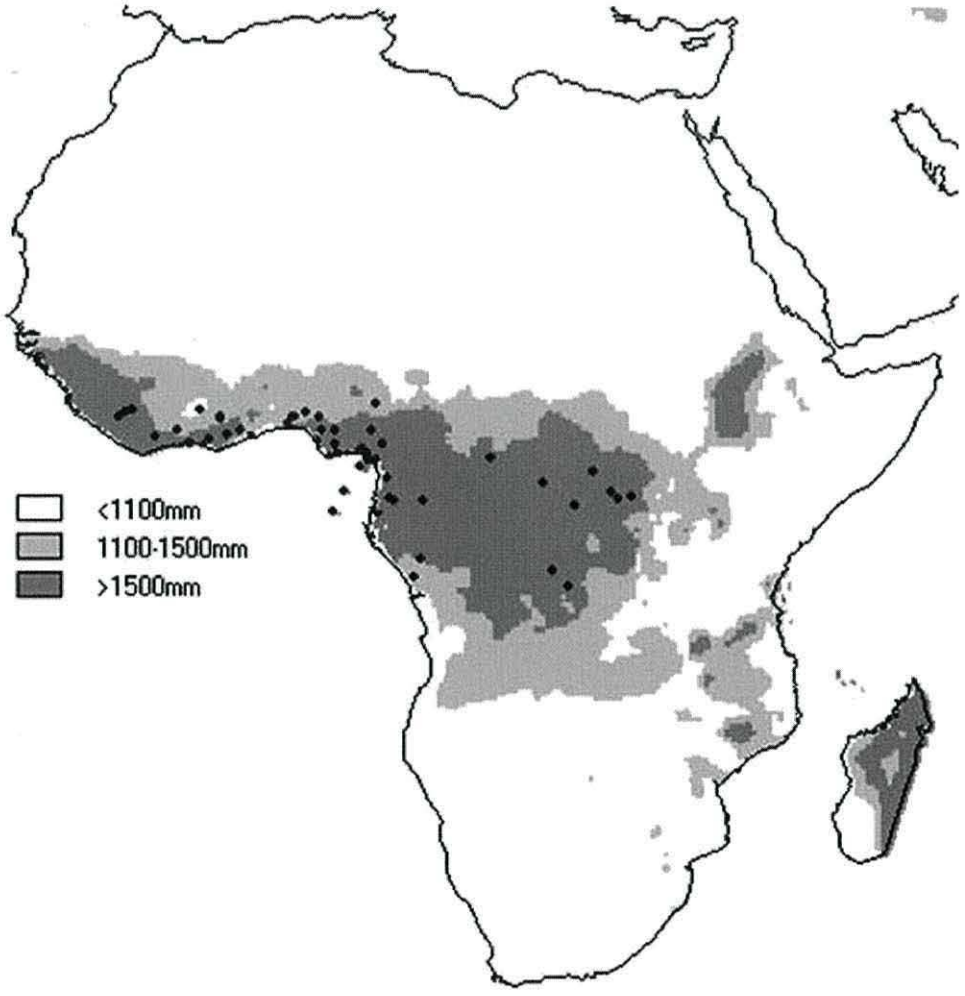

Figure 1.2 Natural distribution of *Thaumatococcus daniellii* in relation to mean annual rainfall (after Waliszewski *et al.,* 2005)

Thaumatococcus daniellii is a robust understorey forest herb forming extensive and dense colonies under mature canopies in virgin forest (Raponda-Walker and Sillans 1961; Waliszewski *et al.,* 2005), secondary forests (Enti, 1975; Nicol, 1976; Isawumi, 1981; Burkhill, 1985), and a variety of other habitats including secondary scrubland and abandoned cocoa farms (Nicol, 1976; Burkhill, 1985). Though it grows in shaded environments, Hawthorne (1996) categorized *T. daniellii* as a "cryptic pioneer", local collectors in Ghana described its occurrence in canopy gaps and open abandoned farms (Waliszewski, 2002). It is known to grow under crops such as cassava, banana, and plantain and perennial species such as coffee, cocoa, rubber and oil palm (Nicol, 1976).

Across its range it is associated with ferralsols (FAO-UNESCO, 1977) though local knowledge of cultivators in Ghana suggested that it grew best in sandy soils, with soils with a high gravel content or highly compacted leading to a reduction in the spread of rhizomes (Waliszewski *et al.,* 2005).

1.3 *Hevea brasiliensis* in West Africa

A major structural change in the natural rubber industry has seen a steady decline in estate involvement and the increasing importance of smallholder rubber (UNCTAD, 2010a). This is especially evident in Ghana, where Ghana Rubber Estates Ltd (GREL), through the Rubber Outgrower Plantation Project (ROPP), planted 4000 ha of smallholder rubber for 900 farmers during 1995-2005 and a further 7000 ha for 1750 farmers between 2006 and 2010 (Owusu, 2007). Cameroon more than quadrupled the rubber area planted in 1960, from 11,000 ha in 1961 to about 45,900 by 2005 (UNCTAD, 2010a), with the Cameroon Development Corporation processing a high proportion of cup lump rubber from smallholders in addition to rubber from its own estates (Cameroon Development Corporation, 2010; Odilous Mbuyeh, pers. comm. 2010).

However, due to the economic downturn of the late 1990s and fluctuations in the international price of rubber, as a result of too much rubber entering the market from South East Asia, the price of rubber fell dramatically to a 30 year low (Buddiman, 2003), with the result that many smallholders without recourse to alternative income

sources or diversification strategies could not, or were reluctant to, remain in rubber production (A. F. S. Buddiman, pers. comm. 2004). In response to this the Common Fund for Commodities (CFC) funded the "Enhancing Incomes of Smallholder Rubber Farmers in West and Central Africa" project in 2006 with US$ 2,980,342. The aim was to transfer technology to Cote d'Ivoire, Ghana and Cameroon in a bid to increase incomes and maintain the supply of rubber from these producers. One technology transfer investigated was alternative crops that could be intercropped with rubber to diversify smallholder livelihoods (Emmanuel Akwasi Owusu, pers. comm. 2009).

1.4 Summary and rationale for the research

The collection of *Thaumatococcus daniellii* fruit has been ongoing for the past 30 years from the forests and secondary forests of Ivory Coast and Ghana. This has been done to supply the international thaumatin market, which relies on the extraction of thaumatin from the fruits' arils. The current world price for thaumatin is ~ US$6000 per kilogram. In 2002 a new competitor within the thaumatin market emerged, leading to a competition for, and a reduction in, the supply of fruit, which according to Parren and Reitze de Graaf (1995) was already under pressure. New avenues for the supply of fruit were sought from producers of thaumatin with two possibilities emerging: the first to collect fruit from another country/area in the range of *T. daniellii* and the second to produce fruit from cultivated sources.

During the same period rubber had been promoted to smallholder farmers in West Africa. By the late 1990s – early 2000s smallholders were beginning to leave the cultivation of rubber as market prices for natural rubber were depressed, thus decreasing the supply of natural rubber from West Africa (A. F. S. Buddiman, pers. comm. 2004).

This research, with fieldwork undertaken in Ghana and Cameroon during 2005 to 2009, aimed to provide answers to the question: could *T. daniellii* be grown with rubber to provide another income source for rubber smallholders and at the same time diversify the supply of *Thaumatococcus* fruit?

1.5 Aims and Objectives

Recognizing the basic question of whether there was potential to diversify the supply of *T. daniellii* fruit and at the same time diversify income and increase resilience in small holder farmers, the current study focused on two broad themes: variation in *T. daniellii* and its growth, its productivity, and its suitability as an intercrop with rubber, with the following specific objectives:

- to assess variation in *T. daniellii* in terms of morphology and thaumatin content and to determine if this had a genetic basis that could be shown using molecular methods and common garden experiments,

- to assess whether variation observed *in situ* would be expressed in common garden experiments,

  and

- to examine the potential of *T. daniellii* as an intercrop of mature rubber, determining if provenance of *T. daniellii* material was a relevant consideration if intercropping affected rubber yield, and if growth and productivity of *T. daniellii* under a mature canopy was affected by the level of shade.

The thesis is in seven principal chapters. The introduction (Chapter 1) is followed by a series of chapters (2-7) giving the results of the different aspects of the investigation: morphological variation and variation in thaumatin content from material collected in the field (Chapter 2); molecular variation using amplified fragment length polymorphisms (Chapter 3); common garden experiments and growth under rubber at the Missellele experimental site (Chapter 4); the effect of intercropping on rubber production (Chapter 5); the effect of shade on the response and growth of *T. daniellii* (Chapter 6). The thesis concludes with a general discussion and recommendations for future research and domestication initiatives (Chapter 7).

CHAPTER 2

NATURAL DIVERSITY IN *THAUMATOCOCCUS DANIELLII*

This chapter is concerned with the natural diversity seen in *Thaumatococcus daniellii* plants from a substantial portion of its range. It attempts to detail differences in vegetative (petiole, lamina), reproductive (fruit, aril) and phytochemical (thaumatin) features among provenances. The first section details the aims and purpose of the chapter. The second section describes the sites where the study was conducted and their selection. The third section details methods used to collect and analyse morphological data. Results are detailed in Section 4 and the discussion follows in Section 5 with conclusions and recommendations in Section 6.

2.1 BACKGROUND

Morphological variation in *Thaumatococcus daniellii* has been discussed in the literature by Dhetchuvi and Diafouka (1993) where they proposed and described a new variety, var. *puberulifolius* Dhetchuvi & Diafouka, distinguished primarily on microscopic hairs on the abaxial (lower) surface of lamina. This was based on an examination of herbaria specimens from populations in Cameroon, Central African Republic, Congo Brazzaville, and the Democratic Republic of Congo (formally Zaire). These observations were made on herbarium specimens from the Herbarium of the National Botanic Garden of Belgium, Belgium; Muséum National d'Histoire Naturelle, Paris, France; and the Royal Botanic Gardens at Kew Herbarium, U.K. (Cameroun: Bates 396 (BR, K), D. Thomas 7328 (BR, K, MO), Letouzey 13314 (BR, K, P), D. Thomas 2277 (K); CAR: Chevalier 10970 (P), 11042 (P), & 11044 (P); Gabon: Hallé 3531 (P), Le Testu 2264 (P); Congo: Sita 3922 (IEC); Zaire: Lescrauwaet 385 (BR), Gerard 1783 (BR) (Dhetchuvi and Diafouka, 1993). Subsequently, little work had been conducted directly on *T. daniellii,* however, a new species has been put forward, *Thaumatococcus* sp. 1. nov. found in Monts de Cristal, Gabon (Ley and Claßen-Bockhoff, (in press); Ley, 2008; Ley and Claßen-Bockhoff, 2009). This species has yellow sepals surrounding bright yellow petals, Ley 56, 201,

202, 218 (LBV, WAG), but otherwise is indistinct from *T. daniellii*. Subsequent works have included *T. daniellii*, in revisions of the taxonomy of the Marantaceae overall (Prince and Kress, 2006a,b).

Amongst collections of fruit it was reported, at the beginning of the current study, that there were populations with gigantic *T. daniellii* plants and fruit found in the Volta Region of Ghana and the subsequent bordering areas of Togo, compared with the typical plant in the Western Region of Ghana and Ivory Coast, (Martin Laasen, pers. comm. 2005). Staff from the Limbe Botanic Gardens, Limbe, Cameroon, suggested a dwarf variety existed near the village of Bomana, close to Mt. Cameroon (Joseph Nkefor, pers. comm. 2005): samples of this plant being grown at the botanic gardens.

To date, no study in the public domain has documented differences between *T. daniellii* from across its pan-African range (Waliszewski *et al.,* 2005) from live specimens and field observations, though authors have described the plant in floras and forest manuals (Exell 1944; Leonard and Mullenders, 1950; Mangenot, 1957; Aubreville, 1964; Koechlin, 1964; Koechlin, 1965; D'Orey, 1981) and from herbarium specimens (Dhetchuvi and Diafouka, 1993).

Variation in thaumatin content between regions has not been previously documented, though authors have frequently cited the sweetening properties of thaumatin proteins to be in excess of 3500 times that of sugar on a weight/weight basis (Nicol, 1976) and more than 100 000 times sweeter than sugar on a molar basis (van der Wel and Loeve, 1972; Farbman and Hellekant, 1989; Daniell *et al.,* 2000) though not mentioning the percentage yield of thaumatin from arils. It is not known whether there is an allometric relationship between plant morphology, fruit and aril size and thaumatin content.

Understanding variation in the phenotypic expression of *T. daniellii*, where phenotype is determined by the combined effect of genotype, environment and the interaction of the two, will underpin the basis for subsequent chapters. Chapter 3 will investigate whether there is a molecular basis for variation that is seen in plants, and

Chapter 4 will investigate the effect on phenotype of growing plants from different populations at the same site.

This chapter therefore examines morphology of *T. daniellii*: lamina, petioles, fruit and arils, and percentage thaumatin yield from four geographically distinct populations within its range. Results will be put into a context of collection effort and production of arils and thaumatin from a given amount of fruit.

The initial hypotheses are that there are no significant differences between plant morphology, based on character traits, lamina length and width, and petiole height, fruit and aril mass and size, and thaumatin content from different populations across the plants range.

2.2 Study area

The current study was conducted in two countries in the range of *T. daniellii*: Ghana and Cameroon (Figure 2.1). Previous work on *T. daniellii* in Ghana made it an obvious choice for one of the study sites and Cameroon was chosen as links existed between the School of Environment, Natural Resources and Geography (SENRGY), Bangor University, and institutions there to facilitate research.

2.2.1 Selection of sites

In Cameroon, the selection of sites was based on the knowledge and experience of the Limbe Botanic Garden (LBG) staff, observation of herbarium specimens at the LBG herbarium, available literature (Hepper, 1968; Dhetchuvi and Diafouka, 1993) and links with local communities established with the Wildlife Conservation Society. It became apparent that Cameroon could potentially be the source of two possible varieties of *T. daniellii* in addition to var. *daniellii*, the already determined var. *puberulifolius* (Dhetchuvi and Diafouka, 1993) and a 'dwarf' variety (Joseph Nkefor, pers. comm. 2005).

In Ghana, where collection of fruit has been conducted for the past 20 years there are two predominant areas for collection, the Western Region, bordering Ivory Coast, and the Volta Region, bordering Togo. The latter area is said to produce a distinct

'variety' of the *T. daniellii* fruit (Charles Boy, pers. comm. 2005) being significantly larger than the typical variety found in the Western Region and Ivory Coast. For this reason it was chosen to reconnoitre an area in the Volta Region. Links with an established fruit supplier, Forest Im-Pex, a company based in Hohoe (7°09'N, 0°28'E), Volta Region, involved with the collection and processing of fruit, and the Afadjato Community Forest Conservation Project, led to the reconnaissance of a site where collection operations had not yet been conducted for the current year. Previous work in Ghana (Waliszewski *et al.,* 2005) enabled links established with Forest Services Division Tarkwa / Asankregwa (FSD), a section of the Department of Foresty, to be renewed, thus facilitating the search for sites in the Western Region.

2.2.1.1 South Province, Cameroon

One of the eight herbarium specimens used to determine *Thaumatococcus daniellii* var. *puberulifolius* Dhetchuvi & Diafouka, was Bates 396 (2°47'N, 10°32'E), collected from Efulan, near Kribi, South Province, Cameroon. This specimen was from the most accessible of all potential locations for this variety in the current study and as such the source of the area was reconnoitred.

A forest area beyond Efulan's 22 hectares of smallholder community rubber plantation was trekked to with guides, at the south western edge of the village, where logging had been conducted 5 years previously. After walking for three hours and observing the areas where guides thought *T. daniellii* was located it was decided to return to the village as none had been seen. Guides were certain that there was a dense colony of *T. daniellii* at a neighbouring village, Mebanga, some 60km from Efulan. (2°49'N, 10°38'E).

This village was visited and two massive colonies of *T. daniellii* were observed. The first some 20 minutes south and above the village was found on an abandoned cassava plantation; it was trapezoid in shape with the long axis being 10 m in length with the depth extending between 15 – 80 m.

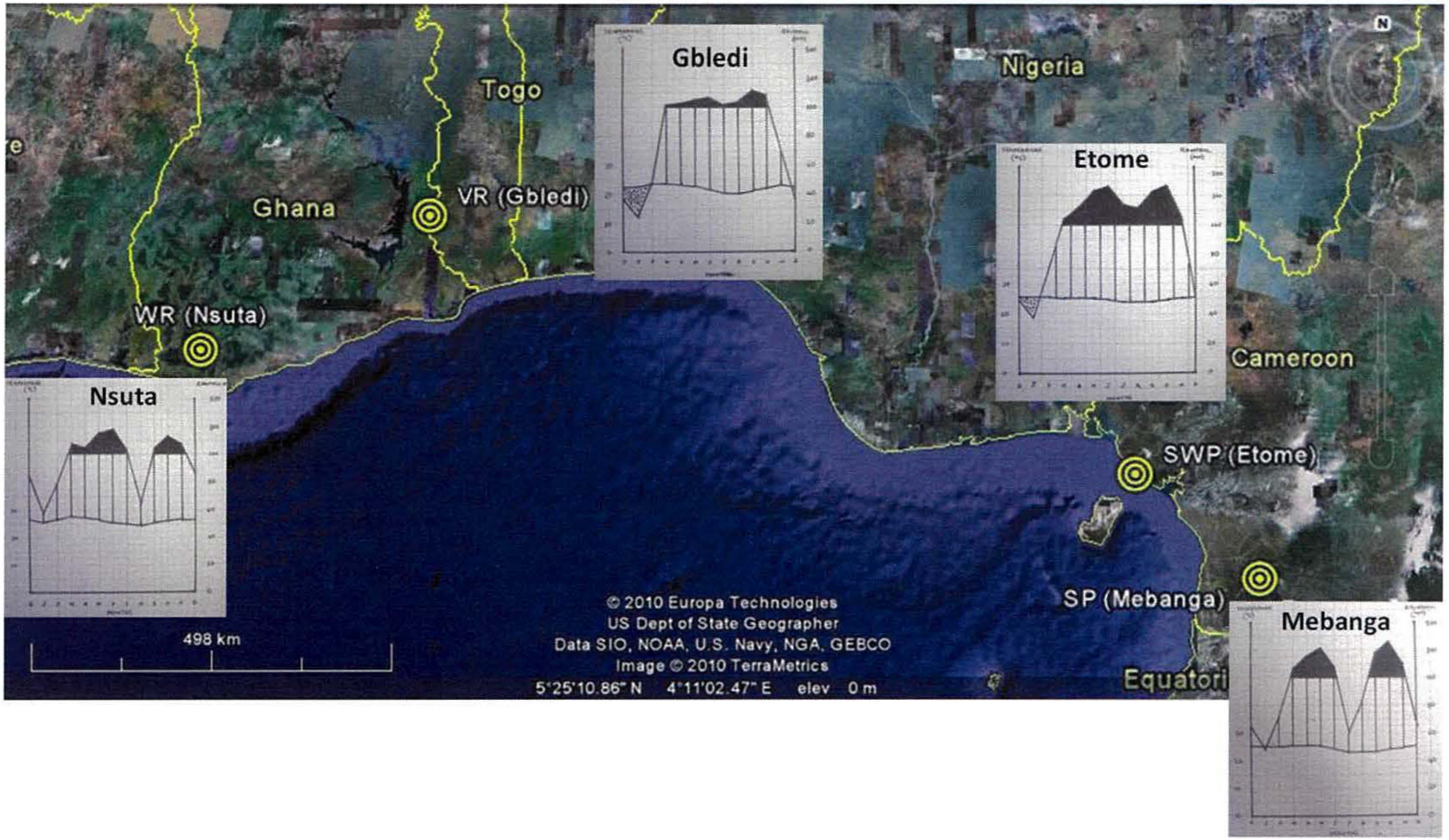

Figure 2.1 Study sites shown on a map of Central West Africa (Google Earth), with Walter Climatic diagrams.

WR: Western Region, Ghana; VR: Volta Region, Ghana; SWP: South West Province, Cameroon; SP: South Province, Cameroon.

There was a continuous patchy stretch of *T. daniellii* for 629 m found following a path up a hill. Patches extended to 2 – 30 m either side of the path at various distances along it. Another large colony was found over the crest of the hill on an abandoned cocoa farm. The large colony began 121 m from the first clump and began 25 m from the path, extending 53 m down the slope and was over 70 m in width.

The mean annual precipitation for Mebanga is 2084 mm with a bi-modal rainfall distribution pattern (Figure 2.1). Mean annual temperature is 24.6 °C with temperatures in the range of 23-25 °C. Temperature and rainfall data for all climate graphs was sourced from New_loc_CLIM ver. 1.10 (Grieser, 2006). The soil in this area, determined using the Harmonised World Soil Database Viewer ver. 1.10, (Verelst, 2009) is typically described as a ferralsol (FAO-UNESCO, 1977), a deeply weathered red or yellow soil with diffuse soil horizons and clay assemblages characterised by low activity clays, kaolinite; weathered soils high in sesquioxides (Ahn 1993). Mebanga is at an altitude of 460 m.

2.2.1.2 South West Province, Cameroon

Nkefor intimated that variation in the length of the petiole may well distinguish other varieties (Joseph Nkefor, pers. comm. 2005). Bomana, near Ideanau, some 42 km from Limbe, South West Province, was suggested as a location of this 'dwarf' variety of plant. Limbe Botanic Garden herbarium specimens also detailed collection of material from a nearby village of Etome on Mount Etinde, some eight kilometres from Limbe. These two villages were an hour's drive from each other, following the western edge of Mount Cameroon. In addition to their close proximity to Limbe, good relations existed between Etome and colleagues and it was therefore decided that these two villages would form the base for material and data collected from the South West Province.

Other potential sites that were considered but not used were locations near Mamfe, (Letouzey 13608 and Mezili, P. 203) and Korup National Park (Thomas, D. 2277). The distance to these locations, the time necessary to gain access to these sites and logistics precluded visiting these sites for reconnaissance.

Three dense patches of *T. daniellii* were found near the village of Etome (4° 03' N, 9° 07'E) at an altitude of approximately 425 m. The first patch was 45 minute walk from Etome, through forest fallow, to a section of *T. daniellii*, 44 m in length, between 2-5 m either side of the path. There was a short gap, 20 m along the path, before the largest of the three patches started. This section was 180 m in length, with dense, non contiguous patches either side of the path that extended in places to over 5 m in width. The last section of *T. daniellii* was a dense patch about 36 m in length, with patches extending to 10 m either side of the path.

A secondary site was visited near Etome Village, Mbwanda, about two hours walk alongside Mt. Etinde. Walking to the village, patches of *T.daniellii* were found, the largest of which was 55 m in length. Just before reaching the village another expanse of *T. daniellii* was seen, 204 m in length.

Bomana Village (4° 17'N, 9° 04'E) was visited to look for the 'dwarf' variety. Two areas were investigated. The first was either side of the road, leaving Bomana towards Idenau. *T. daniellii* was found in patches either side of the road for 2.5 km, from the village. Patches varied in size from 2 m in length with young plants, to 43 m in length with mature dense vegetation. The second area visited was a path towards a local river, River Musingili, some 100 m from the centre of Bomana. The path to the head of the river, about 1600 m, was surrounded on either side by clumps of mature, fruiting, *T. daniellii*, for the first 300 m and the last 200 m.

The mean annual precipitation in Etome is 2509 mm with a uni-modal rainfall distribution pattern (Figure 2.1). Mean annual temperature is 25.10 °C with temperatures in the range of 24.2 – 26.0 °C. The soil in this area is typically described as an andosol (FAO-UNESCO, 1977), a soil that develops in typical volcanic landscapes, being typically black and fertile (Verelst, 2009). Etome is at an altitude of 660 m.a.s.l.

2.2.2.1 Western Region, Ghana

It was decided to find a reconnaissance site in the Western Region that was within the catchment of the Asankregwa FSD, namely the forest reserves of Boitano, Tano and Tano Imri.

FSD Tarkwa controls the exploitation and management of forest reserves (FR) in the Tarkwa area of the Western Region, working closely with timber companies, private enterprises and local communities. With a staff of reserve managers, supervisors and forest guards it was ideally suited to help in the location of potential sites. Range supervisors in the forest reserves serviced by Tarkwa FSD: Bonsa, Ndumeri, Subri River, Benea, Fure Headwaters and Fure, were asked to inform staff at FSD Tarkwa if there were extensive patches of *T. daniellii* within their FRs.

Two forest reserves were visited initially over two days with forest guards and range supervisors, Neung South and Neung North. One small clump of *T. daniellii* (less than $5m^2$) was found in a smallholding of rubber.

Ghana Primewood and Timber, Takoradi, let me join their range manager for a visit to Subri Forest Reserve; the day was spent driving through the reserve and looking for *T. daniellii* in different compartments, specifically where there had been logging operations, as *T. daniellii* tends to grow where there has been disturbance (Hepper, 1968; Enti, 1975; Nicol, 1976). None was found, however, I have been informed that there are dense and extensive colonies deep in the forest reserve.

Fure Headwaters FR and Fure River FR were visited where there had been a definite observation of *T. daniellii*, near Nsuta village (5°22'N, 2°12'E) approximately 15 km from Prestea.

The sites used for reconnaissance were in the Fure FR on abandoned accepted farms. Accepted farms are farms that were in existence prior to the demarcation of the forest reserves. The site for reconnaissance was a 50 minute march from Nsuta village, passing many cocoa farms and agriculturally managed land. The transition between what was currently being managed and what had been left to fallow was marked by the boundary of the Fure River.

The area, part of the flood plain of the Fure River, was mapped using compass and tape measure. Four sites were used: the first two were adjacent sites, the first a larger continuous colony some 75 m in length by 53 m in width formed a tapered oblong shape. The second smaller site was irregular in shape (40 m by 20 m at the widest points) containing isolated patches of *T. daniellii*.

The third site was trekked to through dense vegetation, 20 minutes from the first site. Site 2 was a small patch of *T. daniellii* that contained abundant fruit. The final site, on the other side of the Fure River was 35 minutes trek from site 3. The patch was roughly the shape of a banana being 33 m long by 12 m wide.

The mean annual precipitation in Nsuta Village is 1857 mm with a bi-modal rainfall distribution pattern (Figure 2.1). Mean annual temperature is 26.3 °C with temperatures in the range of 24 -28 °C. The soil in this area is typically described as a ferralsol, (FAO-UNESCO, 1977; Verelst, 2009). Nsuta is at an altitude of 80 m.

2.2.2.2 Volta Region, Ghana

The site in the Volta Region had dense colonies of the plant and fruit. The site 15 km east of Hohoe, Gbledi-Gbogame village (7°02'N, 0°34'E) part of the Afadjato Community Forest Conservation Project (Plate 2.1), is an area from which fruit are routinely collected.

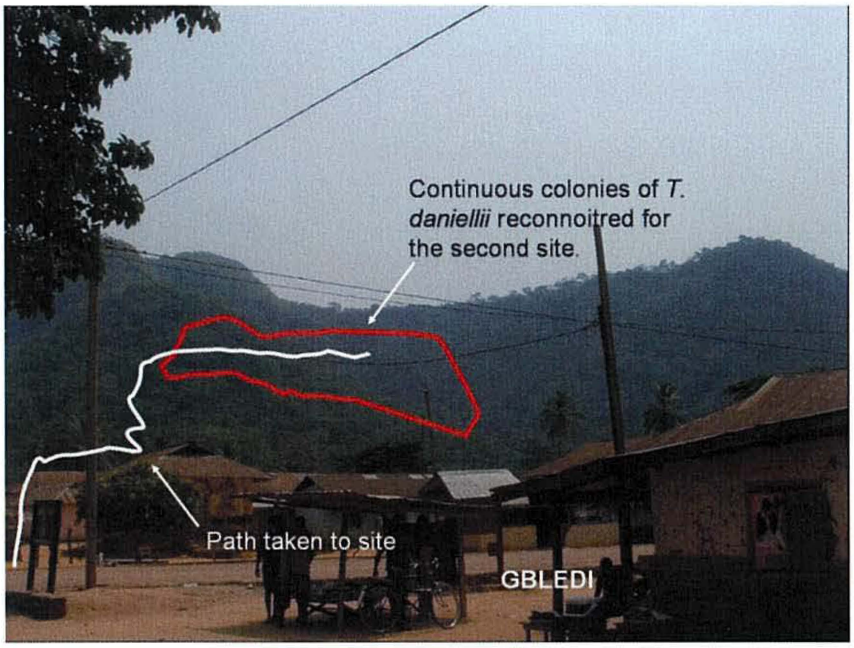

Plate 2.1 Gbledi village in the foreground with the Afadjato-Agamatso Community Conservation area in the background.

The path taken to the second site is shown and the estimated extent of *Thaumatococcus daniellii* that could be seen from the path is enclosed by the red line. This may well be a conservative estimate.

The first site was relatively close to the village; about 30 minutes walk north of the village passing cassava and cocoa plantations and was situated in an abandoned banana farm, either side of a river gulley. The site was extensive, following a path for 125 m and extending either side of the path on the steep slopes to a distance between 8 and 15 m. The other side of the gulley was not reconnoitred but was seen to extend at least 20 to 40 m up the slope.

The second site lay well within the Afadjato Community Forest Conservation area, about an hour and twenty minutes walk to North West of the village (Plate 2.1). This area had very steep, rocky terrain (Plate 2.2) and had massive colonies of *T. daniellii*. Colonies of *T. daniellii* extended to where it was not safe to venture due to the terrain. Walking along the forest path colonies were continuous for almost 300 m. Either side of the path it was estimated that the colony extended well to the base of the gulley and high above the path to at least 50 - 75 m. The area reconnoitred was between 2 - 5 m either side of the path due to the very steep nature of the terrain.

The mean annual precipitation in Gbledi Gbogame is 1432 mm with a bi-modal rainfall distribution pattern (Figure 2.1). Mean annual temperature is 22.8 °C with temperatures in the range of 21 – 25 °C. The soil in this area is typically described as a leptosol (FAO-UNESCO, 1977), a very shallow soil that is very gravelly or stony (Verelst, 2009). Gbledi Gbogame is at an altitude of 300 m.

2.3 Material and methods

This section looked at the decision making process for the collection of plant and fruit data, and how the data was analysed. Further, it described the method for the extraction of arils, and the analysis of extracts of thaumatin solutions. Of the four provenances described above, data on plant morphology was collected from the following areas: Western Region, Ghana: all sites in the Fure River Forest Reserve; Volta Region, Ghana: the second site in the Adjafato Community Forest Area; South West Province, Cameroon: Etome sites and South Province, Cameroon: Mebanga site.

2.3.1 Collection and storage of fruit

Fruit had to be collected in order to be measured to investigate differences in fruit morphology and also to provide sufficient aril for the extraction of thaumatin. It was suggested that an amount of about 150 g of aril would be necessary for thaumatin extraction (Charles Boy, pers. comm. 2005). This equated to about 3 – 5 kg of fruit per provenance. Fruit were collected from the entire area, not just the measurement quadrats, where measurements of petiole and lamina were made. The fruit of *T. daniellii* is very easily identified and as such no real training in the identification of fruit had to be conducted, other than showing collectors what the fruit looked like. It was emphasised that only mature fruit was to be collected. Mature fruit was identified as being fully red, either bright or a deep crimson red colour, and easily removed from the rachis by gently pulling. Immature fruit, orange, yellow, black or green in colour, were to be left in situ. Fruit were placed in sacs, labelled, and carried safely to the processing area. Fruit collected had to be stored in refrigerated environments for until they could be processed.

2.3.1.1 Ghana

There was a delay of two weeks in the delivery of scales from the UK due to courier problems and customs checks. For this reason fruit collected from the Western Region (13th January 2006), was stored in a domestic refrigerator set to 5°C. This temperature was suggested following experience in the collection and storage of fruit (Martin Laasen, pers. comm. 2005): any temperature higher than this made fruit rotten within a few days. Volta region fruit were collected on the 19th January 2006, and transported to Takoradi where they were stored till the arrival of scales (27th January 2006).

Fruit were spread out as evenly as possible on the shelves of the refrigerator following washing of fruits in lukewarm water to remove forest residues, dirt and insects.

2.3.1.2 Cameroon

Due to the processing and planting of rhizomes and the large amounts of fruit collected from both sites had to be refrigerated until there was adequate time to conduct processing.

2.3.3 Establishment of *Thaumatococcus daniellii* sampling plots

Using a calculator random numbers were used to determine the location of plots within dense colonies of *T. daniellii* for measurement. The first number would select the distance walked along the path (m), or around the patch from a given datum point, the next number would select the distance walked (m) into *T. daniellii* colony. A 1 m^2 quadrat was erected with the centre being placed on the point selected, parallel with the path or edge of the patch. Dense colonies of *T. daniellii* had a minimum of eight one meter squared plots assigned for which the number of petioles and fruit present were measured. The height of the petiole was measured and the length and width of the lamina were recorded.

2.3.4 Measurement and analysis of plant and fruit morphology

2.3.4.1 Measurement of petiole height

Using a 5 m tape measure the height of the petiole, from ground level to the upper pulvinus, where the callus became pronounced, was measured to the nearest millimetre (Plate 2.2). The entire length of the plant (properly described as the leaf,

i.e. the petiole plus the lamina), that is its height to the acuminate tip of the lamina blade was not measured for two reasons: the first was that the lamina varied in aspect in relation to the ground surface and could result in the lamina being below the height of the upper pulvinus (this is likely a phototaxic response of the pulvinus to differing light levels throughout the day); the second is that accuracy in the measurement of the petiole when including the callus, was found to be difficult and due to the curved nature of the callus, especially where the height of the petiole was in excess of two meters.

2.3.4.2 Measurement of lamina blade

The adaxial (upper surface) of lamina was measured. The callus of *T. daniellii* has an indented groove at the juncture of the blade. The length of the blade was measured from this groove to the acuminate leaf apex, with the measuring tape being placed along the primary vein (Plate2.3). The width of the blade was measured at the widest point horizontal to the primary vein. This was found by moving the rule along the blade, horizontal to the primary vein, till the maximum width was observed. Both measurements were made to the nearest millimetre.

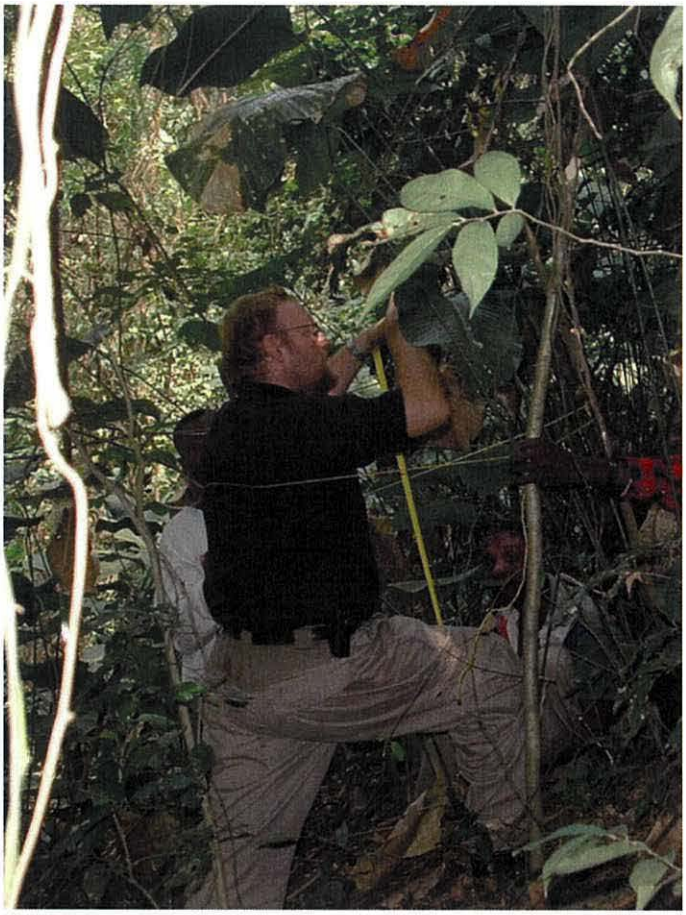

Plate 2.2 The author measuring petiole height on the steep terrain of the Afadjato Community Forest Area. Gbledi Gbogame, 2006.

Plate 2.3 Measurement of the lamina length and width using tape measure. Gbledi, 2006.

2.3.4.3 Measurement of fruit length and width dimensions

Fruit had their length and width measured using a pair of Vernier callipers accurate to 0.02 mm. Length was measured by placing the tip of the callipers at the top of the

fruit, where it had been attached to the rachis, and placing the other tip of the calliper at the apex of the fruit, where a small circular indentation is found (Plate 2.4).

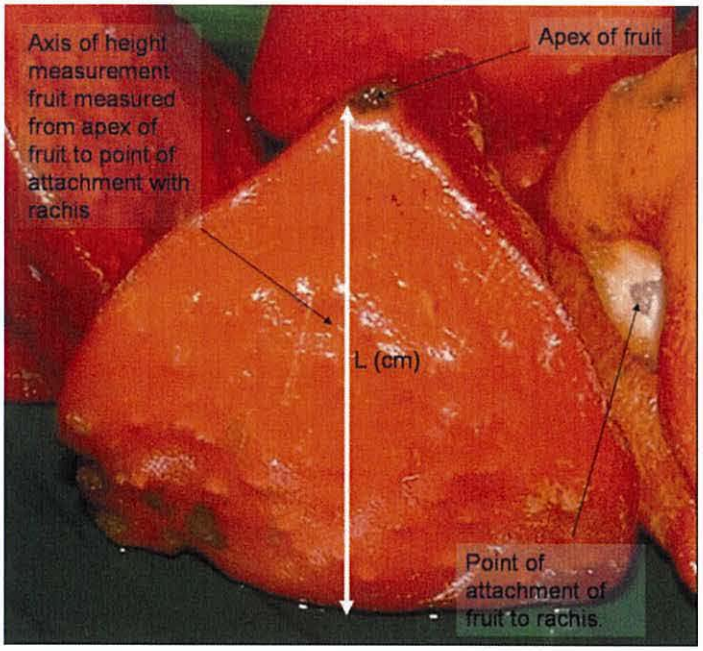

Plate 2.4 *Thaumatococcus daniellii* fruit showing the axis along which length was measured.

Width was measured perpendicular to the length, in parallel with the top of the fruit along the longest side. (Plate 2.5)

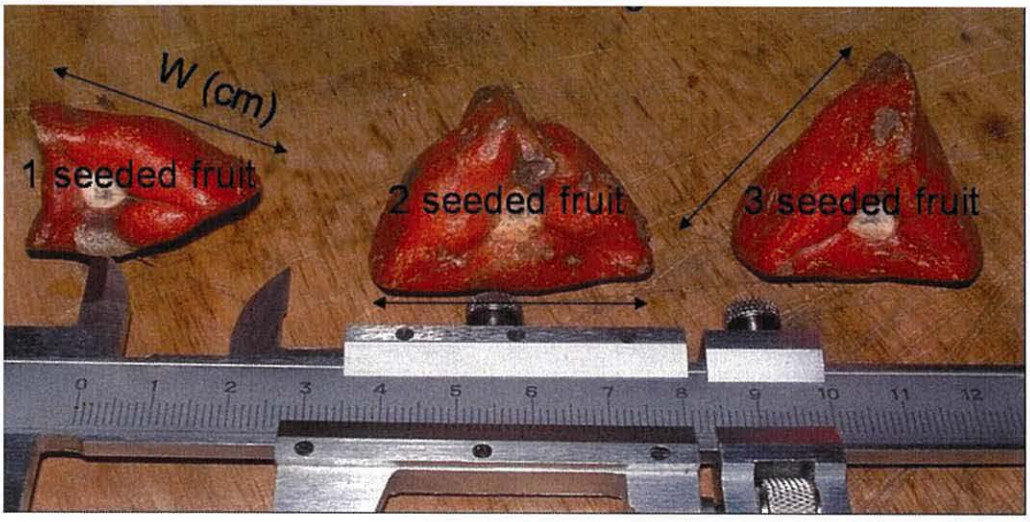

Plate 2.5 *Thaumatococcus daniellii* fruit containing different numbers of seeds. Double ended arrows show the long axis of the fruit where width measurements were taken.

2.3.4.4 Measuring fruit mass

Individual fruit weighed using an Adam Equipment ACB plus 1000 laboratory scale, Adam Equipment Co. Ltd. Milton Keynes, UK. The scales were placed onto a level surface and zeroed before weighing began. Individual fruit were weighed to an accuracy of 0.01 g, the mass being recorded in a table, and removed from the scales.

2.3.4.5 Cutting fruit and excision of arils

Fruit were cut in order to remove the complete aril without damaging it and to record the number of seeds in the fruit. Once cut, seed number and aril number were recorded. Methods were slightly different for fruit with different number of seeds. With experience fruit were identified as being single, two or three seeded before cutting (Plate 2.5).

Single seeded fruit

The short edge of the fruit was cut longitudinally from the edge of the scar, closest to the short edge, to the apex with a sharp knife. A cut was made on either of the long sides about a 1/3 of the way from the top of the fruit to the apex, creating a flap of pericarp. This flap was flipped up gently with the knife to remove it, showing the aril. The aril was removed by placing the knife between the aril and the seed and cutting into the top of the seed, and separating the integuments holding the aril from the seed.

Two seeded fruit

The two short edges of the fruit were cut, about 1/3 of the way from scar to apex. The resulting flap was levered out of the way by placing the knife on the corner of the fruit opposite to the long side and lifting the flap. Exposed arils were removed by cutting between the aril and the seed, on each aril in turn and then separating the integument holding the arils from the third ovary compartment.

Three seeded fruit

Any two of the three edges were cut as for two seeded fruit, from scar to apex. The resulting flap was levered in the same manner as for two seeded fruit. The three exposed arils were removed as a complete item by cutting from one corner of the fruit, between the base of the aril and the top of the seed, to the opposite edge.

2.3.4.6 Measurement of aril mass

Excised arils from each cut fruit were placed onto the ACB plus 1000 Adam Equipment Co. Ltd. Milton Keynes, UK, weighed and mass recorded to the nearest 0.01g. For two and three seeded fruit, two or three arils were weighed together respectively. Mean individual aril mass was determined by dividing the mass of the complete weight by the number of arils.

2.3.4.7 Analysis of plant and fruit morphology

Data collected for each plot were used to generate plot means for petiole height, lamina length, and lamina width. These plot means were used to generate provenance means for the above characteristics. SPSS v 14, was used to run an analysis of variance (ANOVA) on the data set. Where significant differences were observed effect sizes were given following procedures in Appendix 1.

As fruit were collected from the entire population for each provenance, means for length, width, mass and aril mass were based per population not per individual plot. Aril mass as a percentage of fruit mass was worked out for each fruit, and a mean then worked out. The data set had an ANOVA applied to it using SPSS v 14.

Numbers of different seeded fruit per provenance were also noted and are given as a function of the total fruit collected.

2.3.5 Storage and export of arils

Measured arils were placed on a steel tray and placed in a freezer kept below 0°C (usually between -5 °C and -15°C). Once arils were individually frozen they were removed from the steel tray and placed into plastic bags, labelled, and kept in the freezer compartment.

2.3.5.1 Export of arils from Ghana

Frozen arils were packed into thermos flasks that had been kept open in the freezer compartment overnight. Ice was placed into the thermos flasks with the bags of frozen arils fitted into the thermos flasks. Arils were taken to Hohoe to Forest Im-Pex

where they were stored in the company's chest freezers. Frozen arils were shipped together with an aril consignment, with all necessary shipping and export documentation, to the UK via air freight.

2.3.5.2 Export of arils from Cameroon

Frozen arils were packed into a portable cool box, with ice and ice-packs. The box was packed with insulating material and sealed with tape. The cool box was transported in hold luggage with all necessary phytosanitary certificates.

2.3.6 Thaumatin extraction

The extraction of thaumatin used a scaled-down version of the extraction method used by Natex UK Ltd. An aqueous extraction of thaumatin, from arils, was conducted with chromatography to purify and concentrate the resultant solution (Boy and Waliszewski, 2009). Analysis was then conducted using an ultraviolet spectrophotometer. Approximately 50 g of arils were used per extraction.

2.3.6.1 Overview

A 5% (± 0.05%) thaumatin solution, batch number GL05SB1901, was provided by Natex UK Ltd. This was used to calibrate the UV spectrophotometer being used (Vis spectrophotometer – UV1 Thermospectronic.), and work out the protein extinction coefficient. Most protein extinction coefficients (εpercent) fall in the range 4.0-24.0 (Fasman 1992; Pierce Biotechnology, 2002). Using a 1 cm light path, the extinction coefficient for a 1% solution of thaumatin protein at a wavelength of maximum absorbtion around 280 nm is 12 (Charles Boy, pers. comm. 2009). However, spectrophotometer deuterium lamps decay with use and readings for extinction coefficients vary as a result, so the wavelength of maximum absorbance near to λ280 nm was determined and the 5% thaumatin solution used as a standard to determine the extinction coefficient (ε) using this specific equipment (Table 2.1).

Prior to reading absorbance values of thaumatin extracts the UV spectrophotometer had to be calibrated and zeroed using distilled water, the base solvent for the extraction of thaumatin. Four 1 cm^3 quartz ultraviolet cuvettes were used during the analysis. Quartz cuvettes are used in near UV spectrophotometery to measure

protein concentration as they are transparent to about 190 nm, whereas glass is opaque to UV below 300 nm (Harwood and Claridge 1997). They were standardised by reference to the absorption (γ) at λ280 nm through water 0.000 units. Any variance from this value was accepted as cuvette absorbance variation: subsequent readings from cuvettes that showed cuvette absorbance variation, were corrected by the factor that they varied with distilled water.

To read the absorbance value of thaumatin extracts (first the diluted 5% thaumatin solution), they were placed into quartz cuvettes and read at a wavelength of λ279 nm as opposed to the standard value of λ280 nm (Aitken and Learmonth, 1996). It was found following repeated measures that λ279 nm was the wavelength at which maximum absorbance occurred, and was used throughout. The UV spectrophotometer was set to this wavelength (λ279 nm) for all subsequent measurements. The basic procedure for using the UV spectrophotometer is given below:

1. Warm up the UV lamp (about 15 minutes)
2. Adjust wavelength to 279 nm
3. Calibrate to zero absorbance with buffer solution only
4. Measure absorbance of the protein solution

2.3.6.2 Determination of the extinction coefficient at λ279 nm using diluted (5%) standard thaumatin solution.

The standard solution of known thaumatin concentration (5%) was used to calibrate the UV spectrophotometer being used. In this study the solution was dissolved by a factor of approximately 100 as per Aitken and Learmonth (1996) to enable detection by the UV spectrophotometer. Two standards were prepared and the mean percentage thaumatin concentration from diluted standard was used to determine the extinction coefficient (Equation 3).

Table 2.1Description of standard produced for the determination of the extinction coefficient

5 % thaumatin solution (g)	Mass of water (g)	Concentration of thaumatin (mass fraction) – using equation 1	Diluted thaumatin concentration from the standard solution (5%) (%) – using equation 2
2.85	249.31	0.0005	0.05
2.52	252.23	0.0005	0.05
Mean			0.05

To determine the concentration of the diluted standard thaumatin solution Equation 1 was used.

$$\textbf{Conc. dil. thaumatin. standard solution} = \frac{\textbf{Mass of thaumatin standard solution (g)}}{\textbf{Mass of solvent (water)(g)}}$$

(Equation 1)

In order to determine (ε) the result of Equation 1 was used to work out the thaumatin concentration (mass fraction) of the diluted solution as a percentage in two steps: the first to determine the concentration (Equation 2),

$$\textbf{Concentration of thaumatin in solution} = \textbf{Conc. dil. thaumatin. std. sol.} \times \textbf{\% standard thaumatin solution}$$

(Equation 2)

the second to work out the % of thaumatin in the diluted solution (Equation3)

$$\textbf{\% Diluted thaumatin conc. std sol.(\%)} = \textbf{Conc. of thaumatin in solution.} \times \frac{\mathbf{100}}{\mathbf{1}}$$

(Equation 3)

The result from equation 3 was divided by the value of the mean absorbance at λ279 nm of the diluted thaumatin standards, n= 4, (Equation 4) to determine (ε).

$$\varepsilon = \frac{\gamma\ \textbf{Standard Solution}_{(\lambda\ 279)}}{\textbf{\% Dil. Th. conc. standard (\%)}}$$

(Equation 4)

The mean absorbance at γ279 nm was 0.700 units. This gave an extinction coefficient (ε) of 14.0.

2.3.6.3 Calculation of percentage thaumatin yields and concentration from solutions of aril extracts.

Thaumatin was extracted from the arils using the method of Boy and Waliszewski (2009). Thaumatin was extracted from a known mass of arils, AR mass (g), and produced a mass of raw solution, RS mass (g). Four thaumatin extract solutions per provenance were extracted. The constraint on the number of extracts was due to the amount of aril that had been sourced from each of the provenances.

The resultant extracts were immediately analysed with an ultra violet spectrophotometer (Vis spectrophotometer – UV1 Thermospectronic.), using a standard estimation of protein by near UV absorbance at λ 279 nm (modified, Aitken and Learmonth, 1996).

Four samples per extract were measured using the UV spectrophotometer, such that 16 readings were taken from all the provenance samples, plus an additional four extractions were made from a sample (unknown location, Cameroon) provided by Natex UK Ltd. to act as a control and to check the method being used was comparable to the analysis of thaumatin concentration and percentage yield used in industry.

To calculate the percentage of thaumatin that was contained within the raw extract from the arils, the absorbance values for each thaumatin extract (n=4) (γ Raw Solution at λ279 nm) were read and the mean used in equation 5.

$$\textbf{Mean percentage thaumatin from raw solution } \left\{\overline{Th_{RS\%}}\right\} (\%) = \frac{\overline{\gamma\ Raw\ Solution}_{(\lambda 279)}}{\varepsilon} \qquad \textbf{(Equation 5)}$$

To work out the yield of thaumatin, in terms of concentration and percentage from arils, the result of equation 5 was used to work out the mass of thaumatin present in

arils (Equation 6) and then, using this value, concentration of thaumatin in arils was calculated (Equation 7).

$$\textbf{Mass of thaumatin from arils (g)} = \overline{Th_{RS\%}} \times \textbf{RS mass (g)}$$

(Equation 6)

Concentration of thaumatin in arils equals;

$$\mathbf{AR_{(Th.conc.)}} = \frac{\textbf{Mass of thaumatin from arils (g)}}{\textbf{AR mass (g)}}$$

(Equation 7)

With the concentration of thaumatin in arils known, $AR_{(Th.\ conc.)}$, the percentage thaumatin yield (%) generated from arils could be worked out as follows (equation 8)

$$\textbf{Thaumatin yield (\%)} = \mathbf{AR_{(Th..conc.)}} \times 100$$

(Equation 8)

Mean values of thaumatin yield from extracts from the same provenance were used to determine differences in thaumatin yield from provenances.

Results were tabulated and SPSS version 14 and Minitab 16 were used to conduct an analysis of variance between the extracts.

2.4 RESULTS

2.4.1 Plant and fruit variation

The plants collected from the Volta Region, were the largest with 69% taller petioles and lamina length and width larger than the mature plants found in the other provinces, 64% and 65% respectively (Table 2.2). Volta Region plants were also significantly taller than plants from both Cameroonian populations. There was no significant difference between the South Western Province, Cameroon and Western Region, Ghana in mean lamina length or width, or petiole height. South Province plants were significantly shorter and with smaller leaves than all other populations (Plate 2.6).

Table 2.2 Mean ± standard error of *Thaumatococcus daniellii* plant and fruit characteristics by provenance.

Provenance	Lamina length (cm)	Lamina width (cm)	Petiole height (cm)	Fruit mass (g)	Aril mass (g)	Aril mass as % of fruit mass
Western Region	42.71 ± 1.57^{b} (8)	28.57 ± 1.13^{b} (8)	151.36 ± 16.39^{ab} (8)	10.78 ± 0.21^{b} (313)	0.69 ± 0.02^{b} (313)	6.35 ± 0.11^{b} (313)
Volta Region	50.78 ± 2.11^{a} (8)	33.35 ± 1.05^{a} (8)	193.91 ± 15.28^{a} (8)	20.99 ± 0.34^{a} (276)	1.08 ± 0.03^{a} (276)	5.07 ± 0.08^{a} (276)
South West Province	39.99 ± 0.52^{b} (36)	27.17 ± 0.43^{b} (36)	143.40 ± 8.85^{b} (36)	12.63 ± 0.18^{c} (413)	0.46 ± 0.01^{c} (413)	3.55 ± 0.06^{c} (413)
South Province	28.42 ± 1.07^{c} (17)	17.95 ± 0.73^{c} (17)	86.20 ± 5.97^{c} (17)	8.15 ± 0.11^{d} (507)	0.47 ± 0.01^{c} (507)	5.64 ± 0.07^{d} (507)

Lamina and petiole data based on number of plot mean samples, fruit and aril metrics based on number of samples Numbers in brackets refers to the number of plot means used for sampled plants and number of sampled fruit. Means in the same column with different superscript letters are significantly different ($p < 0.05$)

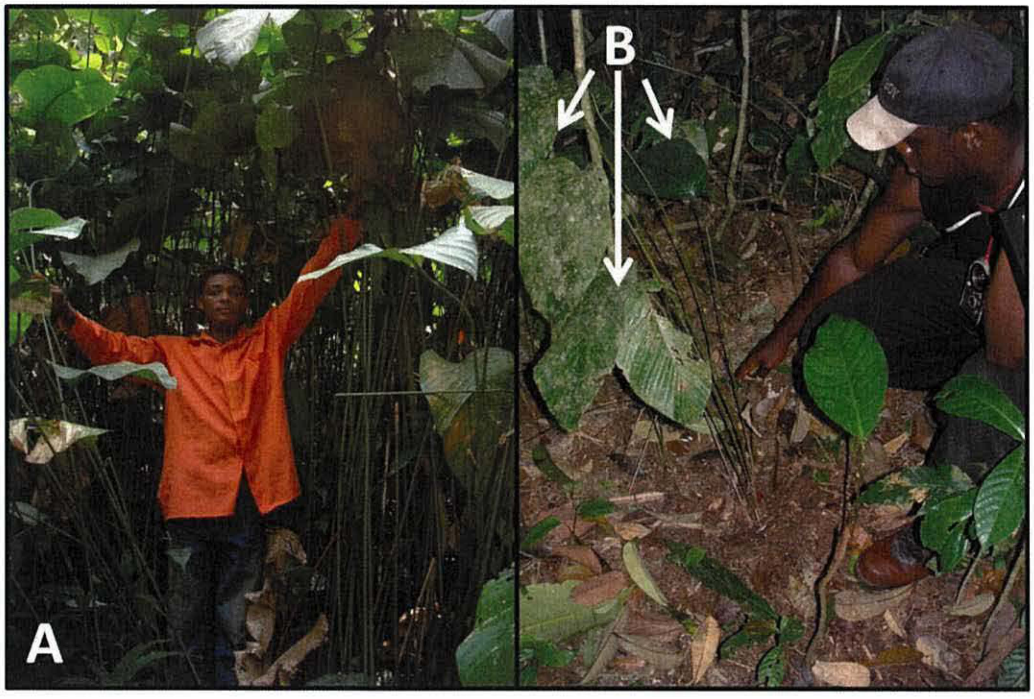

Plate 2.6 Mature *Thaumatococcus daniellii* from the Volta Region, Ghana (A) and the South Province, Cameroon (B).

These two populations display the greatest natural variation between plants in terms of petiole height and lamina size with Volta Region petioles being 69 % taller and lamina size being 64 % greater than plants from the Southern province. (Table 2.2). Arrows point to *T.daniellii* in picture B.

Fruit mass varied significantly with provenance $F(3,1505) = 701.67$, $p<0.000$ $\omega^2 = 0.58$, with mature fruit from the Volta Region being 72% more massive than Southern Province fruit (Figure 2.7) which were the smallest with a mean mass of 8.15 g. Mean aril mass was not significantly different between the two Cameroonian populations, though fruit size was. Mean aril mass as a percentage of fruit mass varied amongst all provenances with the highest in the Western Region of Ghana being more than twice than that of the lowest in the South West Province of Cameroon $F(3, 1505) = 242.97$, $p<0.000$, $\omega^2 = 0.32$.

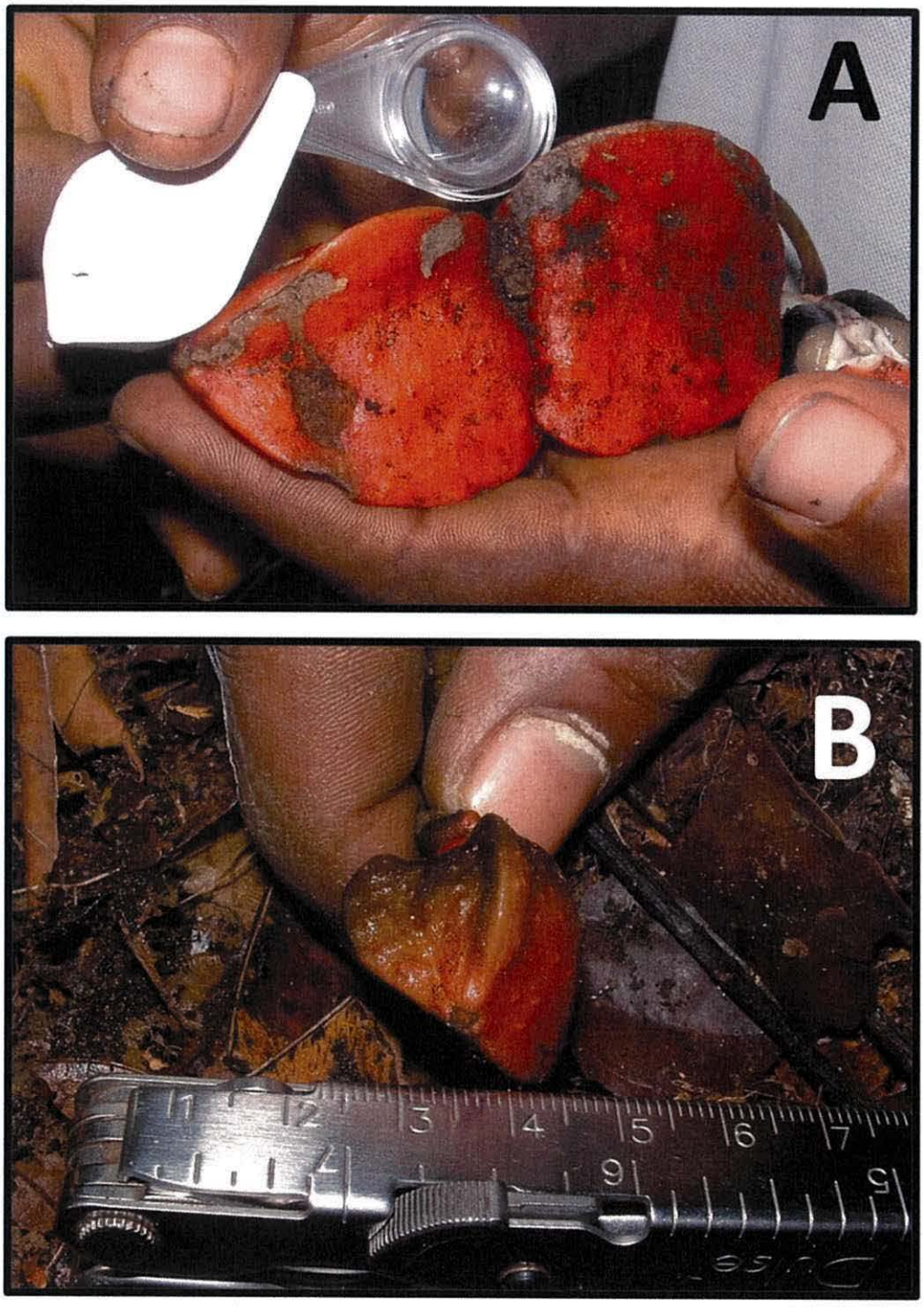

Plate 2.7 Variation in mature fruit of *Thaumatococcus daniellii.*

Fruit shown are from the Volta Region, Ghana (A) and the Southern Province, Cameroon (B). Volta Region fruit are 72% more massive than South Province fruit based on mass (g).

2.4.2 Thaumatin variation

Thaumatin content varied within-provenance extracts, though not significantly. Percentage thaumatin yield, the standard by which thaumatin is measured in industry, varied between 1.08% and 2.00% from individual samples (Figure 2.1) There was significant variation in the mean percentage yield among provenances $F(4,15) = 12.97$, $p<0.000$ $\omega^2 = 0.70$ (Figure 2.1). The mean percentage yield of thaumatin from the South Province, Cameroon, 1.81%, (Figure 2.1) was significantly greater than other provenances. Extracts from the South West Province and the Western Region yielded the lowest percentage yields, 1.33 % and 1.31 % respectively. There were visible differences, in colour, hue and clarity of thaumatin extracts, within-and-among provenances (Plate 2.8).

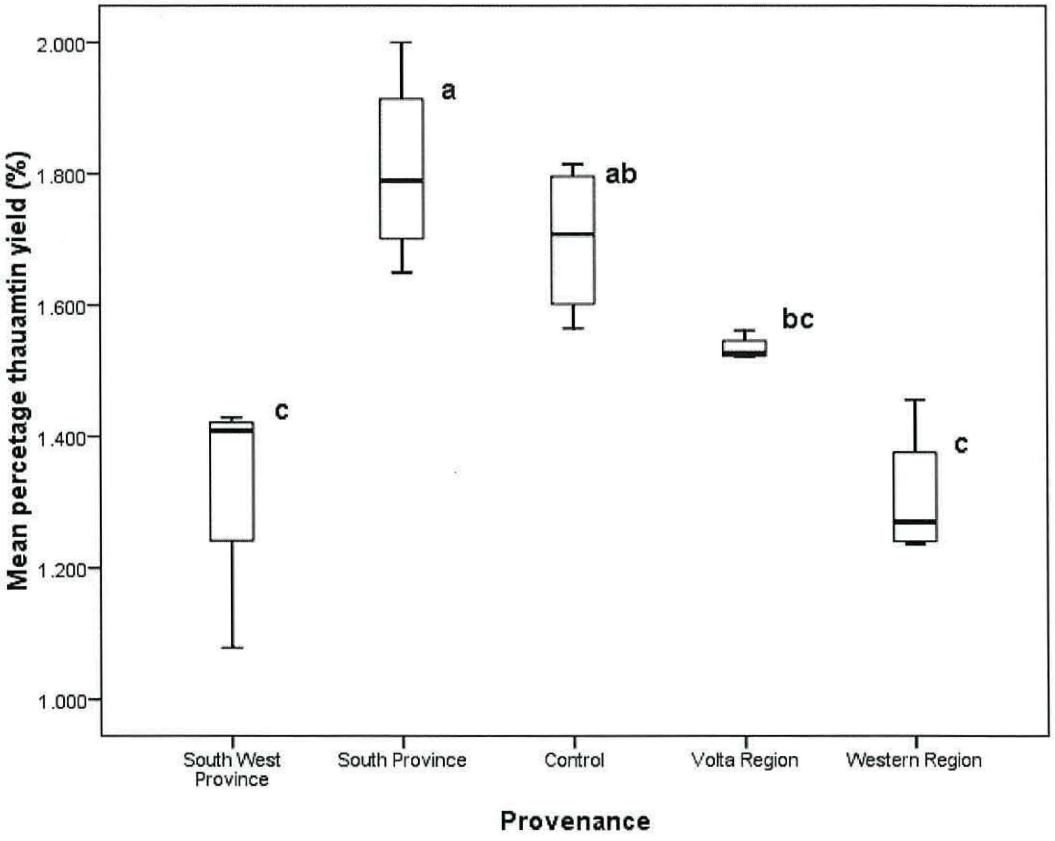

Figure 2. 2 Boxplot showing thaumatin yield (%) by provenance.
Superscript letters indicate significant differences amongst means (p = 0.001).

Plate 2.8 Variation in colour of thaumatin extracts from arils from different populations plus a control group:

1: Western Region, Ghana; 2: Volta Region, Ghana; 3: South West Province, Cameroon; 4: South Province, Cameroon. 5: Control sample, unknown location, Cameroon.

2.4.3 Collection effort as a function of fruit number and aril mass

The number of fruit and mass of fruit required to produce a kilogram of aril varies between populations, due to variance in the mass of fruit, and the aril mass as a percentage of total fruit mass particular to a provenance. At the extremes this varies by 1315 fruit (VR-SWP) and 12.34 kg of fruit per kg of aril (WR-SWP) among provenances. In terms of the number of fruit required the Volta Region, Ghana, requires the least number of fruit to be collected, 951, (Figure 2.3). In terms of mass of fruit the Western Region of Ghana, requires the least mass of fruit, 15.73 kg. Fruit from the South West Province contain the least amount of aril as a percentage of total fruit mass reflected in the high number, 2230, and mass of fruit 28.16 kg required in order to produce 1 kg of aril.

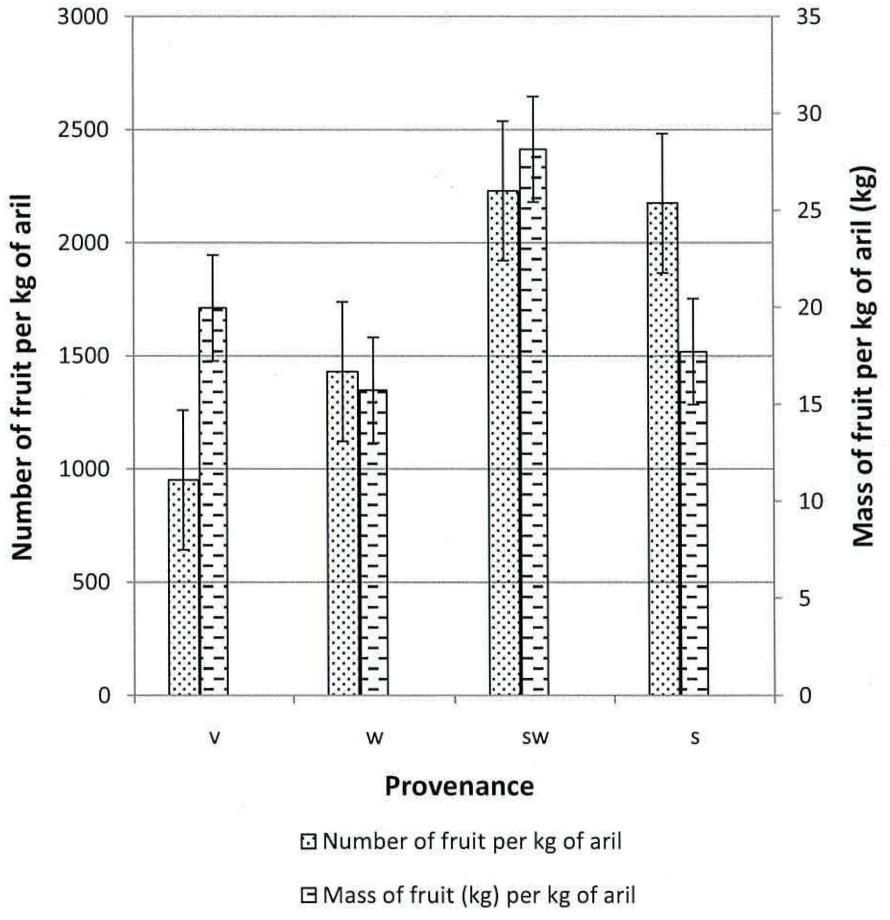

Figure 2.3 Number and mass of fruit needed to produce 1 kg of aril.
V = Volta Region, Ghana; W = Western Region, Ghana; SW = South West Province, Cameroon; SP = South Province, Cameroonn.

2.4.4 Thaumatin content and fruit number

The number and mass of fruit required to produce a kilogram of thaumatin varies according to two factors: the first being the mass of aril per fruit, and the second being the percentage thaumatin yield from extracted arils (Figure 2.1).

The mass of fruit required to produce 1 kg of thaumatin varies by 1131 kg between the provenances, and by 105 524 fruit (Table 2.5). The Volta Region, Ghana requires the least amount of fruit to produce 1 kg of thaumatin, almost 62 000, whereas material from the South Province of Cameroon requires the smallest mass of fruit, at 980 kg to produce a similar amount of thaumatin. With a slightly lower yield of thaumatin the Western Region of Ghana requires 58 183 less fruit, approximately 912 kg less material, than is required by the South West Province of Cameroon to produce 1 kg of thaumatin.

Table 2.3 Mass and number of fruit and mass of aril required to produce 1 kilogram of thaumatin.

Provenance	Thaumatin yield (% of aril mass)	Thaumatin mass kg^{-1} aril (g)	Mass of aril kg^{-1} thaumatin (kg)	Number of fruit kg^{-1} thaumatin	Mass of fruit kg^{-1} thaumatin (kg)
Western Region	1.31	13.08	76.46	109335.58	1202.69
Volta Region	1.53	15.34	65.19	61994.78	1301.17
South West Province	1.33	13.31	75.12	167518.03	2115.38
South Province	1.81	18.07	55.33	120338.61	980.41

2.5 Discussion

Supply issues

Present industrial concern is to diversify supply to ensure that it remains stable and sustainable in anticipation of increasing demand for thaumatin (Waliszewski and Sinclair, 2007), however, increasing supply from managed cultivation or new collection areas may threaten existing collectors and cutters livelihoods, if there is a decrease in the demand for thaumatin.

There are, it is understood two methods of supplying cut arils. The first is described by Bonnehin, (1997) operating in Ivory Coast: it involves intermediaries buying fruit from collectors and extracting arils, which they in turn sell to exporters of the arils. In this situation the price of the fruit is determined by the aril cutters and the quality and quantity of arils cut. The second supply chain method, described by Waliszewski and Sinclair (2007) currently operating in Ghana involves the cutting station buying fruit from collectors and paying staff to extract arils. Both methods produce quality arils for extraction of thaumatin, however, where intermediate aril extractors sell arils to exporters it could be argued that the quality of cutting is better. In this situation the first method of supplying arils, through the use of intermediary cutters, would favour the siting of a cutting station close to where natural populations have a higher percentage of thaumatin in arils.

Variation

In terms of thaumatin content, the highest was from arils from the South Province, Cameroon. This was significantly more than any of the other provenances, but with the caveats that fruit size was the smallest and that its value of 1.8% thaumatin yield was within expected industry values. Furthermore, thaumatin content, which varied significantly amongst provenances was not inversely related to fruit size as results from South Province, Cameroon suggest, that is, smaller fruit contain higher percentages of thaumatin, as Volta Region fruit which are the most massive - both in terms of absolute fruit and aril mass (but with relatively low percentage aril as a proportion of total fruit mass) - have the second highest thaumatin percentage yield.

The Western Region was the most efficient provenance for the production of arils, even though its fruit were the third smallest in comparison with other provenances, as it had the largest arils as a percentage of total fruit size. Conversely, the South West Province, Cameroon, with large fruit and small arils was the most inefficient for the production of arils and thaumatin, as it also had a correspondingly low percentage of thaumatin in the arils.

It is not envisaged that variation in the fruit, aril and thaumatin from the natural resource herein described will adversely affect current collection operations in Ghana

and Ivory Coast that have been ongoing for the past 20 years (Bonnehin, 1997) but rather may complement current collection. These two countries include two of the most important provenances for fruit collection; the Volta Region, Ghana where the largest natural fruit exist, and the Western Region, Ghana which produces the most efficient fruit for the extraction of arils.

Production of fruit managed with agricultural crops is another potential method to diversify the supply and ease the collection of fruit. The difference in petiole height and lamina size is of interest primarily where *T. daniellii* is to be considered as an intercrop with perennial tree crops such as rubber, (*Hevea brasiliensis)*, cocoa, *Theobroma cacoa* L. and oil palm, *Elaeis* spp. Jacq., or for the production of leaves for sale in markets or petioles for mat making (Abbiw 1990; Arowosoge and Popoola, 2006). It is known that Ghanaian cocoa farmers generally considered *T.daniellii* to be a weed because the prevalent tall variety, prevented air circulation in cocoa plantations with potentially negative impacts on disease incidence (Waliszewski *et al.,* 2005). It was thought that plant height was of little consequence under a tall, mature rubber canopy (Waliszewski and Sinclair, 2007). Some farmers expressed concern that having a tall "weed" growing under their rubber plantations would aesthetically displease them (Paul Appiyah, pers. comm. 2009), whilst others were concerned with the effect of tall herbs on tappers access to plants (Pa Enoh Ferdinand, pers. comm. 2009) though, tappers when asked said that it did not affect their access to tapping or collecting of latex and cup lump.

The height of plants did vary considerably among provenances, with the most massive coming from the Volta Region, Ghana. The 'dwarf' variety suggested by Limbe Botanic Garden staff, was not found in the South West Province, Cameroon, but instead near the location of one of Dhetchuvi's (Dhetchuvi, 1996) and Dhetchuvi and Diafouka's (Dhetchuvi and Diafouka, 1993) specimens of var. *puberulifolius*. These plants indeed had pubescent hairs on the underside of leaves but only in young lamina; in mature plants the leaves were glabrous, perhaps indicating that pubescence is a developmental stage in the leaves of this plant and Dhetchuvi and Diafouka's (Dhetchuvi and Diafouka, 1993) determination of var. *puberulifolius* need re-examination. Lamina from other populations did not show pubescence in immature leaves *in situ* lending some credence to Dhetchuvi and Diafouka's

assertions, though perhaps not being relevant to the Western part of West Central Africa (Dhetchuvi and Diafouka, 1993).

Environmental factors

Of interest is the marked similarity between the morphology of plants from the Western Region of Ghana and the South Province of Cameroon, and the differences between them and the other two provenances. It seems that phenotypic similarity may be due to plants sharing a similar latitude and climate, than those further apart. Clinal gradients may be present that affect plants further north or south of the latitudinal plane between 4-5°N. This may result from clinal variations such as rainfall. Interestingly, the Volta Region, which produced by far the largest fruit and arils, was found in the driest region surveyed, and on soil that collectors in the Western Region of Ghana suggested would be the worst for the growth of *T. daniellii* (Waliszewski *et al.,* 2005). Collectors suggested that rocky or gravelly soils (leptosols (FAO-UNESCO, 1977; Verelst, 2009)) would impede the growth of *T. daniellii* which they suggested required soft clay or loamy soils to grow. Adesina (1994) and Enti (1975) supported this supposition, stating that *T. daniellii* preferred loamy soils. Shutt (1986) explained that *T. daniellii* preferred alluvial soil, shunning high primary forests and oxysoils (oxisols / ferralsols) which are described as the most highly weathered tropical soils by Ahn (1993), though the distribution as a whole suggests that *T. daniellii* is assosciated with ferralsols (Waliszewski *et al.,* 2005).

Given that *Thaumatococcus daniellii* generally exists in environments with a mean annual rainfall of >1500 mm, with it being well-distributed (>50 mm in at least nine months) and with a mean annual temperature between 23°C to 26°C (Waliszewski *et al.,* 2005) it is not such a contradiction to find it present in Gbledi-Gbogame, Volta Region, which shares these characteristics (1432 mm of rain per annum; mean annual temperature 23°C and nine to ten months with > 50 mm of rain (Figure 2.1). Thus there is no evidence to suggest that the high rainfall and fertile soils in Etome, South West Province, Cameroon, confer an advantage the plant.

As it seems the climatic conditions are conducive to the growth of *T. daniellii* in Gbledi Gbogame, Volta Region, there is no evidence to suggest that gravelly, stony, or rocky soils have a negative impact on fruit or aril size, contradicting the local knowledge of collectors in Ghana (Waliszewski *et al.,* 2005).

Future production

Opportunities for exploitation of fruit in new parts of the plant's range are clearly higher where aril yield per individual and per kg of fruit are higher (Waliszewski and Sinclair, 2007), and gross fruit size, percentage yields of thaumatin, and aril as a percentage of total fruit size are large. However this is only true assuming that the areas produce the same amount of fruit per hectare and they have broadly the same annual production and similar infrastructure in place. Collection operations from new areas could support the current supply of fruit and diversify its source. Where fruit exist naturally there would be a requirement to establish new supply chains and cutting stations.

Cultivation of *T. daniellii* has been reported under *Hevea brasiliensis* (rubber) in Ivory Coast and under *Elaeis* spp. (oil palm) and *Tectona grandis* L.F (teak) in Ghana (Waliszewski *et al.,* 2005) and is another method to diversify and secure supply of fruit in the long term. The natural variation revealed here suggests both that there is considerable scope for gains through the use of high yielding germplasm and the possibility of selecting different plant morphology for different intercropping niches. In West and Central Africa the inter-rows of mature rubber, are usually found with cover crops, weeds or rubber seedlings.

The perception of what a typical rubber farm looks like may alter if there is to be value gained from intercropping with *T. daniellii*, as suggested by small holders. However, although domestication may seem an attractive option, capable of increasing and diversifying smallholder rubber income and increasing resilience to shocks, there has been high variability in the production of fruit from managed areas largely due to low fruit set (Onwueme *et al.,* 1979). Currently there has been no published data to determine the effect of *T. daniellii* on the production of the primary crop, latex and cup lump rubber till this work (Chapter 5).

2.6 Conclusion and recommendations

Variation in *T. daniellii* is apparent within wild populations and looks to be clinally influenced with variation to typical form and structure of petiole, lamina, fruit size and aril, determined by increased latitudinal distance from 4-5° N. Differences in thaumatin yield have been shown to be statistically significant, though all fall within the expected range of thaumatin yield from industry standards. Differences in aril content of fruit and percentage thaumatin yields may be deciding factors in the placement of new cutting stations and selection of germplasm for intercropping is envisaged to be influenced as much by predicted yield and size of fruit and thaumatin yield as height of mature plants.

Chapter 3 now continues the investigation of variation in this species seeking to determine if it has a molecular basis, whether there is evidence to describe and determine a new species, and whether variation is consistent with the clinal hypothesis proposed above.

CHAPTER 3

MOLECULAR VARIATION IN *THAUMATOCOCCUS DANIELLII* ACROSS CENTRAL WEST AFRICA

This chapter continues the investigation into variation in *Thaumatococcus daniellii,* here using molecular methods. It attempts to detail molecular variation and put this into the context of the morphological variation already described. The first section reports previous work conducted using molecular methods involving *T. daniellii* and the aims and purpose of the chapter. The second section describes the methods used to collect sample material for the extraction of DNA and its analysis using a modified amplified fragment length polymorphism (AFLP) methodology. Results are detailed in Section 3 and the discussion follows in Section 5, with conclusions and recommendations in Section 6.

3.1 BACKGROUND

Knowledge of the genetic relatedness, variation and diversity within *T. daniellii*, will be key to identifying whether there is a need for extensive sampling of germplasm from across wide areas of its range for potential future domestication under *Hevea brasiliensis*, or, whether this can be restricted to collections from local material. Understanding the diversity within the species will also be relevant to whether specific populations of the plant should be conserved for specific traits and whether genetic diversity/differentiation is consistent with, and informs, current views on its reproductive biology. Also the debate about the now-in-question monospecificity of *T. daniellii* can be more informed and broadened with data from this current study, which looks specifically at molecular variance within the genus.

The distribution of *T. daniellii* is pan-African, conforming to the broad Guineo-Congolian phytochorion (White, 1979; White, 1983). Within the wide distribution range of the species (Dhetchuvi and Diafouka, 1993; Dhetchuvi, 1996; Waliszewski *et al.,* 2005) there is marked phenotypic variation: Onwueme *et al.* (1979) used two

strains in their experimental trials, a Kajola strain and an Ikeji-Ile strain, the former never reaching more than 1.5 m in height and the latter growing to 2-3 m; Dhetchuvi and Diafouka (Dhetchuvi and Diafouka, 1993) and later Dhetchuvi (Dhetchuvi, 1996) proposed a new variety, *puberulifolius*, based on rachis length and minute hairs on the underside of lamina (Chapter 1); Ley and Claßen-Bockhoff (2005) displayed the first photograph of a yellow *Thaumatococcus* flower - this has subsequently been termed *Thaumatococcus* sp.1 nov. (Ley and Claßen-Bockhoff, (in press); Ley, 2008), and seems to be restricted to the Monts de Cristal montane area of Gabon *(Thaumatococcus* sp.1 nov. Gabon. Monts de Cristal, 0° 22.11'N, 10° 14.52'E, 14 Nov 2004, *Ley 56* (LBV, WAG)) but at the time of writing has not been formally described. Even in the restricted range studied, either side of the Dahomey (Benin) Gap, variation in the natural resource was noted (Chapter 2). In field work carried out for this thesis it was noted that flower colour varied between planted wild specimens (Chapter 4).

However, no comparative studies have been made using genetic data from samples within the species: examples where studies have been conducted using plastid and nuclear *T. daniellii* DNA have always been conducted in isolation, with one exemplar sample being used within the context of phylogenetic analyses of the family and or order, Marantaceae and Zingiberales, respectively. These are discussed after a brief explanation of the types of DNA material used in phylogenetic studies (3.1.2).

3.1.1 DNA USED IN PHYLOGENETIC STUDIES

Different genomes in the same organism mutate at different rates. Rapidly mutating genes will show recent evolutionary changes, whereas a slowly mutating gene will show evolutionary change over a long time period (Willis and McElwain 2002). In plants there is a choice of three genomes to target: mitochondrial DNA (mtDNA), and plastid DNA (cpDNA termed as such because it is found in the chloroplast genome) are found in the cytoplasm; and nuclear DNA (DNA) found in the nucleus (Willis and McElwain 2002). The first two, mtDNA and cpDNA develop mainly through the process of maternal or cytoplasmic inheritance, that is, the genes are only transmitted through the female parent (the exception being in most gymnosperms

where cpDNA is paternally transmitted): the male parent contributes nuclear material (Grierson and Covey 1984). cpDNA, though smaller than mtDNA, is far more prevalent than mtDNA in plant cells (Rollo *et al.,* 1994). Both evolve very slowly with low mutation rates and both are valuable in systematic studies above the species level (Weising *et al.,* 2005). As nuclear DNA is composed of both maternal and paternal DNA, it has a much higher rate of evolution than either cpDNA or mtDNA (Grierson and Covey 1984). Ribosomal DNA and RNA are also used in phylogenetic analyses as they are highly conserved (Rieger *et al.,* 1976; Smit *et al.,* 2007), for example, 18S rRNA is commonly used in phylogenetic analyses (Meyer *et al.,* 2010).

3.1.2 The placement of *Thaumatococcus daniellii* within the Marantaceae and the order Zingiberales.

The evolutionary relationships of the Zingiberales have been the focus of investigations by Tomlinson: (1961; 1962; 1969), Dahlgren and Rasmussen (1983), Kirchoff: (1983; 1988; 1991) and notably Kress and co-authors: (Kress, 1990; Smith *et al.,* 1993; Kress, 1995; Kress *et al.,* 2001; Kress *et al.,* 2002; Kress and Specht, 2005; 2006a; Prince and Kress, 2006b). Tomlinson (1961; 1962) described information on anatomy - Kirchoff focussing mainly on floral morphology (1983; 1988; 1991) - in the Marantaceae (as part of the work on the order Scitamineae) referring eight families to the order, and in more detail as the order Zingiberales, after Hutchinson (1959). Two informal groups of families were recognised, the first being the Strelitziaceae, Musaceae, Heliconiaceae, and Lowiaceae relationship based on them all possessing raphide sacs and guard cells with outer and inner ledges equal except for the Lowiaceae, and the second comprising the remaining families, with close relationships between two unrelated pairs of families, the Cannaceae and Marantaceae, and the Costaceae and Zingiberaceae (Tomlinson, 1969). Takhtajan (1980) described the position of eight families within the order, stating that the Marantaceae were "the most advanced family in the order."

Kress (1990) recognised eight families in the Zingiberales: Cannaceae, Costaceae, Heliconiaceae, Lowiaceae, Marantaceae, Musaceae, Strelitziaceae, and the Zingiberaceae, distributed consistent with the first cladistic analysis by Dahlgren and

Rasmussen (1983) in three major clades: a ginger group (Cannaceae, Costaceae, Marantaceae, Zingiberaceae); a banana group (Heliconiaceae, Musaceae); a bird-of-paradise group (Lowiaceae, Strelitziaceae).

Smith *et al.,* (1993) ushered in a new era in the investigation of the order when they began using genetic markers together with morphological characters to determine "phylogenetic" relationships. This work was continued by others, (Kress, 1995; Andersson and Chase, 2001; Kress *et al.,* 2001; Kress *et al.,* 2002; Prince and Kress, 2006b) (Table 3.1)

Table 3.1 DNA used in phylogenetic analyses of the order Zingiberales

DNA type	Origin of DNA	Genes
Cytoplasmic	Chloroplast	atpB[4]; matK[5,6,7*]; ndhF[6,8*]; rbcL [1,2,4,6]; rps16 intron[3,6]; trnL-trnF IGS[6,7*]; trnL/trnL-F [9*]; 3'- end IGS[7*]
	Mitochondria	cox1[6]
Nuclear	Nucleus	ITS [5, 6, 9*]; 5'-end of 26S [6]; 18S rDNA[4]; 5S [9*]

[1](Smith *et al.,* 1993); [2](Kress, 1995); [3](Andersson and Chase, 2001); [4](Kress *et al.,* 2001); [5](Kress *et al.,* 2002); [6](Prince and Kress, 2006b); [7](Prince and Kress, 2006a); [8](Givnish *et al.,* 2005); [9](Ley and Claßen-Bockhoff, (in press)). ITS – internal transcribed region; IGS – Intergenic spacer region. * *Thaumatococcus daniellii* DNA used

Thaumatococcus daniellii DNA was first used in a cladistic analysis by Prince and Kress (2006a) (Figure 3.1) following Anderson's (1998) cladistic revision of the Zingiberales and subsequent description of *T. daniellii* as a species of uncertain affinity: Anderson (1998) had proposed five informal groups to subdivide the order with *T. daniellii* being one of five species of uncertain affinity, following his dissatisfaction of the historical classification of the order during the 18th-19th centuries.

(Prince and Kress, 2006a) determined, as other authors had, that *T. daniellii* is monotypic and monophyletic, and determined a strong sister relationship between *T. daniellii* and *Megaphrynium macrostachyum* (Benth.) Milne-Redh., with 100% maximum parsimony bootstrap and posterior probability values of ≥0.95 (Fig 3.1). Furthermore they stated that there was a striking vegetative similarity between the

two genera, that there were structural nectaries located at the tips of cataphylls (modified foliar leaves/scales), yet differences in the caryopsis-like fruit type were present, *Thaumatococcus* having indehiscent fruit whilst *Megaphrynium* had dehiscent fruit (Prince and Kress, 2006a). Ley and Claßen-Bockhoff ((in press); 2008) support both the monophyletic nature of *Thaumatococcus* and its sister relationship with *Megaphrynium*, but question the monospecificity of *Thaumatococcus*.

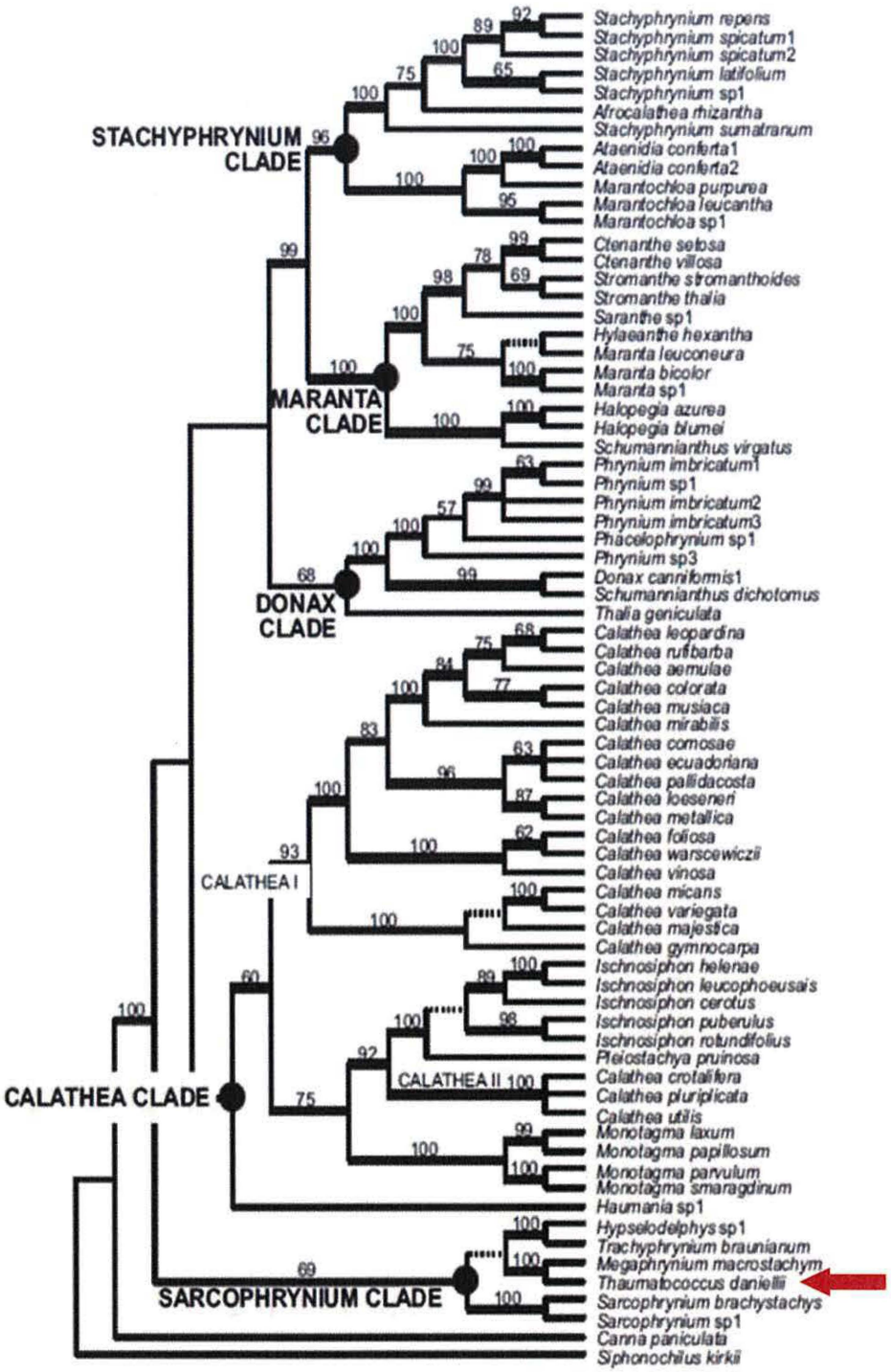

Figure 3. 1 One of the shortest maximum parsimony (MP) trees for combined analysis of the trnL-F IGS, *mat*K coding, and *trn*K 3'IGS data for taxa within the Marantaceae (after Prince and Kress, 2006b).

Note the placement of *T. daniellii* in the *Sarcophrynium* clade and its close sister relationship with *M. macrostachyum*. Numbers above branches are MP bootstrap percentages. Bold lines indicate braches with posterior probabilities of 0.95 or higher. Dashed lines indicate branches not found in the MP strict consensus tree.

3.1.3 AFLP analysis

There are many methods for the estimation of genetic diversity, which complement existing strategies for the evaluation of genetic relatedness, such as comparative anatomy, morphology, embryology and physiology (Weising *et al.,* 2005), including the use of isozymes and allozymes in early genetic studies. The use of co-dominant markers such as microsatellites (simple sequence repeats (SSRs)), restriction fragment length polymorphisms (RFLPs) and polymerase chain reaction RFLPs (PCR-RFLPs) require previous sequence knowledge to produce specific primers for the target organism and are based on a single locus. This means that they yield high information content per locus, but their development is expensive; primers can only be used in specific species and they do not necessarily yield genomic-wide diversity in the target organisms (Bensch and Åkesson, 2005).

The use of dominant marker methods such as random amplified polymorphic DNA, RAPD, (1993) and amplified fragment length polymorphisms (AFLP) (Vos *et al.,* 1995), do not require prior sequence information of the target organisms. They are based on numerous multi-locus markers that cover the entire genome of an organism, thus providing better estimates of genetic diversity (Meudt and Clarke, 2007; Pattanaik, 2008) and are highly useful in discriminating within species and between species (Weising *et al.,* 2005). Meudt and Clarke (2007) stated that AFLP is now a firmly established molecular marker technique with broad applications in population genetics, shallow phylogenetics, single locus PCR development, linkage mapping and parentage analysis.

AFLPs are as good as, if not superior to other genetic marker techniques for determining genetic diversity and differentiation within-species and within-and between populations and determining genetic similarity and expected values of heterozygosity (Table 3.2). Woodhead *et al.* (2005), stated

> *"that the brute force of large numbers of dominant markers (AFLPs) can outperform smaller numbers of hypervariable co-dominant markers (SSRs) ... and that the approach should not be considered a 'poor second best' when time prevents SSR primer isolation."*

AFLP markers and have been specifically used within the Zingiberales for genotype identification and phylogentic relationships among banana cultivars (Musaceae) (Venkatachalam *et al.,* 2008); genetic relationships among *Calathea* spp. and hybrids (Marantaceae) (Chao *et al.,* 2005); genetic diversity within between populations of *Heliconia bihai* (Heliconiaceae) (Meléndez-Ackerman *et al.,* 2005) and species authentication in *Boesenbergia* (Zingiberaceae) (Techaprasan, *et al.,* 2008). However, due to the fact that these markers are dominant, the loci investigated are bi-allelic, that is the homozygous (AA) and heterozygous (Aa) states cannot be distinguished, which makes frequency-based estimates of genetic diversity more complex (Lynch and Milligan, 1994). Despite this, many studies go on to estimate average heterozygosity, genetic similarity, genetic distance, including the use of AMOVA with AFLP markers to investigate within-population and within-individual variance and principle components analysis to establish relationships within cultivars and populations (Table 3.2).

Table 3. 2 Examples of the use of genetic markers in studies focussing mainly on AFLP methodologies (1992-2008)

Study	Genetic Markers	Average heterozygosity	Nei Genetic distance	Genetic similarity	Φ RT (%)	Φ PR (%)	ΦPT (%)	Species
Chalmers *et al.* (1992)	RAPD	0.160-1.754		0.50-0.81	60	40		*Gliricidia maculata*
Travis *et al.* (1996)	AFLP	0.13-0.04		0.31-0.71	62.77	10.56	26.67	*Astragalus cremnophylax* var. *cremnophylax*
Cardoso *et al.* (1998)	RAPD			0.19-0.45	28.5	29.6	42	*Caesalpinia echinata*
Muluvi *et al.* (1999)	AFLP	0.026-0.099	0.040-0.122		18.59	44.53	36.88	*Moringa oleifera*
Russell *et al.* (1999)	AFLP	0.249-0.315	0.103-0.020		5.9	4.1	90.0	*Calycophyllum spruceanum*
Cardoso *et al.* (2000)	AFLP	0.083-0.160	0.157-0.031		42.6	57.4		*Euterpe edulis*
Drummond *et al.* (2000)	AFLP	0.18					90	*Metrosideros bartlettii*
Dutech *et al.* (2002)	SSR	0.42-0.54						*Vouacapoua americana*
Maguire *et al.* (2002)	AFLP SSR	0.193 0.78			11 35	9 19	80 46	*Avicennia marina*
Belaj *et al.* (2003)	RAPD AFLP SSR	0.28 0.25 0.42		0.28-1.00 0.48-1.00 0.00-0.93	7.35 9.21 7.19	92.65 90.79 92.81		*Olea europaea*
Tang *et al.* (2003)	AFLP			0.76-0.88	4.7	10.5	84.8	*Hibiscus tiliaceus*
Gaudeul *et al.* (2004)	AFLP SSR	 0.148	0.007-0.477					*Eryngium alpinum*
Cardoso *et al.* (2005)	AFLP	0.108-0.136	0.038-0.290	0.09-0.16	51.67	10.39	37.94	*Caesalpinia echinata*
Choa *et al.* (2005)	AFLP			0.14-0.99				*Calathea sp*
Meléndez-Ackerman *et al.* (2005)	AFLP	0.13-0.30		0.24-0.46	11.2	34.62	54.26	*Heliconia bihai*
Assogbadjo *et al.* (2006)	AFLP	0.26-0.37			14.7	5.02	80.28	*Adasonia digitata*
Ellis *et al.* (2006)	AFLP	0.122-0.215			4.21	30.81	64.97	*Argyroderma sp*
Mwase *et al.* (2006)	AFLP	0.223-0.322	0.27±0.141		1.21	6.8	92	*Uapaca kirkiana*
Yonemoto *et al.* (2007)	RAPD AFLP			0.45-0.94 0.49-0.92				*Casimiroa edulis*
Barracosa *et al.* (2008)	RAPD AFLP			0.72-0.90 0.72-0.86				*Ceratonia siliqua cultivars*
Techaprasan *et al.* (2008)	AFLP SSCP			0.44-0.94				*Boesenbergia sp.*
Venkatachalam *et al.* (2008)	RAPD ISSR			0.32-0.78 0.18-0.80				*Musa sp*

AFLP: amplified fragment length polymorphism; RAPD: random amplified polymorphic DNA; SSR: simple sequence repeat (microsatellite); ISSR: inter simple sequence repeats; SSCP: single strand conformational polymorphism; Φ RT: the proportion of variation between regions relative to total variance; Φ PR: the proportion of variance between populations within regions relative to variance among and within populations; ΦPT: the proportion of variance between individuals from within a population relative to total variance

The AFLP methodology is efficient at detecting polymorphism in previously un-investigated species, has high reproducibility, can generate many makers per primer pair and has no development costs (Bensch and Åkesson, 2005; Pattanaik, 2008). For this reason, plus the fact there were no microsatellite markers yet developed for *T. daniellii*, AFLPs were used in this study, an investigation to estimate the genetic diversity within the species.

3.1.4 AIMS

Given this context an investigation was carried out to:

1) estimate the genetic diversity within *T. daniellii* at the within-and between-population level;
2) test whether or not variation within natural populations is determined by latitude;
3) whether there is evidence to suggest that the large phenotypic variation seen in the natural population is a basis for recognising further species within the genus.

3.2 MATERIALS AND METHODS

3.2.1 Site description

Leaf material was collected from natural populations of *T. daniellii* from four study sites (Section 2.2): Fure River FR, nr. Nsuta Village, Western Region, Ghana; Gbledi Gbogame Village, Volta Region, Ghana; Etome Village, South West Province, Cameroon; Mebanga Village, South Province, Cameroon.

3.2.2 Sample material:

The first collection of leaf samples, August 2006 and September 2006 (Ghanaian and Cameroonian material respectively), from the study sites was not successful, as the amount of leaf collected was too great for the silica gel to dehydrate completely. DNA extracted was of poor quality and quantity, so a further collection of leaf samples, was conducted in February 2007 and September 2007, (Cameroonian and Ghanaian material respectively).

40 individual leaves of *T. daniellii* were harvested at random from each site (Section 2.2, Figure 2.1) with the proviso that leaves collected had fruit attached, to ensure collection of the correct plant material, avoiding possible confusion with plants of *Megaphrynium macrostachyum*. All leaves at collection were swabbed with ethanol prior to having a 5 cm^2 section excised from the lamina. Leaves were swabbed with ethanol to remove dirt and any contagions present. Leaf sections were immediately bagged in plastic sealable bags containing self-indicating silica gel packs (25 g) to dry the leaves (Chase and Hills, 1991). If the silica gel packs changed colour (from blue to pink) they were removed from the sealed plastic bags and dried in an oven prior to being re-introduced to continue the dehydration of the leaf samples. Two samples of *M. macrostachyum* were also collected to be used as outgroups for the DNA analysis. These were collected from specimens found at Missellele Rubber Estate, nr. Tiko, South West Province, Cameroon in May 2009. Phytosanitary certificates for all leaf samples were obtained from the appropriate authorities and the material was transported to the Plant Genetics Laboratory, Bangor University, where it was frozen prior to DNA extraction. In total, there were 168 leaf samples, 160 of *T. daniellii* and eight of *M. macrostachyum*.

3.2.3 DNA extraction and quantitation

DNA was extracted from 50 mg of dried leaf tissue using a modified CTAB protocol, (Kerényi *et al.,* 1999) after (Murray and Thompson, 1980). The amended protocol is given in Appendix 2.

The quality and quantity of the extracted DNA was checked by running 1% agarose minigels stained with Safeview TM (NBS Biological Ltd) and viewed under ultraviolet light. The 168 samples were compared with known size standards of cut λ DNA at concentrations of 100; 50; 25; 12.5 and 6.25 ng /µl.

More precise quantification of total genomic DNA and its quality was conducted using a Nanodrop ND1000 Spectrophotometer (Thermo Fisher Scientific) to confirm the results, following repeated attempts to restrict, ligate and amplify extracted DNA without success. DNA extracts were diluted to 1:20 prior to being quantified using

the Nanodrop ND1000 Spectrophotometer (Thermo Fisher Scientific). The protocol for the use of this equipment is given in Appendix 3

Five samples were selected randomly from each population for the AFLP analysis (Table 3.3) where the samples met or were very close to the following criteria: the ratio of absorbance at 260:280 nm was between 1.70 and 2.00, indicating high purity (Wendy Grail, pers. comm. 2009), and the quantity of DNA was above 50 ng/µl, to enable dilution of the extract. Two samples were selected from the outgroups.

Table 3. 3 Nanodrop DNA concentrations for *Thaumatococcus daniellii* from four provenances and two out group samples of *Megaphrynium macrostachyum* used in the AFLP analyses

Region	Provenance	Sample ID	DNA conc (ng/µl)	DNA quality (260:280nm)
Ghana	WR	nsu05	125.20	1.69
Ghana	WR	nsu09	105.25	1.75
Ghana	WR	nsu16	47.10	1.72
Ghana	WR	nsu26	91.50	1.71
Ghana	WR	nsu38	185.30	1.72
Ghana	VR	gbl06	356.40	1.74
Ghana	VR	gbl20	357.80	1.63
Ghana	VR	gbl30	316.44	1.95
Ghana	VR	gbl37	114.10	1.87
Ghana	VR	gbl39	265.70	1.87
Cameroon	SWP	eto42	303.59	2.03
Cameroon	SWP	eto57	80.30	1.97
Cameroon	SWP	eto63	151.70	1.99
Cameroon	SWP	eto69	131.40	1.97
Cameroon	SWP	eto80	143.00	1.97
Cameroon	SP	mbe03	154.90	1.57
Cameroon	SP	mbe04	193.86	1.61
Cameroon	SP	mbe10	108.90	1.62
Cameroon	SP	mbe22	79.40	1.55
Cameroon	SP	mbe30	218.00	1.64
Outgroup	OG	mm1_1	111.16	1.78
Outgroup	OG	mm2_1	61.76	1.68

WR: Western Region; VR: Volta Region; SWP: South West Province; SP, South Province; OG, outgroup.

3.2.4 Generation of AFLP markers and optimisation of AFLP procedures.

The AFLP assay was conducted using a modified version of the protocol set out by (Vos *et al.,* 1995), and adapted for use with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.). Modifications to the procedure reduced quantities of primers and end-labelling of primers with fluorescent dyes rather than radioactive labels (Debener and Mattiesch, 1999; Myburg *et al.,* 2001). The full protocol is provided in Appendix 4 and Figure 3.2 summarises the procedure.

Firstly, the genomic DNA was digested with a pair of restriction enzymes *EcoR1* (recognition site GAATTC) and *Mse1* (recognition site AATT). *Mse1* is termed the frequent cutter as it generates many small fragments of genomic DNA (It is also known as *Tru9*). The rare cutter, *EcoR1* reduces the number of fragments digested. These enzymes digest the genomic DNA at specific sequences of nucleotides, recognition sites, resulting in thousands of DNA fragments with three types of specific 'sticky' overhangs, namely, Eco-Eco; Mse-Mse; and Eco-Mse.

In the next step, the ligation reaction, specific double-stranded adaptors, termed universal adaptors as they provide a basic template of nucleotides to which specific primers are annealed to during the PCR, were attached to the 'sticky' ends of the DNA (Table 3.2). The restriction and ligation reactions were run sequentially, though they can be run at the same time (Weising *et al.,* 2005).

A pre-amplification PCR was run to anneal universal primers *E00* and *M00* (pre-selective primers) to the universal adaptors (Table 3.4). This reduced the number of fragments of the Eco-Eco and Mse-Mse subset and increased the subset of Eco-Mse fragments produced, as the primers only prime DNA synthesis of fragments with bases flanking the restriction sites that are complementary to the selective nucleotides of the primers. This reduces the number of fragments to ~ $1/16^{th}$ of the initial amount (Meudt and Clarke, 2007). The thermo-cycler (a PCR machine) regimen for pre-amplification consisted of 30 cycles of: 30 seconds denaturing at 94°C; 60 seconds annealing at 56°C and 60 seconds of extension at 72°C. There was a final 600 seconds extension at 72°C to complete the program.

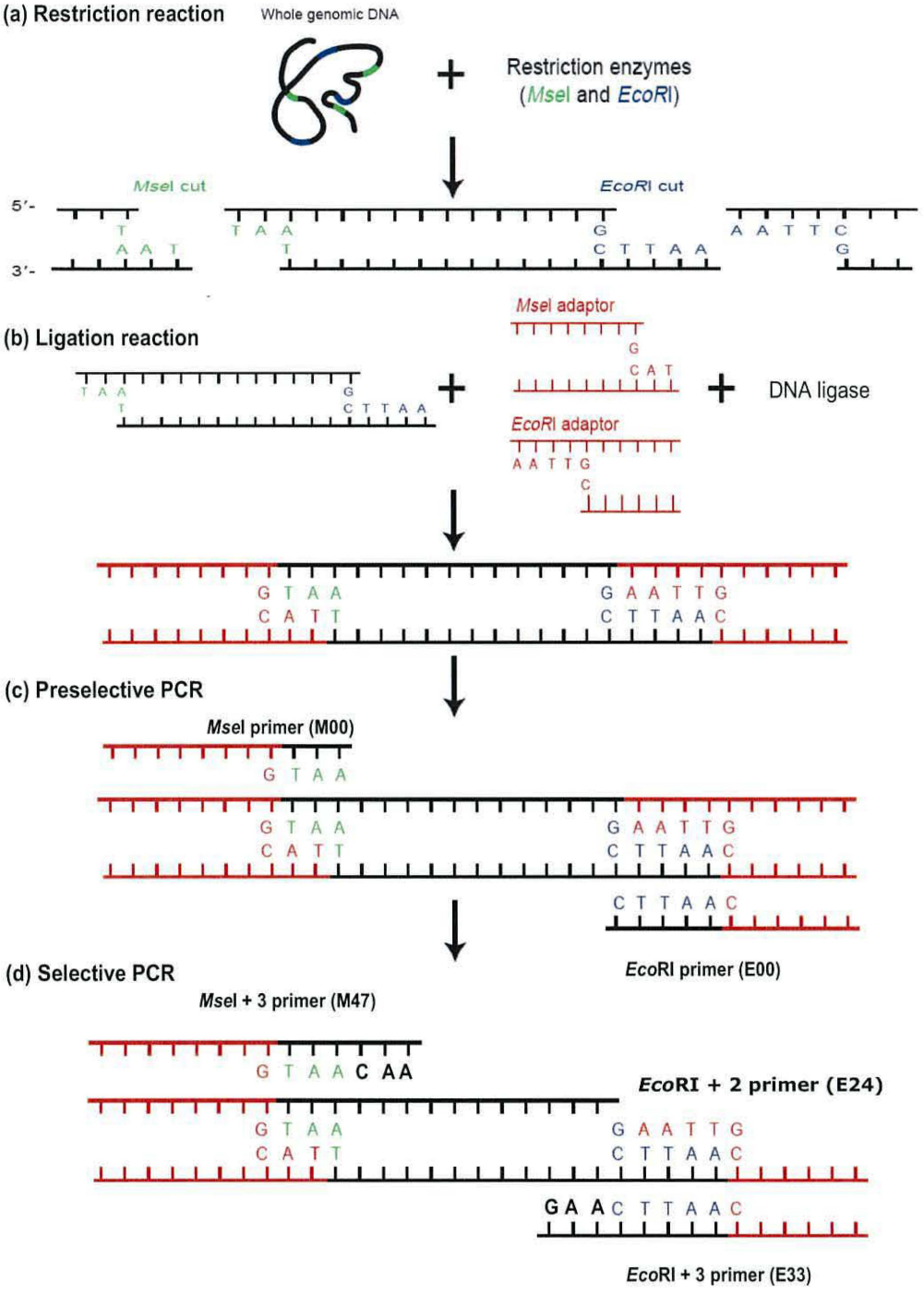

Figure 3. 2 Diagrammatic representation of steps involved in generation of AFLP markers (in *Thauamtococcus daniellii*).
(a) digestion of genomic DNA with two restriction enzymes, (b) ligation of adaptors to the digested DNA, (c) pre-selective amplification with universal primers, (d) selective amplification with selective primers Genomic DNA was digested with restriction enzymes, followed by selective polymerase chain reactions (PCR) amplification, and electrophoresis of a subset of the fragments resulting in unique, reproducible fingerprints (Meudt and Clarke, 2007). Diagram adapted from Mueller and Wolfenbarger (1999) and after Pattanaik (2008).

5µl of the pre-amplification product was run on 1% mini agarose gels using the same procedure as DNA quantification on minigels. This was done to check that restriction and ligation and pre amplification were successful, and latterly to optimise the experiment.

Table 3.4 Nucleotide sequences of the adaptors and primers used in the ligation and pre-amplification stages of the AFLP process with *Thaumatococcus daniellii* and out group species.

Adaptor/Primer	Adaptor/Primer sequence
***Eco* RI adaptor**	5'..CTC GTA GAG TGG GTA CC.....3' 3'.....CAT CTG ACG CGA GG TTAA...5'
***Mse* 1 adaptor**	5'..GAC GAT GAG TCC TGA G.....3' 3'.....TA CTC AGG ACT CAT.....5'
E00 universal primer	5′ GAC TGC GTA CCA ATT C 3′
M00 universal primer	5′ GAT GAG TCC TGA GTA A 3′

In the selective amplification PCR, the number of fragments was further reduced to a suitable number to be viewed by the gel electrophoresis system. To optimise visualisation of the fragments, the resultant pre-amplification product was initially diluted by factors of 1:10, 1:30 and 1:50, and then by 1:75, 1:100 and 1:200 for running in the selective amplification.

5µl of the diluted product from the pre-amplification PCR had selective primers annealed to the DNA fragments, with extension and amplification using a thermo cycler. The regimen used in the second PCR was as follows: an initial 30 seconds of denaturing at 94°C, followed by 13 touchdown cycles where the annealing temperature was gradually reduced by 0.7°C per cycle from an initial starting temperature of 65°C: this was done to avoid amplifying non specific sequences, with a view to increasing PCR specificity (Don *et al.,* 1991); after each annealing cycle there were 60 seconds of extension at 72°C. After the 13 cycles, there were 23 cycles of 30 seconds denaturing at 94°C; 60 seconds annealing at 56°C and 60 seconds of extension at 72°C. There was a final 600 seconds extension at 72°C to complete the program. The selective primers had two or three additional bases depending on the primer (Table 3.5) attached to the DNA fragments and replicated. The Eco-selective primers with two selective primers were end-labelled with a fluorophore, a

fluorescent dye, D4, to enable detection by the CEQ 8000 sequencer (Beckman Coulter, Inc.). Mse selective primers were not end-labelled.

Table 3.5 Selective AFLP primers and their nucleotide sequences used during the selective amplification of fragments in *Thaumatococcus daniellii* and outgroups.

Primer	Primer sequence
E15 + 2 (E + CA)	5' GACTGCGTACCAATTC **CA** 3'
E16 + 2 (E + CC)	5' GACTGCGTACCAATTC **CC** 3'
E24 + 2 (E + TC)	5' GACTGCGTACCAATTC **TC** 3'
M47 + 3 (M + CAA)	5' GATGAGTCCTGAGTAA **CAA** 3'
M50 + 3 (M + CAT)	5' GATGAGTCCTGAGTAA **CAT** 3'
M61 + 3 (M + CTG)	5' GATGAGTCCTGAGTAA **CTG** 3'

All Eco-primers (E) contain two selective base-pairs and were end-labelled with the fluorescent dye D4. Mse selective primers (M) have 3 selective bases.

The final step was to analyse DNA fragments using the gel electrophoresis system. To increase the detection level of fragments selective amplification product had to be diluted in sample loading solution (SLS) to establish the optimal dilution for maximum fragment resolution. The selective amplification product was diluted with SLS in ratios of 1:2, 1:4; 1:8 and 1:10 and analysed with the CEQ 8000 sequencer (Beckman Coulter, Inc).

It was found that the optimal dilution of pre-amplification product with *T. daniellii* and out-group samples was 1:100 and the optimal level of dilution of selective amplification product, with SLS, for *T. daniellii* and outgroup samples was 1:4.

3.2.5 Optimisation of procedure

Initial experimentation with available primers (universal primers and selective primers: E23 (E+TA-3); E26 (E+TT-3); E22 (E+GT-3); M47 (M+CAA-3) and M50 (M+CAT-3) (Various sources); buffers (Buffer W (WebScientific)), enzymes (*Eco*R1 and *Tru* 9, (WebScientific)) and master mixes (BiomixRed TM, (Bioline)) led to non-reproducibility of results and noise in the signal ratio evidenced from the initial AFLP analysis results using the CEQ 8000 sequencer (Beckman Coulter, Inc.). The former has many possible causes including: the use of adaptors with a single base-pair mismatch resulting in ligation failure; the use of enzymes that were inactive; the use of existing primers which may have been out of date, and the use of

an inferior buffer. Through a process of elimination and repeated testing, these issues were resolved and the process was optimised (Table 3.6).

Table 3. 6: Optimisation and resolution of issues during the AFLP analysis of *Thaumatococcus daniellii* and outgroup samples.

Nature of problem	Issue	Resolution
Adaptors	Mismatch in base pair sequences of single stranded adaptors leading to formation of double stranded adaptors failing to ligate properly to DNA resulting in failed ligation.	New double stranded adaptors produced (as shown in Table 3.2) from fresh single stranded adaptors purchased from MWG (MWG-Eurofins.
Enzymes	Newly purchased *Tru 9* (Web Scientific) inactive.	Purchase of new enzyme, *MseI* (New England Biolabs Ltd).
Out of date primers	Use of existing universal and selective primers: i.e. that had been stored at -20°C for > 1 year.	New primers ordered and used (MWG –Eurofins)
Buffer	Use of W buffer in restriction digest	New buffer, NEB4, from (New England Biolabs) tested and found to work better than W buffer with existing Eco R1 and new MseI restriction enzymes.

The noise issue was due to three possible causes: primer-dimer effects, where an excess of primers results in the signal to noise ratio being artificially high; the purity and quality of available primers, and the master mix used in the pre- and selective amplification, BiomixRed™ (Bioline), containing a dye (Paul Kayser, pers. comm. 2009). These were resolved by reducing by 50% the quantity of primers used in the pre- and selective amplification, and using a master mix that did not contain a dye Biomix™ (Bioline). New primers were selected based on Chao *et al.'s* (2005) AFLP primer selection (Table 3.5) from work on species and cultivars of *Calathea*, a genus in the Marantaceae (2001; Prince and Kress, 2006a; Prince and Kress, 2006b).

A sub-set of five samples, one from each of the provenances and an outgroup sample (nsu09; gbl30; eto42; mbe04; mm2_1), was used to screen nine primer-pair combinations (Table 3.7), from which three were selected. Those selected produced clear, reproducible bands and displayed variation between populations. To assess the repeatability of the data, all restriction-ligation, pre-amplification PCR and selective amplification PCR reactions were replicated using the same extracted DNA samples.

It was found that the bands were 100% reproducible. Blanks were run together with samples as a negative control to confirm that no contamination had occurred.

Table 3. 7 AFLP primer combinations, tested on a sub-set of 5 samples

Primer combination	Total number of bands scored	Total number of polymorphic bands	Total number of usable polymorphic bands	% polymorphic bands
E15:M47	142	96	69	48.56
E15:M50	103	72	51	49.51
E15:M61	112	91	76	67.86
E16:M47	89	81	78	84.64
E16:M50	118	101	100	84.74
E16:M61	76	76	63	82.89
E24:M47	121	88	86	71.07
E24:M50	112	82	80	71.43
E24:M61	102	89	89	87.25

Total number of fragments generated by each primer set, number of polymorphic fragments detected, and percentages of polymorphic fragments used produced from *Thaumatococcus daniellii* and outgroup, *Megaphrynium macrostachyum*, samples.

The following three primer combinations were selected: E16:M50; E24:M47; E15:M61. These primer combinations were chosen for a full analysis of samples based on the highest number of polymorphic fragments that could be seen with 5 samples and that produced large numbers of fragments, ensuring that all six primers would be used. These were then run on all the selected samples (Table 3.3).

3.2.6 Scoring of AFLP fragments.

0.5 µl of undiluted final PCR products together with 0.5 µl of size standard, and 40 µl of sample loading solution (SLS), which is deionised formamide, were added to individual wells on a 98 well loading-plate and covered with 2-3 drops of mineral oil to prevent evaporation of the well contents whilst the sequencer was running. The software package of the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.) was used to input the data detailing well contents, prior to loading the plate into the sequencer and running the analysis. The selective PCR products were separated using capillary gel electrophoresis in the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.) and analysed with the included software. A size standard was used to size fragments (PA400). This is the red trace seen in Figure 3.3. The laser detects fragments present in the spectrum of each fluorophore, producing an

electronic profile of relative fluorescence units (RFUs) versus fragment size (Meudt and Clarke, 2007). Peaks which are present in all samples are termed monomorphic, whereas if they are present in some samples yet absent in others they are termed polymorphic. This is a difference in the DNA sequence and can be caused by the loss or gain of a restriction site, a change in the selective primer binding site or a length polymorphism (Meudt and Clarke, 2007) – that is an insertion or a deletion of nucleotides. The traces of samples were compared, and visually scored (Figure 3.3). Peaks were scored if they were between 1000 and 130 000 RFUs (vertical axis). A peak was scored as 1 and its absence as 0. Bin width size was restricted to a maximum of 1 bp and peaks were scored between 60 and 410 bp see Figure 3.3 for an explanation and example of scoring peaks. A binary data matrix was constructed in Microsoft Office Excel 2007, based on the presence or absence of peaks.

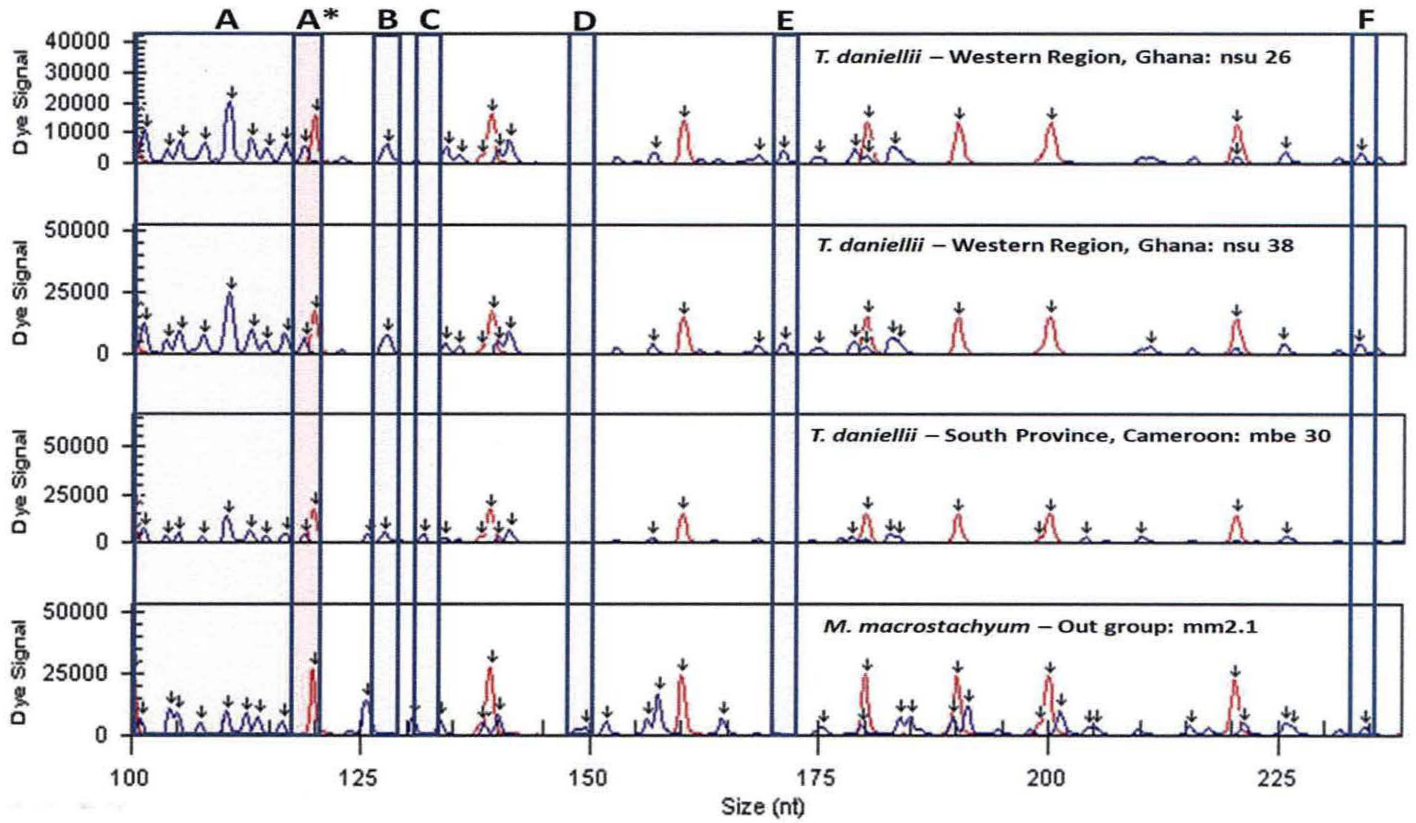

Figure 3.3 : AFLP profile traces showing polymorphic and monomorphic fragments, between 100 and 230 bp, using primer set E24:M47 (E+TC:M+CAA).
Three traces of *Thaumatococcus daniellii* samples are shown, together with an out group trace: two *T. daniellii* traces are from the same population, but different samples, (nsu 26, nsu 28) Western Region, Ghana (WR); the other *T. daniellii* sample is from the South Province, Cameroon (SP) (mbe 30); the last trace is of the out group *Megaphrynium macrostachyum*, (OG) (sample, mm 2.1). Presence and absence of one peak (fragment / locus) indicates a polymorphism between relevant samples. Shaded bars indicate the bin width used, 1 nucleotide (nt), to distinguish between peaks except when highlighting the cluster of peaks (A). A: Cluster of 8 peaks which are monomorphic between the OG and *T. daniellii* samples; A*: The last peak of the A cluster is absent from the OG, but present in all *T. daniellii* samples.; B: Present in all *T.daniellii* populations, absent in the OG; C: Present only in the South Province, Cameroon, sample; absent in both the OG and WR *T. daniellii* samples; D: Double peak present in the OG sample. Absent in all *T .daniellii* samples; E: Present in WR samples only. Absent in SP and the OG sample; F: Present in the WR samples and the OG sample. Absent in the SP sample.

3.2.7 Analysis

3.2.7.1 Percentage polymorphic loci

Monomorphic loci generated in the AFLP binary matrix were removed from the matrix, as they are not useful for characterising differences between samples (Page and Holmes 1998). Tables were produced showing percentage polymorphism, determined as the number of polymorphic loci divided by the total number of loci analysed multiplied by 100.

3.2.7.2 Principle components analysis (PCA)

A principle components analysis was performed to visualise the data from the binary matrix produced from fragment analysis: a protocol for the production of a PCA is given in Appendix 8. This is a common way to analyse multivariate data and usually helpful with AFLP analysis. A binary matrix of presence/absence of fragments was produced, and was transformed into a matrix of covariance. The AFLP binary data matrix (306 loci x 22 Operational Taxonomic Units (OTUs)) was reduced by ordinating the data to produce a co-variance matrix. An OTU can be an individual, a cultivar, a population or a species (Weising *et al.,* 2005). In this study an OTU is an individual sample. This was transformed to a correlation matrix using NTSYSpc ver 2.11 (Rohlf, 2000). This new matrix was used to produce a table of eigen vectors and values. The resultant eigen values and vectors were plotted to show the degree of similarity between OTUs and provenances.

3.2.7.3 Phenetic analysis: SM coefficient and the NJ algorithm

The binary AFLP data matrix (306 x 22) was converted to a similarity matrix of pairwise distances between OTUs using the Simple Matching (SM) distance coefficient using the software package NTSYSpc v 2.11 (Rohlf, 2000) and Excel 2007 (Microsoft Corporation Ltd., 2007). Kosman and Leonard (2005), suggest the use of the SM coefficient rather than the Jaccard's (1908) or Dice's (Nei and Li, 1979) coefficients for dominant diploid organisms. All of these analysis methods were run to see if there would be differences between using alternative coefficients, though only the SM coefficient was used in the following cluster analysis.

The simple matching (SM) coefficient (Weising *et al.,* 2005) is derived from:

$$S_s = \frac{n_{ab} + n_{AB}}{N}$$

where:

s_s = simple matching coefficient

n_{ab} = the number of bands shared by OTUs *a* and *b*

n_{AB} = the total number of bands that are absent between OTU *a* and *b*, (but present in other OTUs)

N = the total number of bands

Cluster analysis of the SM similarity matrix was carried out using NTSYSpc v 2.1 (Rohlf, 2000) using the more robust neighbour-joining (NJ) algorithm of Saitou and Nei, (1987), rather than the unweighted pair group method using arithmetic average (UPGMA) which assumes similar evolutionary speed: the NJ algorithm, which does not assume similar evolutionary speeds, overcomes this by producing additive trees (Weising *et al.,* 2005). The input for the NJ algorithm was the dissimilarity matrix constructed from the complement of the above similarity diagonal matrices. The dissimilarity matrix is the complement to the similarity matrices; that is (1- δi) values. The resulting tree was rooted to an outgroup sample, *M. macrostachyum* (mm 2.1).

3.2.7.4 Expected heterozygosity and genetic differentiation

As AFLP markers are assumed to be dominant and diploid (Kosman and Leonard, 2005) it is difficult to estimate the expected frequency of heterozygosity within populations. The Lynch and Milligan (1994) estimation, used within the software program GenAlEx6 (Peakall and Smouse, 2006) was used to determine expected heterozygosity and assumes, the above, that the species is diploid and dominant, displaying independent AFLP loci with two alleles, namely the dominant homozygote, AA, or the heterozygote condition Aa (the recessive homozygote, aa would not be seen as a peak), that there is random mating within the population and that the population conforms to the Hardy-Weinburg equilibrium.

3.2.7.5 Genetic differentiation

Genetic analysis was performed using GenAlEx6 (Peakall and Smouse, 2006). The AFLP binary data matrix used for the PCA and cluster analysis was reduced by the removal of outgroup samples and monomorphic loci. This matrix was subjected to the Lynch and Milligan (1994) algorithm whereby loci with an observed peak frequency of less than 1- (3/N), where N is the sample size, were rejected. This was done to reduce bias in the diversity statistic attributed to estimating heterozygosity when using dominant diploid species, *i.e.* where it is not possible to directly distinguish between the heterozygote and homozygote condition. The matrix produced (106 loci x 19 OTUs) was converted into a pairwise tri-matrix of genetic distances (GDs) following the method of Peakall *et al.,* (1995), using GenAlEx6 (Peakall and Smouse, 2006) where:

$$GD = n\left\{1 - \left(\frac{n_{xy}}{n}\right)\right\}$$

n = total number of polymorphic peaks

n_{xy} = total number of peaks shared by two individuals

The genetic distance matrix (triangular matrix 19 x 19 OTUs) showed genetic distance values between each pair of OTUs.

An analysis of molecular variance (AMOVA) was conducted as per Excoffier *et al.* (1992). The analysis investigated variation at regional (ΦRT), population (ΦPR) and within population, (ΦPT) levels using the random permutation method of Peakall *et al.,* (1995). One analysis was performed with data from all the populations of *T. daniellii* (19 OTUs). Two regions were designated in the analysis: these were based on the country of origin of the populations, Ghana and Cameroon. Variation was expressed in terms of total variation. Equations for the determination of ΦRT, ΦPR and ΦPT are given below (Peakall and Smouse, 2006), where:

AR = Estimated variance among regions

AP = Estimated variance within populations

WP = Estimated variance among populations

ΦRT is the proportion of variation between regions relative to total variance

$$\Phi RT = \frac{(AR)}{(AP) + (WP) + (AR)}$$

ΦPR is the proportion of variance between populations within regions relative to variance among and within populations.

$$\Phi PR = \frac{(AR)}{(AP) + (WP)}$$

ΦPT is the proportion of variance between individuals from within a population relative to total variance. This is a measure of a population's genetic differentiation for binary data that is analogous to F_{st} (Peakall and Smouse, 2006)

$$\Phi PT = \frac{(AR) + (AP)}{(AP) + (WP) + (AR)}$$

3.3 RESULTS

3.3.1 AFLP polymorphism

AFLP profiles of the *T. daniellii* samples and two outgroups of *M. macrostachyum* were generated using three primer-pair combinations. A total of 364 loci were visualised, of which 84.6 % (306) were polymorphic.

AFLP fragments ranged in size from 61 - 420 base pairs (bp), with the majority being distributed between 62 bp and 360 bp. Primer-sets produced varying numbers of scorable fragments: scorable fragments were those with a peak of more than 1000 relative fluorescence units (RFUs) and within a 1 bp bin width. (Table. 3.8), varying between 125 to 114 with a mean of 121. Primer-set E15:M61 detected more polymorphic bands (108) than the either E16:M50 (100) or E24:M47 (98), though primer set E24:M47 detected more scorable fragments within the *T. daniellii* populations (125) (Table 3.9).

The mean number of polymorphic loci per primer-pair was 102 with the total number of polymorphic loci being 306 (84.6%).

Table 3.8 The number of scored fragments, number of polymorphic fragments and percentage polymorphism using the selected primer combinations with all samples, including outgroups.

Primer combination	Scorable fragments	Polymorphic fragments	Monomorphic fragments	% polymorphism
E16: M50	114	100	14	87.71
E24:M47	125	98	27	78.4
E15:M61	125	108	17	86.4
Total number of loci	364	306	58	84.06

The Western Region, Ghana population (NSU) showed the maximum number of bands (194) though the lowest rate of polymorphism within *T. daniellii* populations, at only 8 %. The highest rate of polymorphism was seen within the Ghanaian Volta Region population (GBL) at 29%. The outgroup, *M. macrostachyum* showed overall the lowest level of polymorphism at only 0.52%: this could be due to the effect of using only two samples, rather than five per the other populations.

Table 3. 9 Percentage polymorphism within four populations with different primer combinations.

	Population														
PC.	NSU			GBL			ETO			MBE			OG		
	B	P	%	B	P	%	B	P	%	B	P	%	B	P	%
E16:M50	44	0	0	49	5	10.2	48	6	12.5	52	15	28.8	62	0	0
E24:M47	70	2	2.9	63	7	11.1	71	6	8.5	75	10	13.3	80	0	0
E15:M61	50	15	30	52	24	48	50	15	30	50	10	20	51	1	1.97
Total	194	17	8	164	36	29	169	27	15	174	35	20	193	1	0.52

B is the total number of bands; P is the number of polymorphic bands within the population. PC: Primer combination. NSU: Western Region, Ghana; GBL: Volta Region, Ghana; ETO: South West Province, Cameroon; MBE: South Province, Cameroon; OG – Out-group – *M. macrostachyum*.

There were 143 loci that were only present in the outgroup samples and hence polymorphic between *T. daniellii* samples. These accounted for 39.3 % of all loci visualised and 46.7 % of all polymorphic loci.

3.3.2 Genetic relationships among and within populations

3.3.2.1 Principle Components Analysis (PCA)

A table of eigen vectors and values was produced (Table 3.10) containing 21 OTUs and 306 loci using NTSYS- pc ver 2.1 (Rohlf, 2000). The first three components of the multidimensional data set explain 81.08 % of the variation in the observed relationships. This large value shows that the analysis was successful. (Weising *et*

al., 2005). These first two eigen vectors were plotted to show the degree of similarity among entries Figure 3.4.

Table 3. 10 Eigen values produced from a reduced correlation matrix of observed relationships in the original binary data matrix and the individual proportion of variation they explain.

	Eigenvalue	Percent	Cummulative
1	11.51	54.8	54.8
2	3.27	15.6	70.4
3	2.25	10.7	81.1
4	1.32	6.3	87.4
5	1.22	5.8	93.1
6	0.42	2.0	95.1

The first two axes account for 70.4% of the variation in the observed relationships in the data (Table C). The first principle axis reflects the species separation of *T. daniellii* and the outgroup *M. macrostachyum*; the second principle axis corresponds to variation between and within the populations of *T. daniellii*. There is a negative correlation between outgroup samples and *T. daniellii* samples shown by outgroup samples being to the left of the plot midpoint. There is a positive correlation between *T. daniellii* samples, as all are towards the right of the midpoint (Gabriel, 1971; Rohlf, 2000) . There is distinct grouping of samples from the same *T. daniellii* populations shown by samples enclosed within ovals. The least variation within a population is that shown by the Western Region, Ghana samples (NSU grouping). Samples NSU 05 and NSU 16 are virtually indistinguishable: similarly there are extremely close relationships between GBL 20 and 30; ETO 63, 57 and 80; and MBE 03 and 04.

There appears to be a similar level of variation within the populations of the South province and South West Province, Cameroon: respectively MBE and ETO clusters. The Volta Region samples, (GBL grouping) show the greatest variation between samples based on angular distances between vectors. Interestingly, two of the Volta Region samples show very low variation, i.e. high similarity with samples from the NSU grouping.

There seem to be two distinct groups of populations. This seems to be based on the origin of the samples region. Ghanaian populations show a closer relationship to each other than to either of the two Cameroonian populations, and *vice versa*.

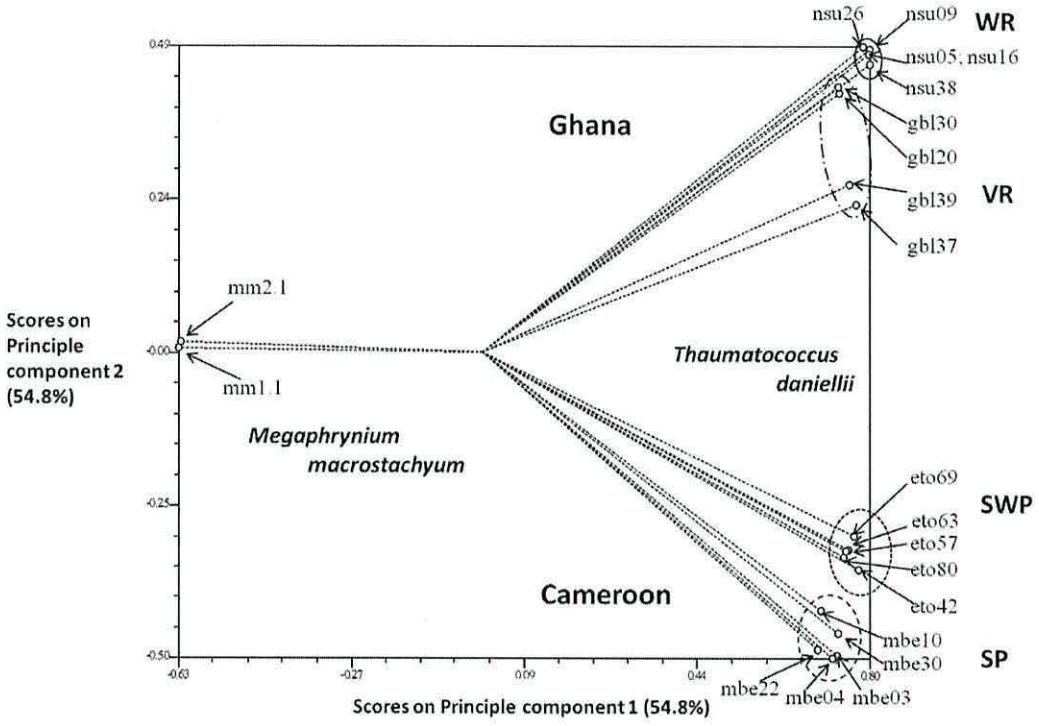

Figure 3.4 Visualisation of the first two axes from a principle components analysis (PCA) illustrating population clusters of *Thaumatococcus daniellii* from four populations and two outgroup samples, based on 306 polymorphic AFLP loci.

The first two axes account for 54.82 and 15.56% of the variance within the data set. The first three axes account for 81% of the variation. Clusters to the right of the midpoint are positively correlated with each other; clusters to the left are negatively correlated. Distances between vectors (angular distances) illustrate variation between individuals within populations and variation between populations: increased angular distance between vectors indicates increased variation between samples. Ovals have been placed around populations and numbered 1- 4: 1: Western Region Ghana (NSU); 2: Volta Region population (GBL); 3: South West Province, Cameroon (ETO), and 4: South Province, Cameroon (MBE).

3.3.2.2 Phenetic analysis

The pairwise Simple Matching coefficients among OTUs (Table 3.11) ranged from 0.29–0.32 (between outgroups *M. macrostachyum* and *T. daniellii* samples) to 1.00 (between NSU 05 and NSU 09 and GBL 20 and GBL 30). Within populations the coefficients were typically high ranging from 0.95 to 1.00 in the Western Region population (NSU); 0.89 to 1.00 in the Volta Region population (GBL); 0.93 to 0.98

in the South West Region population (ETO) and 0.92 to 0.97 in the South Region population (MBE). There was no variation between the outgroup samples with a value of 1.00. Between populations differences ranged from 0.66 (between MBE 22 and GBL 37) to 0.87 (between GBL 20 and NSU 38). There was more similarity between populations from similar countries, than between populations from different countries, as was shown in PCA plot above.

A cluster analysis using the Neighbour-Joining algorithm (NJ) (Saitou and Nei, 1987) using the simple matching dissimilarity matrix produced a phenogram (Figure 3.5) showing relationships between the outgroup and the four populations of *T. daniellii*. The tree was rooted to one of the outgroup samples.

The tree shows that there are two distinct groups both completely distinct from the outgroup populations. Branches indicate that Ghanaian (GBL and NSU) and Cameroonian (ETO and MBE) populations are more similar to one another than between each other. Horizontal distances on the tree indicate variation between individuals and populations.

The NSU cluster shows the formation of four nests within the group, with NSU 05 and NSU 09 in the same nest. This NSU cluster is more distinct from the GBL cluster, than the two Cameroonian clusters are from each other, with a 0.036 coefficients difference between the two regions, Ghana and Cameroon.

M. macrostachyum was included in this study as an outgroup. It is sufficiently related to be useful in this study as a sister species, being placed into the same clade by (Prince and Kress, 2006a). The outgroup was properly positioned outside of the *T. daniellii* clusters, being negatively correlated to them on the PCA plot. *M. macrostachyum* shares genetic similarity of between 0.29 and 0.32 with *T. daniellii* using the SM coefficient.

Table 3.11 Half matrix of pairwise Simple Matching (SM) distance coefficients between 21 operational taxonomic units (OTUs) and 306 polymorphic AFLP loci.

All values above ≥ 0.90 (for *Thaumatococcus daniellii*) have been embolded

	NSU05	NSU09	NSU16	NSU26	NSU38	GBL20	GBL30	GBL37	GBL39	ETO42	ETO57	ETO63	ETO69	ETO80	MBE03	MBE04	MBE10	MBE22	MBE30	MM2_1	MM1_1
NSU05	1.00																				
NSU09	**1.00**	1.00																			
NSU16	**0.99**	**0.99**	1.00																		
NSU26	**0.99**	**0.99**	**0.99**	1.00																	
NSU38	**0.95**	**0.95**	**0.95**	**0.95**	1.00																
GBL20	0.83	0.83	0.83	0.82	0.87	1.00															
GBL30	0.83	0.83	0.83	0.83	0.87	**1.00**	1.00														
GBL37	0.82	0.82	0.81	0.81	0.82	0.89	0.89	1.00													
GBL39	0.81	0.81	0.81	0.81	0.82	**0.91**	**0.91**	**0.95**	1.00												
ETO42	0.73	0.73	0.73	0.73	0.73	0.72	0.72	0.78	0.75	1.00											
ETO57	0.73	0.73	0.72	0.72	0.73	0.72	0.72	0.79	0.76	**0.96**	1.00										
ETO63	0.72	0.72	0.72	0.71	0.73	0.74	0.74	0.77	0.76	**0.96**	**0.94**	1.00									
ETO69	0.73	0.73	0.73	0.72	0.74	0.75	0.75	0.76	0.76	**0.95**	**0.92**	**0.98**	1.00								
ETO80	0.72	0.72	0.72	0.71	0.72	0.74	0.73	0.76	0.76	**0.96**	**0.93**	**0.98**	**0.98**	1.00							
MBE03	0.71	0.71	0.71	0.71	0.71	0.67	0.67	0.72	0.72	0.79	0.77	0.76	0.76	0.75	1.00						
MBE04	0.71	0.71	0.71	0.70	0.70	0.67	0.66	0.72	0.71	0.78	0.76	0.75	0.75	0.75	**0.97**	1.00					
MBE10	0.72	0.72	0.72	0.72	0.72	0.68	0.67	0.71	0.71	0.75	0.74	0.72	0.73	0.73	**0.93**	**0.93**	1.00				
MBE22	0.70	0.70	0.70	0.70	0.71	0.67	0.66	0.70	0.70	0.76	0.75	0.73	0.74	0.73	**0.95**	**0.95**	**0.95**	1.00			
MBE30	0.72	0.72	0.72	0.71	0.72	0.70	0.70	0.75	0.74	0.79	0.78	0.76	0.76	0.75	**0.96**	**0.94**	**0.92**	**0.94**	1.00		
MM2_1	0.29	0.29	0.29	0.29	0.29	0.31	0.30	0.30	0.31	0.33	0.32	0.31	0.31	0.32	0.28	0.28	0.30	0.29	0.30	1.00	
MM1_1	0.29	0.29	0.29	0.29	0.28	0.30	0.30	0.30	0.31	0.32	0.32	0.31	0.31	0.31	0.28	0.28	0.30	0.30	0.30	1.00	1.00

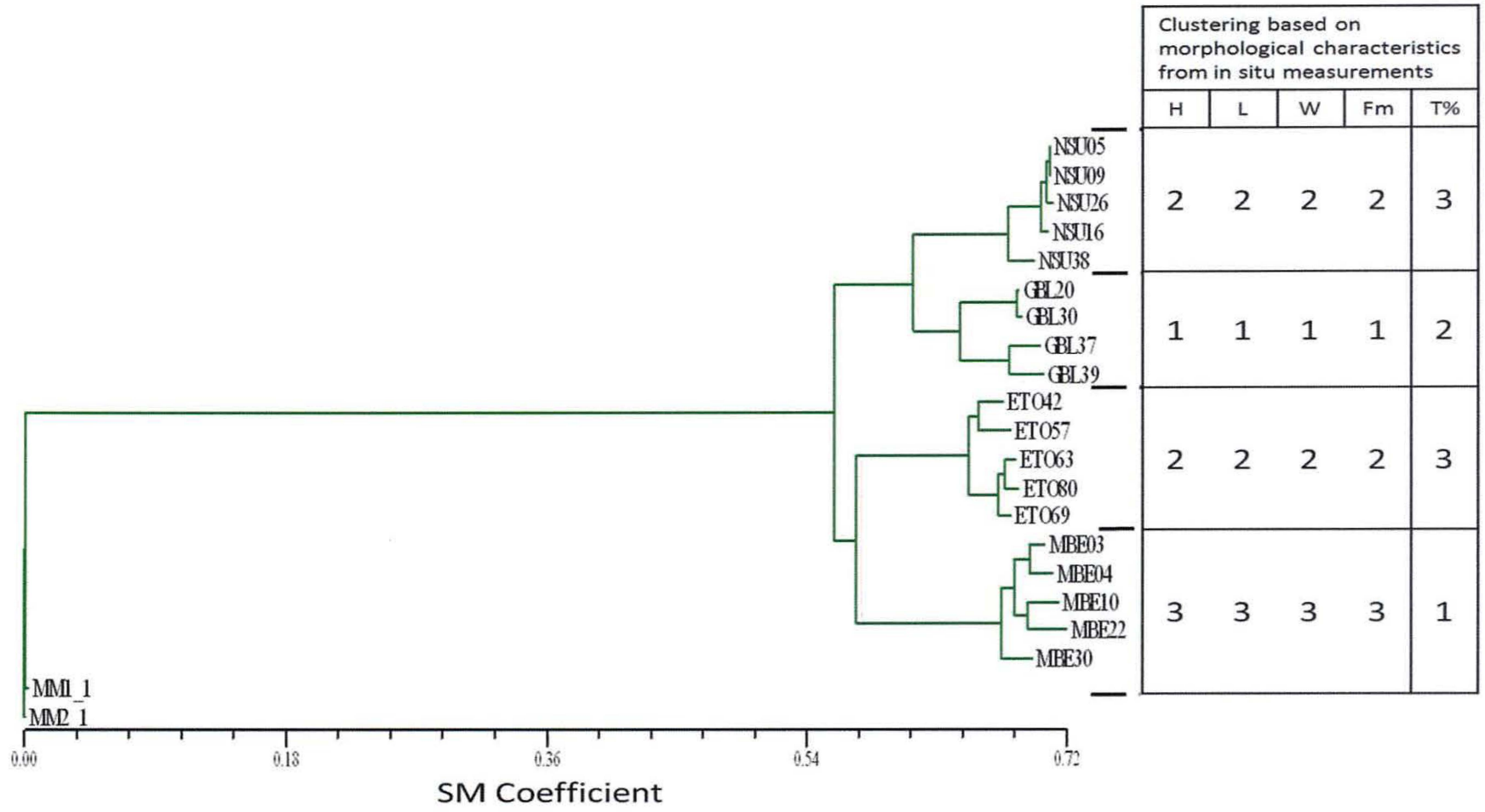

Figure 3. 5 Phenogram of 19 specimens of *Thaumatococcus daniellii* with 306 polymorphic AFLP loci using the neighbour joining (NJ) algorithm cluster analysis based on a simple matching coefficient dissimilarity matrix with morphological clustering.

NSU: Western Region, Ghana; GBL: Volta Region, Ghana; ETO: South West Province, Cameroon; MBE: South Province, Cameroon. The phenogram was rooted to one of the out group species. Morphological clustering from in situ measurements, based on mean values from Table 2.1(Chapter 2), structured into three ordinal groups: 1: largest mean value; 2: intermediate mean value; 3: smallest mean value. Morphological characteristics: H: petiole height; L: lamina length; W: lamina width; Fm: fruit mass; T%: thaumatin percentage

3.3.2.3 Nei genetic distance measures

Nei Genetic Distance (Ds) varies from 0.303 to 0.69 among *T. daniellii* populations (Table 3.12).

Table 3. 12: Pairwise population matrix of Nei Genetic Distance based on 19 operational taxonomic units (OTUs) and 106 loci

	Pop1NSU	**Pop2GBL**	**Pop3ETO**	**Pop4MBE**
Pop1NSU	0.000			
Pop2GBL	0.303	0.000		
Pop3ETO	0.647	0.516	0.000	
Pop4MBE	0.675	0.693	0.522	0.000

Pop1NSU: Western Region, Ghana; Pop2GBL: Volta Region, Ghana; Pop3ETO: South West Province, Cameroon; Pop4MBE: South Province, Cameroon.

The lower the value of the Nei Genetic Distance Ds, indicates closer relationships between populations (Nei, 1971). NSU and GBL are more closely related than the ETO and MBE populations (Table 3.12).

3.3.3 Genetic diversity

For the analysis of genetic diversity of *T. daniellii*, the outgroup samples were removed from the analysis with a corresponding removal of 133 resultant monomorphic loci (either all present or all absent null alleles) from the analysis. Further use of the Lynch and Milligan correction (1994), resulted in one band being rejected as it had a frequency greater than ≥ 0.85 ((1-(3/N), where N = 20). 162 loci (52.94%) were therefore used in the analysis to estimate heterozygosity of populations (Table 3.13)

Table 3.13. Genetic diversity estimates for *Thaumatococcus daniellii* populations across West Africa, estimated with AFLP markers from combined primer sets; 19 operational taxonomic units (OTUs) and 162 loci. Figures in brackets indicate standard error

Region	**Population**	**% polymorphic loci**	**Mean expected heterozygosity (He)**
Ghana	NSU	10.49	0.032 (± 0.008)
Ghana	GBL	23.46	0.095 (± 0.014)
Cameroon	ETO	16.67	0.067 (± 0.012)
Cameroon	MBE	20.37	0.082 (± 0.013)
Mean		17.75 ± 2.79	

NSU: Western Region, Ghana; GBL: Volta Region, Ghana; ETO: South West Province, Cameroon; MBE: South Province, Cameroon

The two populations from Ghana showed the largest difference in percentage polymorphic loci and expected mean heterozygosity (He). Cameroonian populations had percentage polymorphic loci and He values within the range of the Ghanaian populations. Mean percentage polymorphic loci was 17.75% (SE 2.79%). Interestingly, the greatest heterozygosity and percentage polymorphism within populations is seen in the two populations that are spatially distinct latitudinally: the Volta Region and the South Province populations.

3.3.4 Genetic differentiation at regional and within population levels.

Table 3.14, summarises the analysis of molecular variance of the pairwise genetic distances among individuals and amongst populations from the combined total of primer sets. The majority of variance is apportioned to among population variation, 58%, that is between populations; a quarter of the variance is determined as regional variation and the remaining variance, 17%, is within-population variance. All are significant, that is within and among populations and among regions is significant.

Table 3.14 Summary AMOVA table of pairwise genetic distances from *Thaumatococcus daniellii* samples across West Africa.

Source	df	SS	MS	Estimated Variance	% Molecular variance	ΦStat	Value (Sig.)
Among Regions	1	227.625	227.625	10.798	25%	RT	0.253 (0.010)
Among Pops	2	248.483	124.242	24.799	58%	PR	0.777 (0.010)
Within Pops	15	107.050	7.137	7.137	17%	PT	0.833 (0.010)
Total	18	583.158		42.733	100%		

Φ statistics are given with values in brackets indicating the probability of significance. There were five OTUs per population with the exception of the Volta Region, Ghana (GBL) population, which had four OTUs. 106 AFLP loci were used in the analysis.

3.4 Discussion

3.4.1 Within-population genetics

A total of 306 (86.4%) polymorphic loci were detected from across 21 samples using three primer combinations suggesting that the AFLP marker system is efficient in detecting polymorphism in the Marantaceae. Of these, 163 loci (53.2%) were

polymorphic for *T. daniellii*. Similar studies of the same order have revealed percentage polymorphism ranging from 67.8% (Chao *et al.,* 2005) to 99.78% (Techaprasan *et al.,* 2008), and high percentage polymorphisms for other woody plants (Cardoso *et al.,* 2000; Tang *et al.,* 2003; Cardoso *et al.,* 2005).

The estimated total mean expected hetereozygosities (He) were very low, ranging from 0.032 to 0.095, indicating very little diversity within populations (Table 3.13). Such levels of heterozygosity are seldom seen in woody perennials (Chalmers *et al.,* 1992; Russell *et al.,* 1999; Drummond *et al.,* 2000; Dutech *et al.,* 2002; Belaj *et al.,* 2003; Gaudeul *et al.,* 2004; Cardoso *et al.,* 2005; Meléndez-Ackerman *et al.,* 2005; Ellis *et al.,* 2006; Mwase *et al.,* 2006) (Table 3.2), which are mainly out-crossing and long lived (Hamrick *et al.,* 1991). The most genetically diverse population was from the Volta Region (VR), Ghana, (code GBL), population (He = 0.095, and 29% polymorphic loci) (Table 3.13) though this value is still low when compared to woody perennials. The least diverse population, was the Western Region (WR), Ghana, (code NSU), population with only 8% (Table 3.13) AFLP polymorphism and an expected mean heterozygosity (*He*) of 0.032.

The pattern of genetic diversity and similarity detected at the within-population level in *Thaumatococcus daniellii,* is broadly consistent with its biology. Based on allozyme studies (Hamrick *et al.,* 1991), suggested that low levels of genetic diversity would be seen in plants that were: dicotyledons; short lived perennials; annuals; endemic species, showed mixed-mating; animal-pollinated and selfing. *T. daniellii* is not dicotyledonous; however, it is an understorey perennial weed that propagates vegetatively by rhizomes, and is sexually pollinated by sunbirds (Ley and Claßen-Bockhoff, 2009) producing fruit with relatively large seeds. The Olive Sunbird, *Cyanomitra olivacea* (A.Smith). pollinates *Thaumatococcus* where dense patches of the fruit are found (Ley and Claßen-Bockhoff, 2009). The Western Olive Sunbird, *Cyanomitra obscura* (Jardine), forms a superspecies with *C. olivacea*, which has a low density per hectare, 4 ha^{-1} and a known range of between 2.5 and 3 km (Fry and Keith, 1982-2004.) leading one to expect that pollination would be very localised, leading to low diversity within a population. Ley and Claßen-Bockhoff (2008) confirmed that self pollination was possible in *T. daniellii*, concurring with

Kennedy's assertion (1978) that most species of Calathea were self compatible, but, that it was not autogamous. Ley and Claßen-Bockhoff (2008) also suggested that seed dispersal via primates and pigs intestines was the method by which colonisation of new areas was achieved, though this would result in a relatively small dispersal area: Eastern Lowland Gorillas (*Gorilla beringei graueri* (Matschie, 1914) have a range of about 30 km2, with Lowland Gorillas having a range between 7-31 km2 (Ymke Warren, pers. comm. 2010). Rats (*Rattus* spp. (Fischer de Waldheim, 1803) and grasscutters (*Thryonomys swinderianus* (Temminck 1827)), take *T. daniellii* fruits to their nests as suggested by farmers in Ghana (Waliszewski *et al.,* 2005). Andersson suggested that herbaceous species of the Marantaceae persist in a vegetative state under closed canopy, being ready to flower when there was a disturbance to the habitat (Andersson, 1998). Thus colonisation by a population can be seen to be principally vegetative rather than driven by sexual reproduction (mainly after a habitat disturbance).

Distance estimates based on the Simple Matching coefficient support the low estimates of mean heterozygosity. These were typically high within populations indicating that genetic variation within populations was narrow: 0.95 to 1.00 in the WR population; 0.89 to 1.00 in the VR population; 0.93 to 0.98 in the South West Province (SWP), Cameroon, population (code ETO) and 0.92 to 0.97 in the South Province (SP), Cameroon, population (code MBE). Chao *et al's*., (2005) *Calathea* sp and cultivars, Marantaceae, and the majority of Techaprasan *et al.'s* (2008) 15 *Boesenbergia* (Kuntze) spp., Zingiberaceae, OTU pairings produced genetic distance estimates approaching the levels shown in this particular study, though other species in the Zingiberales, such as *Heliconia bihai* (L.) L., Heliconiaceae, showed much lower genetic distances (Meléndez-Ackerman *et al.,* 2005) indicating a wider genetic variation within the species similar to most tropical woody perennials (Chalmers *et al.,* 1992; Travis *et al.,* 1996; Cardoso *et al.,* 2000) (Table 3.2).

The Simple Matching coefficient was the selected algorithm chosen to estimate genetic similarity, following arguments put forward by Kosman and Leonard (2005). They suggested that although no coefficient was ideal when working out genetic similarity of dominant diploid organisms - the Marantaceae, according to Mahanty

(1970) is diploid - they preferred the simple matching coefficient as it was more conservative at estimating genetic similarity in haploid organisms, and that when dealing with a single diploid species one could regard it as a haploid organism, especially if it was asexual: as absence of a shared AFLP band was a good indication that organisms were genetically similar for a particular trait (Kosman and Leonard, 2005). Use of the Jaccard's (1908) and the Dice's coefficient (Nei and Li, 1979) showed similar patterns and estimates of genetic similarity - results not shown.

3.4.2 Inter-population and regional genetics

Genetic differentiation at the within-population level (ΦPT), though significant, is low at 17% (0.833), in comparison to the other two levels of measured genetic differentiation at regional level, (ΦRT) 25% (0.253), and at the between-population level (ΦPR) 58% (0.777). This shows that there is greater differentiation between populations than within populations.

The values for ΦRT and ΦPR are inconsistent with comparable studies except where variation at the within-population level is not measured (Chalmers *et al.,* 1992; Cardoso *et al.,* 2000; Belaj *et al.,* 2003). It is usually the case that in woody perennial tree species the majority of genetic differentiation is seen at the individual level with values of well over 80% for (ΦPT) Table 3.2, in contrast to that seen in *T. daniellii*, as these are generally longer lived species that are outcrossing (Russell *et al.,* 1999; Maguire *et al.,* 2002; Tang *et al.,* 2003; Assogbadjo *et al.,* 2006). Another herbaceous perennial, *Astragalus cremnophylax* Barneby var. *cremnophylax*, (Table 3.2) differed from *T. daniellii* in that it had lower within-population variation, but higher regional variation, leading the authors to suggest that there had been severely limited gene flow between populations (Travis *et al.,* 1996).

The differences in genetic differentiation shown above are supported by similarity distances (Table 3.11), the phenogram constructed using the simple matching coefficient (SM) of Sokal and Sneath (1963) using the neighbour joining (NJ) algorithm (Figure 3.5), and a principle components analysis (PCA) (Figure 3.4). Principle components analysis works by clarifying relationships between characters

by reducing the data and dividing the total variance of the original characters into a limited number of uncorrelated new variables (Mohammadi and Prasanna, 2003).

The PCA and the phenogram both showed clustering within populations, supporting the above analyses that there was high genetic relatedness within populations. In the phenogram the outgroup species are clearly separate from *T. daniellii* specimens. The angular distance between vectors, in PCA, illustrate the level of between sample variation (Rohlf, 2000), with the VR population showing the greatest difference between samples and the WR population showing the closest clustering, supporting the 8% AFLP polymorphic loci value stated earlier. The phenogram and the PCA also showed the marked differences between species: the PCA displayed this variance (54.8% of the total variance in the sample of 306 polymorphic AFLP loci) on the primary axis with *T. daniellii* samples on the right hand side of the figure and outgroup samples on the left. This value is extremely close to the value of polymorphic AFLP loci attributed to *T. daniellii* earlier, 53.2%.

The distinct clustering within populations and obvious difference to outgroup species show two very important findings:

1. Firstly, the diversity patterns deduced from morphological characterisation of populations differ from the patterns of diversity from molecular evidence.
2. Secondly, regardless of the vast phenotypical variation seen in plants from the natural resource (Chapter 2), there is no evidence to suggest that speciation has occurred in *T. daniellii*, within the geographic range studied here.

It was not possible to formally compare the morphological with the genetic variation because different sample sets were used to generate them. Fruit were collected from the entire population of plants in a provenance, not from individual plants that were measured, thus this data cannot be used to produce a PCA based on morphological characteristics to be compared with molecular data. However, the PCA based on AFLPs clearly shows clustering of two distinct regions, the Ghanaian cluster, WR-VR; and the Cameroonian cluster, SWP-SP. Ranked morphological plant traits, common between Western Region and South Western Province populations, labelled

'2' on the phenogram of SM coefficients distances (Figure 3.5), (petiole height, lamina size, fruit size and the lower level of percentage thaumatin in arils) (Table 2.2, Chapter 2), are not clustered together. Aril size and aril mass as a percentage of total fruit mass, though showing different relationships between populations (Table 2.1, Chapter 2) are also not clustered according to the phenogram (not shown).

If speciation had occurred the phenogram would have shown separation between some or all of the four populations of *T. daniellii* as great as, if not greater than, that which is currently seen between it and the out-group species. This is not evident! The clustering of samples from similar populations does indicate isolation between populations has occurred in the recent past. It is known from work conducted by Kress and Specht, (2005) that divergence within the ginger families of the Zingiberales occurred some 88 Ma, with the Marantaceae diverging from the Cananaceae 80 Ma and divergence within the Marantaceae beginning some 57 Ma.

As can be seen from the table of SM distance coefficients, the similarity between populations is greatest between WR-VR populations (0.81-0.87): followed by SWP-SP (0.72-0.79) with similarity between WR-SWP, WR-SP and VR-SWP being broadly in the same range (0.70-0.79). In contrast, the lowest similarity distances are between individuals from the two populations showing the most phenotypic differences, SP and VR (0.66-0.75). Nei Genetic distances, produced from a reduced subset of AFLP polymorphic loci (not including outgroup loci) (Table 3.12) showed that most divergence was between the VR and the SP populations (0.693) and showed strong support for the close relationship between Ghanaian populations with a low genetic distance of 0.303.

3.4.3 The Dahomey (Benin) Gap

Differences between populations at the molecular level indicate a weaker relationship across the Benin Gap than between the populations restricted to each side, where *T. daniellii* is absent (Dhetchuvi and Diafouka, 1993; Dhetchuvi, 1996; Waliszewski *et al.,* 2005). The Dahomey Gap or Dahomey Interval (White, 1979) is a mixed area of high forest, Guinea savanna and coastal scrub and grassland, bounded by the Volta and Wemé Rivers (Booth, 1958). It separates the main blocks

of the Guineo-Congolian vegetation zone (Dupont *et al.,* 2000), being some 250 km in width (White, 1979). It was formed by a fall in the sea surface temperature which led to aridification and cooling of the adjacent land masses (Maley, 1996). The last major arid event which still influences present day vegetation formations, occurred between 3000-2500 yr BP (Maley, 2002) and caused a massive destruction of the African forests, almost certainly leading to the loss of *T. daniellii* from the Dahomey Gap, if it had not already been displaced by earlier aridification events during the quaternary (Dupont *et al.,* 2000; Marret *et al.,* 2006).

These events could be responsible for the isolation from each other of the populations discussed in the current study. *T. daniellii* is thought to have adapted to different ecological and environmental conditions, with the manifestation of phenotypic variation between different sites. White (1979) suggested that some of the hardiest Guineo-Congolian species retained a foothold in the Dahomey interval, in swamp forest, riparian forest and, in Togo, in small patches of forest on the slopes of low mountains: this could be demonstrated by the population of *T. daniellii* sampled from the Volta Region, bordering Togo on the extreme western side of the interval.

Studies conducted in Cameroon to determine if the phenotypic variation seen *in situ*, was due to environmental conditions (Chapter 4) found that fruit size from different populations, a key factor in the variation seen in the natural resource, did not seem to vary significantly between three of the provenances, though the locally adapted material (SWP), grew to a larger size, and petiole height and lamina dimensions did not reproduce *in situ* results, suggesting that *T. daniellii* is clearly influenced by the environment.

The genetic component of the variation has apparently not changed at the same pace as the morphological variation, thus leading to a discrepancy between morphology and genetic variation.

3.5 Conclusions

The AFLP methodology is more than adequate to identify polymorphic loci in *T. daniellii*, and will distinguish plant material between populations. This has potential

application if material needed to be traced in the future. PCA and phenograms produced from matrices of genetic similarity suggest that speciation has not occurred between geographically isolated populations, which display obvious morphological variation *in situ*, and that West African *Thaumatococcus* is of a single species only. One hypothesis for this isolation could be the repeated bouts of aridification in West and Central Africa during the Holocene, when the Dahomey Gap formed and where it has remained as a formidable barrier to *T. daniellii's* cross-gap contact.

Phenotypic similarity seen between SWP and WR, is not paralleled by molecular evidence. Results from the experimental trials in Cameroon, where locally adapted plant material grew significantly better than any of the other provenance materials (which had larger fruit *in situ*) seems to indicate that control of phenotype is primarily a response to environmental conditions or, that perhaps, it is a consequence of the source populations having been effectively isolated for a very long period. The close similarity between plants grown at a foreign site and reduced mean size of plant and fruit, suggests that plants are not as well adapted to new environments as their own. Labelling of dwarf and gigantic plants therefore must be put into a paradigm of adaption to specific local environmental conditions rather than variation within *T. daniellii* due to speciation.

3.6 RECOMMENDATIONS.

Of primary interest is whether even more morphological variation exists when account if taken of the entire geographic range (Waliszewski *et al.,* 2005). If the Dahomey interval indeed, has separated populations in Ghana and Cameroon, could a barrier further east such as the Sangha River or the Congo Basin have played a comparable role? AFLP should be able to distinguish between such accessions. As an economically important species further work into the variation of thaumatin content and fruit size from other populations in the natural range is merited, especially if these populations exhibit morphological variation not herein mentioned. It would be prudent to grow material, from populations range-wide across a range of sites, to see the adaptability of material and the expression of traits of interest. Significant genetic variation between populations would indicate potential to make crosses allowing selection of desirable traits for particular environments.

CHAPTER 4

THAUMATOCOCCUS DANIELLII GROWTH TRIALS UNDER *HEVEA BRASILIENSIS* (WILLD. EX A. DE JUSS) MUELLER-ARGOVIENSIS IN CAMEROON

This chapter continues the investigation of variation within *Thaumatococcus daniellii* through the use of common garden experiments to determine growth and fruit production under *Hevea brasiliensis* (Willd. Ex A. de Juss) Mueller-Argoviensis (hereafter referred to as rubber). The first section introduces the importance of intercropping rubber, discusses previous cultivation of *T. daniellii* and gives a basis for intercropping it with rubber. The second section details the selection of the experimental site and the materials and methods used in this study. The results section reports information about the survival, growth and fruit yield of *T. daniellii* under rubber, and highlights provenance variation in these characteristics. Discussion and conclusions follow in sections 5 and 6 respectively.

4.1 BACKGROUND

4.1.1 Elasticity in the international price of rubber

The price of rubber is highly elastic and is determined by various factors. Long term price changes are caused by three factors: prices of other goods, demand and supply (Burgers and Smit, 2009). The interaction of supply and demand (the rubber balance), natural rubber stock accumulation and currency movements are among the underlying causes of historic price fluctuations (UNCTAD, 2010b). Higher oil prices increase the price of synthetic rubber, making natural rubber more attractive (UNCTAD, 2010b); a strong dollar also makes rubber more expensive in dollar-dominated markets (Buddiman, 2003). There has been great variability historically in the price of rubber (Figure 4.1).

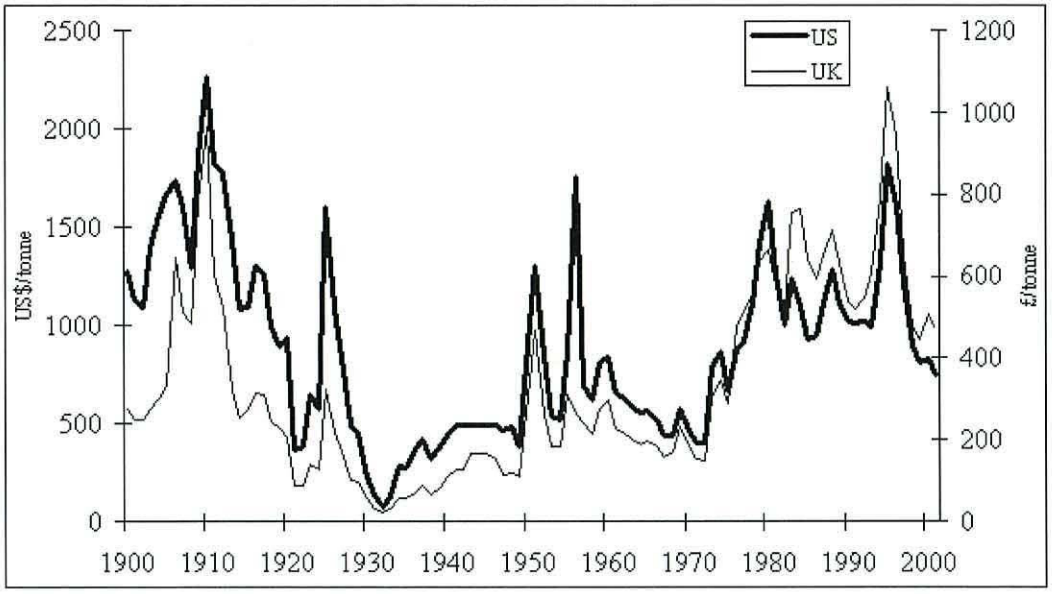

Figure 4.1 Ribbed Smoked Sheets (RSS1) prices in New York and London from Buddiman (2003)

The decline in World rubber stocks increased rubber prices between 1993 and 1994, with a glut of rubber causing the subsequent dramatic fall of prices between 1995 and 1999 (Buddiman, 2003). In real terms the price of rubber fell from 1960 to 2000 from a high of US$ 2 500 tonne^{-1} to US$ 600 tonne^{-1} (Buddiman, 2003).

The downturn in the price of rubber in the late 1990s saw a decline in the number of rubber smallholders remaining in rubber production (A. F. S. Buddiman, pers. comm. 2004). Given this background the Common Fund for Commodities (CFC) project "Enhancing Incomes of Smallholder Rubber Farmers in West and Central Africa" got approval in 2006, and with US$ 2,980,342 aimed to transfer technology to Cote d'Ivoire, Ghana and Cameroon in a bid to increase incomes and maintain supply of rubber from these producers.

After the decline in the late 1990s the price of rubber steadily grew, the primary driver being increased demand for natural rubber from the Chinese market (UNCTAD, 2010b). However, in the current global economic crisis the price of rubber has decreased from a peak of US$3.22 kg^{-1} in June 2008 to US$1.20 kg^{-1} by December 2009 (Heintz, 2009), with the majority of this decrease occurring within the quarter after June 2008. This was primarily due to the effect of the global crisis

on the automotive market, which in turn affected the tyre market – the price of natural rubber being very sensitive to changes in the automotive market (UNCTAD, 2010b). In Liberia the decline in the rubber price negatively affected workers and employees, directly impacting the livelihoods of 60 000 smallholders (Heintz, 2009).

With production of rubber from West Africa accounting for a small proportion of world rubber production - currently 2.22 % of natural rubber (2007) is produced in West Africa (FAO, 2010) - shocks to the market have major effects on rubber producers, both large estates and smallholders.

In the South West Province of Cameroon smallholders complained to the researcher, during farm visits and a smallholder meeting at the experimental site (June 2009), of reduced income from cup lump rubber sales and late payment by the principal buyers in the region, CDC (Happy Daniel, pers. comm. 2009; Pa Enoh Ferdinand, pers. comm. 2009). This was due to an across-the-board reduction of 5-10 % in the amount of rubber purchased by tyre manufactures; this affected smaller producers such as CDC who are reliant on stable and regular incomes and have low resilience to major fluctuations in demand or decreased market prices such as these caused by the current economic crisis (S. Namijo, pers. comm. 2009). Farmers also complained that traders were exploiting the situation by purchasing and storing cup lump rubber at reduced market prices: meetings held with smallholders in Bombe Bakundu and Malende Villages, Ndian Division, South West Province, Cameroon during June 2009, highlighted the plight of some smallholders, who, not resilient to the shock of the economic downturn, had no option but to sell cup lump rubber at reduced rates to traders.

4.1.2 Intercropping rubber

Intercropping provides an important means of raising not only productivity and land-use efficiency of smallholder rubber lands, but also income generation during the unproductive immature phase of the rubber tree (Rodrigo *et al.*, 2001).

Intercropping rubber with companion crops is a method of increasing smallholder's resilience to stresses (small, regular, predictable disturbances with a cumulative

effect (Scoones, 1998)) and shocks (large infrequent, unpredictable disturbances with immediate impact (Scoones, 1998)) by reducing their vulnerability to external factors that affect the price of rubber, making them more resilient in the face of external pressures (Williams, 2000).

There has been a wide range of crops tested with immature rubber lands (smallholdings and estates) (Rodrigo, 2007) including *Musa sp* L. (banana), *Passiflora edulis* Sims (passion fruit), *Ananas comosus* (L.) Merr. (pineapple), *Cinnamomum verum* J.Presl (cinnamon), *Zingiber officinale* Roscoe (ginger), and *Curcuma longa* L. (turmeric) (RRISL, 1996; Williams, 2000; Rajasekharan and Veeraputhran, 2002). There is evidence of intercropping rubber with Cucurbitaceae Juss. (cucurbits/gourds) and *Pogostemon cablin* (Blanco) Benth (patchouli), grown as cash crops, and with *Zea mays* L. (maize), Fabaceae Lindl. (beans), and *Oryza sativa* L. (dry-land rice), grown as subsistence crops, in West Africa (Mellis, 1978 quoted in Watson, 1983). Farmers in Nigeria reportedly intercropped immature rubber with single crops and mixtures of crops including *Ananas comosus* (L.) Merr. (pineapple), Dioscoreaceae R.Br. (yam), *Manihot esculenta* Crantz (cassava), *Z. mays*, vegetables, *Colocasia esculenta* (L.) Schott (cocoyam) and *Musa* × *paradisiacal* L (plantain) (Mesike *et al.,* 2009). Delabarre and Serier ((2000) commented that *Glycine max* (L.) Merr. (soya bean), *Arachis villosulicarpa* Hoehne (groundnut) and *Crocus sativus* L. (saffron) were also intercrops, with Punnoose *et al.* (2000) adding that vegetables such as *Vigna unguiculata* (L.) Walp. (cowpea), *Abelmoschus esculentus* (L.) Moench (okra), *Cucumis sativus* L. (cucumber), and *Amaranthus sp* L. (amaranthus) can be grown with the addition of organic manures and *Sesamum indicum* L. (sesame) in some areas. *Amorphophallus konjac* K. Koch (elephant yams) have also been cultivated under rubber (Rajasekharan and Veeraputhran, 2002). Leguminous cover crops are typically sown from seed and in mixtures, as individual cover crops do not have all the attributes of an ideal cover crop; leguminous cover crops include *Pueraria phaseoloides*, *Mucuna bracteata*, *Calopogonium mucunoides*, *Centrosema pubescens*, *Mimosa invisa* var. *inermis* and *Calopogonium caeruleum* (Punnoose *et al.,* 2000). A review of all the leguminous cover crops used with rubber is beyond the scope of this introduction: Edgar, in his seminal work on rubber management provides a detailed examination of leguminous

cover crops in rubber (Edgar 1958). Wibawa *et al.* (2006) investigating rubber based agroforestry systems (RAS) planted rubber with multipurpose trees (MPTS) *Calliandra sp*, Wingbean and *Gliricidia sp*, and fast growing trees (FGTs) *Gmelina arborea* Roxb., *Paraserianthes falcataria* (L.) I.C. Nielsen, *Acacia mangium* Willd., and other tree species including *Tectona grandis* L. F, *Eucalyptus sp*, *Nephelium lappaceum* L. (rambutan), and *Durio zibethinus* Rumph. ex Murray (durian). For a list of timber and fruit tree species that are currently been examined for their potential to be grown with rubber in agroforestry associations refer to (Wibawa *et al.,* 2006).

Elettaria cardamomum (L.) Maton (cardamom) in India, *Euterpe oleracea* Mart. (heart of palm ~ palmito) and *Coffea* L (coffee) in Brazil (Williams, 2000), provided the shade from the rubber is not too excessive (RRISL, 1996), and *Theobroma cacao* L. (cocoa) (Rodrigo, 2007) have all been intercropped with mature rubber, though Punnoose *et al.* (2000) state that *E. cardamomum* was not high yielding. Shade tolerant medicinal plants have also been found to be successful: *Stobilanthus haenianus*, *Adhatoda vasica*, and *Plumbago rosea* did not affect the yield of rubber (Punnoose *et al.,* 2000). Without a move to alternative spacing regimes to enhance light penetration through rubber canopy as suggested by Rodrigo (2007), the number of crops grown in mature rubber plantations will remain relatively small compared with crops grown with immature rubber.

Other strategies employed to diversify land use under rubber include grazing of livestock (Delabarre and Serier 2000). Shelton and Stür (1990) give a comprehensive overview of the opportunities for the integration of ruminants in rubber plantations (Chong *et al.,* 1990), the benefits of doing so (Chee and Faiz, 1990), the forage species that can be used with rubber (Ng, 1990) and improvements that have been made to these (Tajuddin *et al.,* 1990). Bee keeping with rubber in some areas of India provides small holders with additional income (Delabarre and Serier 2000).

4.1.3 Agronomic studies of *T. daniellii*

The interest in finding alternatives to artificial sweeteners in the late 1960s and early 1970s (Adansi, 1970) led to three collaborative studies of the cultivation of *T. daniellii* (Adansi and Holloway, 1977; Most *et al.,* 1978; Onwueme *et al.,* 1979) and further field planting in Liberia and Malaysia (Witty and Higginbotham, 1994). Since this period of interest there has been one further study, part of a Master's thesis conducted in Ghana in 2002 (Yeboah *et al.,* 2003). Grey literature suggests there has been cultivation of *Thaumatococcus daniellii* in Côte d'Ivoire (Gnagne *et al.,* Unpublished).

Adansi and Holloway (1977) made preliminary investigations with seedlings and rhizomes in 1970 at the Crop Research Institute at Bunso, in Ghana, which has since been abandoned (Abbiw 1990). They planted 0.1 ha of rhizomes and seedlings and determined that mature fruit had been produced 42 months after planting with seedlings (n=4) and 40 months after planting from rhizome material (n=68). They determined that plant height at the end of the experiment was between 1 and 2 m, though it is not clear how this was measured. Work conducted at the same time at the University of Reading, UK (Most *et al.,* 1978) investigated protected cropping, providing detailed information on propagation in glasshouses; they also found that close spacing did not inhibit flowering and that fruit set was very low. The final research from this collaborative project (Onwueme *et al.,* 1979) was done in Nigeria at the University of Ife. This was the largest research project conducted in West Africa, and looked at two local 'strains' of the plant grown in open and shaded conditions. Plots were shaded by a mixture of *Manihot glaziovii* (casava), *Musa* sp. (plantains), *Cola nitida* (Kola) and isolated stands of forest trees. Onwueme *et al.* (1979) established plots of *T. daniellii* with seedlings and rhizomes and recorded fruit yield over three years. Plots were established between November 1973 and June 1975 and results of the research were used to formulate recommendations for future establishment of plots, some of which were used in the current study.

Following interest in *T. daniellii* by Samartex Timber, Samerboi, Ghana, a small study was done to investigate the effect of artificial shade levels on *T. daniellii* (Yeboah *et al.,* 2003). This showed that juvenile growth of *T. daniellii* was affected

by light, as shown by differences in the number of emergent petioles of *T. daniellii* under three different shade conditions during a 10-week period.

This interest reduced the supply of *T. daniellii* fruit and led competitors of Samartex to focus on developing new sources of supply. Samartex planted seedlings of *T. daniellii* on sidings where forest operations had been ongoing (pers. obs), and diversification of supply from other countries was explored by other producers.

4.1.4 Aims of the study

For this research production of fruit from plants grown with a sustainably managed perennial crop, rubber (other crops considered were cocoa, oil palm, and community woodlots) was suggested as a way of augmenting the supply of fruit whilst diversifying incomes and livelihoods of rubber smallholders, thus potentially retaining them as key producers of rubber in West Africa.

Following research conducted in the natural populations in the natural range (Chapter 2) a new question arose: would material showing marked variation in morphology retain the different phenotypes if planted at one site and, if so, would one particular germplasm be best suited to growth under rubber?

Against this background, an investigation was conducted with the principal objectives of determining

- whether *T. daniellii* can be successfully grown under mature rubber
- how long does it take for fruit to be produced and what quantity of fruit it produces
- whether fruit yield can be predicted from plant characteristics
- if phenotypic variation seen in natural populations is maintained under common garden conditions
- if any particular germplasm is better suited to growth under rubber
- the growth pattern of *T. daniellii* plants produced from rhizome sections.

4.2 Methods and Materials

4.2.1 Experimental site selection

Preliminary discussions with the Cameroon Development Corporation (CDC) began in early 2005 to establish an experimental site within their rubber plantations. CDC is an agro-industrial company, a parastatal with head offices in Limbe, South West Province, Cameroon. CDC's major focus is on tropical export crops, mainly oil palm, banana and rubber. It has approximately 39, 000 hectares of planted rubber across 11 rubber estates, with three main rubber sheet and latex factories based at Tiko, Mukonje and Pendamboko. Once CDC had agreed to experimental sites being established in their rubber plantations, selection of a suitable plantation was conducted with the Group Rubber Manager's (GRM) office. Rubber estates between Tiko and Mukonje were visited: they were Malende, Likomba, Mbonge, Meanja, Missellele and Mukonje. The Missellele Rubber Estate was chosen as the location for the experiment, due to its closeness to Tiko, where the Rubber Head Offices and Laboratory were located, and Limbe where logistical support for the research was located. Its easy access by road also made it an ideal location.

The Missellele Rubber Estate has a total area of 2 317 hectares (Kips *et al.,* 1984) of which 1 848 hectares are planted with rubber, with 1 761 hectares being mature rubber (Cameroon Development Corporation, 2010)

The selection of the experimental site within the plantations was completed in conjunction with staff from the GRM's office. Factors that were considered for site selection were:

- productive, uniform rubber trees
- road access
- access to labour
- clonal type : ideally a clone used by both CDC and smallholders
- distance away from settlements / farm animals
- sufficiently hidden to not attract attention
- water availability

Four sites within the rubber estate were visited. The site selected was a section of Missellele I, part of the estate planted in 1996 with rubber clone PR107 at a density

of approximately 510 trees per hectare spaced at 7 m x 2.7 m. It was approximately 9 km from the Tiko rubber factory and 33 km from Limbe on the main Limbe-Doula Road (Figure 4.2). The block was the second one north of the Limbe-Doula Road, and was therefore hidden from casual traffic. The nearest homesteads were more than a kilometre from the site, and there was a stream approximately 250 m north of the site. Bamboo was freely available to construct a specific shed for the site, stakes for planting and carrying vessels for rubber. The rubber trees on the site had been tapped for two years prior to being planted with *T. daniellii*. The centre of the site was at grid point N 04.13558, E 009.434390.

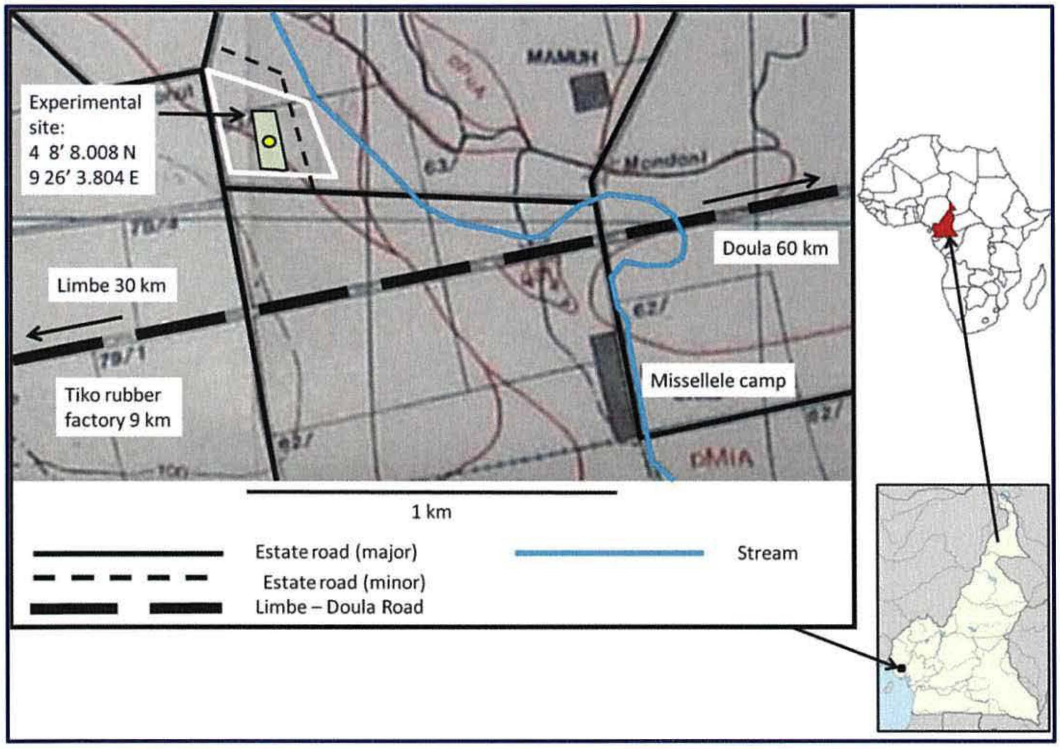

Figure 4.2. The experimental site at the Missellele Rubber Estate, Cameroon.

Inset picture shows Cameroon, and Africa. The outlined white polygon shows the extent of the experimental site, with the shaded green polygon showing the provenance trial. The shed at the centre of the site is highlighted with a yellow circle.

4.2.2 Experimental design

A randomised block design with non-contiguous, multi-plant plots was used for the provenance trial. (Figure 4.3). There were two blocks and four treatments: Western Region (WR); Volta Region (VR); South West Province (SWP) and South Province

(SP). In each block, each provenance was represented by five plots. Each plot was planted with 100 rhizome sections planted at 1 m x 1 m spacing.

Each rhizome section, 20 ± 5 cm, was one of ten sections cut from an individual rhizome collected from each of the study sites, Section 2.2. Each replicate plot therefore would have one section from each of the collected rhizomes.

The reason for such a large experiment was that it was not known how well rhizomes would grow under rubber, nor if sufficient numbers of fruit would be produced to enable an analysis of thaumatin content. At the time of planting the thaumatin extraction procedure had not been established, and it was envisaged that a minimum of 0.25 kg of aril would be needed in order to run an analysis. By planting large numbers of rhizomes in two blocks (effectively, each acted as an 'insurance' block for the other) it was hoped that enough fruit for analysis would be produced.

Initially the experiment was designed to investigate growth and variation of *T. daniellii* grown under *Hevea brasiliensis*, with a secondary objective to investigate the effect of planting *T. daniellii* on the production of cup lump rubber. This is the reason for the gaps in the schematic layout of plots (Figure 4.3). These blank plots were envisaged to be control plots to assess the effect of not planting *T. daniellii* on cup lump rubber production, solely the effect of normal management practices. However, after planting the rhizome sections it was realised that in order to investigate the effect of *T. daniellii* on cup lump rubber production it would have to have been planted around the rubber trees, and not as had been done, in-between the rows of rubber trees. Because of this the rubber trial experiment was established later in 2006, Chapter 5.

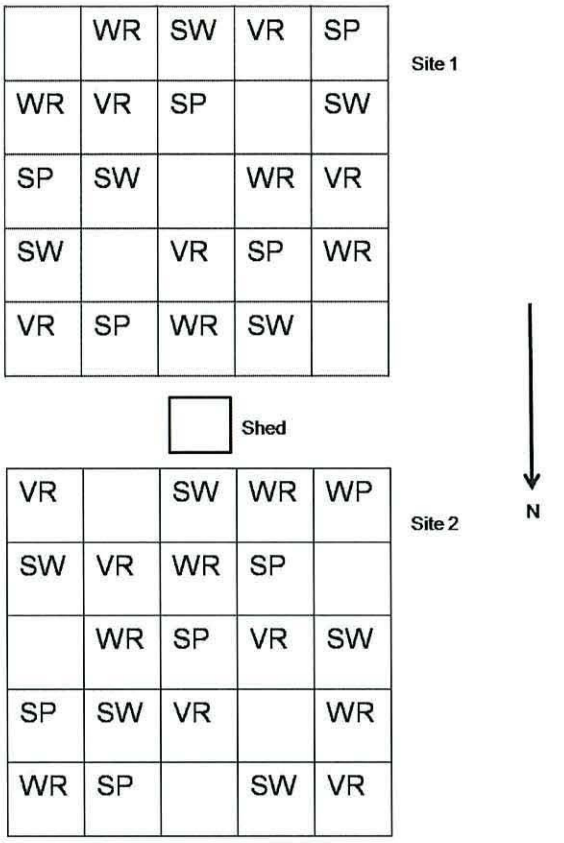

Figure 4.3 A schematic representation of the experimental design of the provenance trial, Missellele experimental site, Missellele Rubber Estate.
Provenance labels as for Table 2.2. Note the figure is not to scale. Plots were rectangular not square and were approximately 7 m x 21.6 m in size.

4.2.3 Experimental site preparation

The whole site was checked for brown bast trees (rubber trees that were not being tapped), fallen trees and missing trees. Locations for the two blocks were identified in areas which contained the most productive trees with the minimal number of gaps. Each block measured 145 m x 63 m, and the two blocks were separated by an area for the construction of a site shed, (approximately 25 m x 63 m) (Figure 4.4).

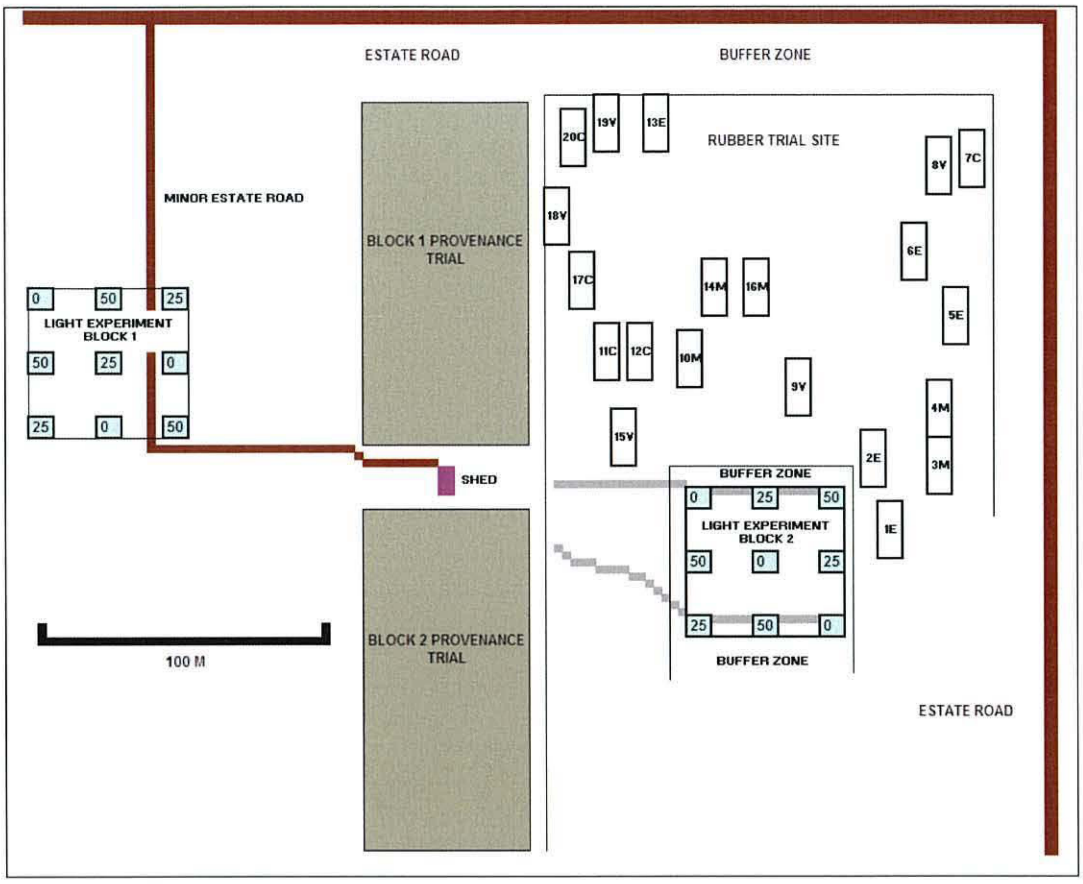

Figure 4.4: The Missellele experimental site with all three experiments shown: provenance trial; rubber trial site and the light experiment blocks.

Block locations were cleared of vegetation with the use of machetes. Woody material was removed; leaf material was left in place. String was used to mark out 25 plots per block. Each plot was the width of one row of rubber (7 m) by eight rubber trees (7 x 2.7 m = 18.9 m): the plot was slightly extended into the short axis space to enable the last rhizome row to be planted. There was a space of three rubber trees (8.1 m) between short axes of plots and a one rubber tree row space between the long axes of plots (7 m). This was done to make sure that rhizomes from one plot would not grow into adjacent plots. The planted area of each plot was 20 m x 5 m, with a one meter gap between the planted rhizomes and rubber trees to allow access to rubber trees by tappers.

Cleared plot areas were staked out with 100 bamboo stakes at 1 m x 1 m spacing, producing a 20 x 5 grid (Plate 4.1). Prior to planting with rhizomes each of the plots was watered and covered with a leaf mulch.

Plate 4.1 Missellele experimental site, provenance trial. Plots staked and covered in leaf mulch prior to planting, February 13-15 2006.

4.2.4 Rhizome collection, storage and transport

Rhizome sections were collected from each of the four study sites (section 2.2) between the 1st and 21st February 2006. The number of rhizomes collected per site was 120, to allow selection of 100 good quality rhizomes following transport. Labourers from a local village were hired and asked to collect individual rhizomes not less than 2 m in length. Differences between *Megaphrynium macrostachyum* and *T. daniellii* were explained to staff by using examples of the plants *in situ* in order to ensure that the correct material was collected; it was made clear that should there be any confusion the author or assistant should be approached. During collection the author and assistant were constantly roaming the site making sure that rhizomes of the correct species were being collected. Incorrectly selected rhizomes were rejected. Rhizomes were dug from the soil using machetes, cut into two pieces, tied with string and placed into rice sacks for transport.

4.2.4.1 Ghana.

Rhizomes were collected from Fure Forest Reserve, Western Region, Ghana, on 1st February 2006 (Plate 4.2) and transported on the same day via Prestea to Takoradi, where they were kept on shaded areas on a lawn and watered each day to prevent them drying out. The following day they were transported to Accra and kept moist by the same method until shipment to Cameroon.

Rhizomes were collected from Gbledi Gbogame, Volta Region, Ghana, on 6th February 2006. Rhizomes were transported the same day to Accra, watered and kept on a lawn separately with WR material until they were transported to Cameroon.

Plate 4.2 Extraction and packing of rhizomes from Fure River Forest Reserve, Ghana, 1st February 2006.

Rhizomes had the necessary phytosanitary certification and were transported from Ghana by Belle View Airlines through Lagos (Nigeria) to Doula (Cameroon) on 9th February 2006 and onward to Limbe on 10th February 2006. WR material was planted on 14th February 2006, 13 days after harvest; VR material was planted on 15th February 2006, 10 days after planting.

4.2.4.2 Cameroon

Rhizomes were collected from Etome Village, South West Province, Cameroon, on the morning of 17th February 2006. This material was processed the next day, kept in a shaded area and watered, prior to planting on 20th February.

Collection and transport of rhizomes from Mebanga Village, South Province, Cameroon, was done on 21st February 2006. Rhizomes were sectioned the following day and planted on 23rd February 2006.

4.2.5 Rhizome sectioning

All rhizomes were inspected and the best 100 selected for planting. The criteria used for this selection were that rhizomes had a large girth, good root mass, many budding points, were not already fraying at the ends and were not damaged from

transportation. Selected rhizomes were cut into ten sections each 20 cm ± 5 cm long, using a pair of secateurs. Rhizomes were sectioned such that pieces contained apical buds and, where present, sylleptic buds (lateral buds that form new lengths of rhizome orthogonally to the original rhizome). *T. daniellii* is a sympodially branching rhizome, with axillary buds forming new regions for continuing growth and development.

The rhizome, now cut into ten sections, was placed in a pile on the floor; sectioning all selected rhizomes there were 100 piles. One rhizome section from each pile was placed into one of ten sacks and watered. This ensured that each sack contained one section of each of the 100 rhizomes (Plate 4.3).

Plate 4.3 Processing and sectioning of rhizomes.

A: Rhizomes from the field; B: One rhizome length sectioned using secateurs; C: Each pile consists of one entire rhizome in ten sections; D: Each sack has one of the ten sections placed on it. One sack of rhizomes is planted in one experimental plot.

4.2.6 Planting rhizomes at the experimental site

Sacks containing sectioned rhizomes were taken the following day to the site for planting. The ten sacks of each provenance were randomly allocated to the ten plots

of each provenance (five in each block). At each staked point a small hole approximately 30 cm in length, was dug to a depth of 10 cm and refilled with 2.5 cm of soil. Rhizomes were taken out of the bag at random, placed in holes (one rhizome per hole), covered with soil and watered (Plate 4.4).

Plate 4.4 Planting of sectioned rhizomes, Missellele experimental site.

4.2.7 Establishment of sample quadrats

Five sample quadrats per plot were established. This was done by allocating a number to each stake (point) and using a random number generator on a calculator to select five points. Once points had been selected a 1 m^2 quadrat was placed with the point and the planted rhizome section at its centre, and the corners marked with bamboo poles (4 cm diameter), to which string was attached.

4.2.8 Monitoring and data collection

Two types of monitoring were done at the site: monitoring of plots and monitoring of sample quadrats.

4.2.8.1 Monitoring and data collection in plots

This was done to establish the extent of growth and spread of rhizomes. Each plot was walked through and a note made of the number of emergent points from a rhizome section, and the type and nature of an emergent petiole. It was found that rhizomes produced mulitple petioles from different points on the rhizome and when it grew, which was expected, that they also produced multiple petioles from single points. Where rhizomes produced new petioles they were termed for example, single, double, triple, and quadruple and so on. Where rhizomes produced petioles with multiple leaves they were termed for example, singlet, twin, triplet, quadlet, and quintlet.

Early monitoring involved labourers on watering duties noting when petioles began emerging. Once the first petiole emerged it was decided to monitor the site once every two days. After 25 days it was decided to reduce this to once every four days as it was clear that once-every-two-day monitoring was too frequent. Due to the high numbers of petioles, monitoring of sample quadrats and budget considerations, monitoring was again reduced to monthly monitoring from the beginning of July 2006 to the end of the monitoring period, October 2008. When it was not possible to complete monitoring and data collection for both blocks in one day, only one block was measured, and the second was measured the following day. Care was taken when monitoring and collecting data not to destroy petioles, especially newly emerged petioles, nor to damage any flowers or fruit produced.

4.2.8.2 Monitoring and data collection in sample quadrats

The five sample qudrats within plots were monitored at the same frequency as the plots themselves. Whilst petioles were still curled their height was measured, using a rule, from the soil to the tip of the leaf (a sharp point). When unfurled, petioles were measured from the ground to the lower pulvinus. Lamina length was measured from the leaf groove, where the lamina was attached to the pulvinus, to the tip of the leaf blade. Lamina width was measured at the widest point of the leaf. Intially a 50 cm rule was used to measure dimensions; thereafter a builder's tape measure was used.

All measurements were made to the nearest millimetre. All petioles arising from a sample plot were measured.

4.2.9 Maintenance of the experiment

As rhizome sections were planted during the dry season, watering of rhizome pieces was done once every two days until the main rains began. This enabled close monitoring of the experiment and established a presence at the site. The GRM's office made an announcement to staff, families and local communities that the experimental site was not to be used as a shortcut and was to be generally avoided.

During periods of rubber senescence and self pruning, large branches that fell into plots were removed. CDC weeded the interrows between plots but did not weed between the replicate plots; this was done by experimental site personnel.

4.2.10 Destructive sample

At the end of the experiment a destructive sample was taken. Ten petioles plus attached laminae were selected at random and removed from each of the replicate plots across both blocks. They were immediately wrapped in a plastic bags and taken to the Tiko Rubber Factory Laboratory, Tiko. There, petiole length, lamina length and lamina width were measured; the lamina was then removed from the petiole and weighed using an Adam Equipment ACB plus1000 laboratory scale (Adam Equipment CO. Ltd. Milton Keynes, U.K.). Leaves were then photocopied and the photocopied image was cut from the paper. The leaf area was then calculated from the cut out photocopied image. A known area of paper had a certain mass x (g). A corresponding mass of paper (with an image of the lamina) had a mass y. The area of the leaf (m^2) was equal to mass y (g) divided by mass x (g).

Leaves were dried in an oven at 70°C for 48 hours following Poorter *et al.,* (2006) and weighed to obtain their dry mass (g).

4.2.11 Fruit collection and metrics

At fruit harvest (the first fruit harvest was on 24th May 2009: the second harvest was on 6th March 2010), all mature fruit from each plot were collected in the second harvest, however, during the first harvest if the number of fruit in a plot was very low, they were left *in situ,* as were unripe fruit during both harvests. Fruit were classed as mature if they were red. Fruit were collected by replicate and stored seperately. Fruits were washed, weighed, measured and cut as described in section 2.3.6.

4.2.12 Treatment of data

4.2.12.1 Plot data

Data collected at the plot level was used to show survival and early growth of rhizomes after planting.

Data collected in May 2006, November 2006, May 2007 and the destructive sample data collected in May 2009 were chosen for analysis.

Analysis of variance (ANOVA) was used to test for differences in petiole height (PH), lamina length (LL) and lamina width (LW) between blocks and among provenances. As there were no significant differences in plant characteristics between blocks, it was decided to use one-way ANOVA to analyses the combined data from both blocks (four treatments (provenances) and ten replicates (plots)). This increases the numbers of useful pieces of information, effectively partioning the degrees of freedom differently, thus increasing variability, lowering the possibility of overfit and increasing the degree of falsifilabilitty (Yu, 2003). All analyses were done with SPSS 14 for Windows (SPSS Inc. Release 14.0.0, September 2005). Effect sizes for significant values were calculated using the protocols in Appendix I.

4.2.12.2 Sample quadrat data

Sample quadrat means were computed for each of the three characteristics PH, LL, and LW. These five sample quadrat means were then used to calculate a plot mean for each characteristic at each sampling date.

One-way ANOVA was used to test for differences in means among and within provenances, at each of the sampling dates.

4.2.12.3 Calculation of SLA and leaf thickness

Specific Leaf Area (SLA)

SLA was calculated using the following formula

$$SLA = \frac{Total\ leaf\ area\ per\ plant\ (LA)}{Total\ leaf\ dry\ weight\ per\ plant\ (LW)}$$

(Hunt 1990)

In this calculation total leaf area was taken to be that of an individual lamina per petiole, rather than that of all the petioles per individual rhizome. The aim of the analysis was to determine the significance of differences among provenances in lamina leafiness, using a modified SLA. Botanically the petiole is part of the leaf; however, in *T.daniellii* it is very much lignified and it is widely accepted that, when one talks of leaves one generally refers to the lamina. In this case, therefore, total lamina area per petiole divided by total lamina dry weight gives specific lamina area defined as the ratio of leaf blade area to its dry weight (Dingkuhn *et al.,* 2000).

$$\mathrm{SLA}_{m^2kg^{-1}} \times \mathrm{LDMC}_{(mg\ g^{-1})}$$

Leaf thickness

Leaf thickness was calculated using the methodology of Vile *et al.* (2005) as:

$$LT_{(\mu m)} = \frac{1}{\mathrm{SLA}_{(m^2kg^{-1})} \times \mathrm{LDMC}_{(mg\ g^{-1})}}$$

Where SLA is the specific leaf area (in this case specific lamina area) and LDMC is the leaf dry matter content. LDMC is calcualted as:

$$LDMC_{(mg\ g^{-1})} = \frac{Leaf\ dry\ mass_{(mg)}}{Leaf\ fresh\ mass_{(g)}}$$

Lamina width to length ratio

This ratio was calculated by dividing the lamina length by the width. As with SLA and LDMC, means were calculated for each plot and these plot means were used in a one-way ANOVA, to test for differences among provenances.

4.2.12.4 Fruit yield prediction

In order to predict fruit yield from plant characteristics a linear multiple regression analysis was carried out with total fruit yield (kg ha^{-1}) as the dependent variable and the following factors as predictors: provenance of material; block; mean petiole height; mean lamina length; mean lamina width; and mean lamina area.

A step wise linear multiple regression was then run with only significant predictors input as factors, in order to determine which factors were the most significant, thus providing a more efficient model.

4.3 RESULTS

This section begins with observations on the growth and form of laminae. This is followed by descriptions of variation in rhizomal survival, petiole production and plant characteristics among provenances. Variation in fruit yield among provenances and the prediction of fruit yield are then reported. The section concludes with a short report of disease and pest attack.

4.3.1. General observations of petiole growth and lamina form

The first leaves to emerge from rhizomes were broader than the typical ovate-elliptic mature leaves. Leaves emerged from the rhizome with furled laminae, unfurling over a period of a few days. (Plate 4.5) Not until they were unfurled was it possible to distinguish between lamina and the petiole.

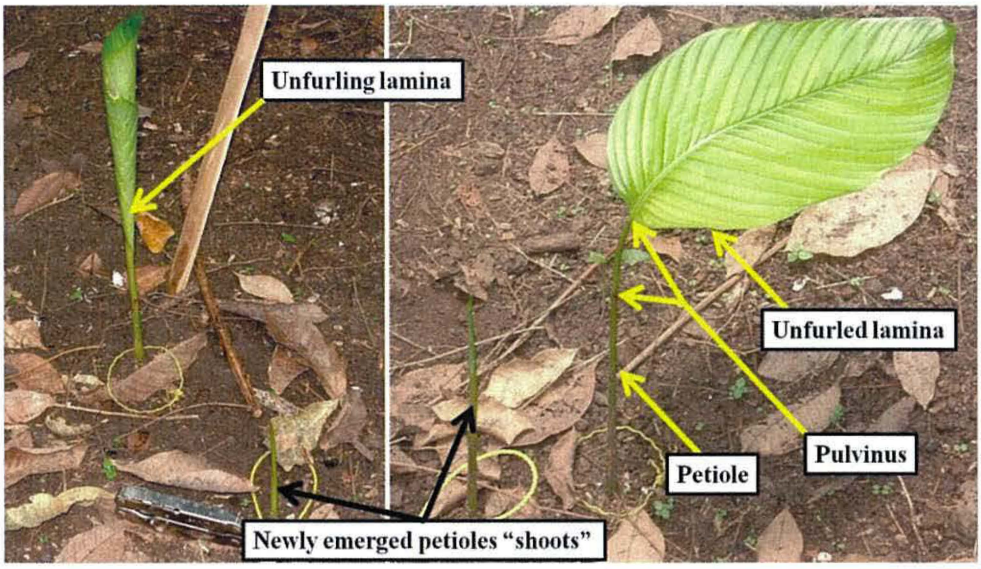

Plate 4.5 Emergence of petioles from rhizome sections.

In the left hand picture the lamina of the shoot has begun to unfurl whilst the younger shoot has the lamina still tightly curled. In the right hand picture a fully unfurled lamina is seen, as can the pulvinus and the petiole, plus a new shoot with a tightly curled lamina. (1st April 2006, Missellele Experimental Site).

Multiple leaves emerging from the same point on a rhizome were commonly observed (Plate 4.6), with upwards of seven leaves being seen growing from one point; by June 2006 almost all leaves arising from rhizomes were emerging as multiple leaves, (Plate 4.6 and Plate 4.7).

The undersides of young leaves were noticeably pubescent. One could feel the hairs if laminae were stroked across lips. Microscopic hairs, more akin to small spikes, were seen with a hand lens on the underside of lamina, in greater density towards the central midrib of the leaf. The undersides of young leaves were dusted with soil, as a result of splash back from rainfall and soil being trapped by the microscopic hairs. As leaves matured, however, they became glabrous, losing the juvenile hairs.

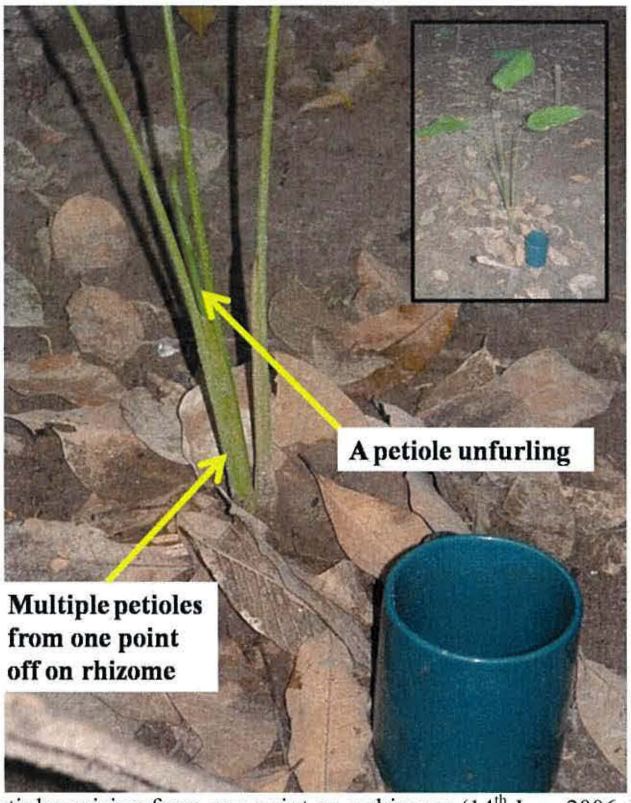

Plate 4.6 Multiple petioles arising from one point on a rhizome (14th June 2006, Missellele Experimental Site)

Plate 4.7 Multiple leaves arising from two single points from a planted section of rhizome (block 1, plot 10, South West Province provenance material).
This picture shows two emergent points, each with multiple petiole/lamina assemblages, from the same rhizome section indicating rhizome spread. (14th June 2006, Missellele Experimental Site).

4.3.2 Rhizome survival and establishment of petioles

After the first two months of growth petiole assemblages were established. Following this there was a decrease in the number of new multiple petiole assemblages arising from single points on rhizomes but more points on the rhizome producing single petioles, with the result that there was an increase in the number of petioles per rhizome. A number of rhizomes in each plot failed to support continuing emergence and growth of petioles; these rhizomes had probably died. As a result there was a reduction in the total number of surviving rhizomes, from the peak at about 50-59 days; the number of surviving rhizomes then reached a plateau which was maintained (Figure 4.5).

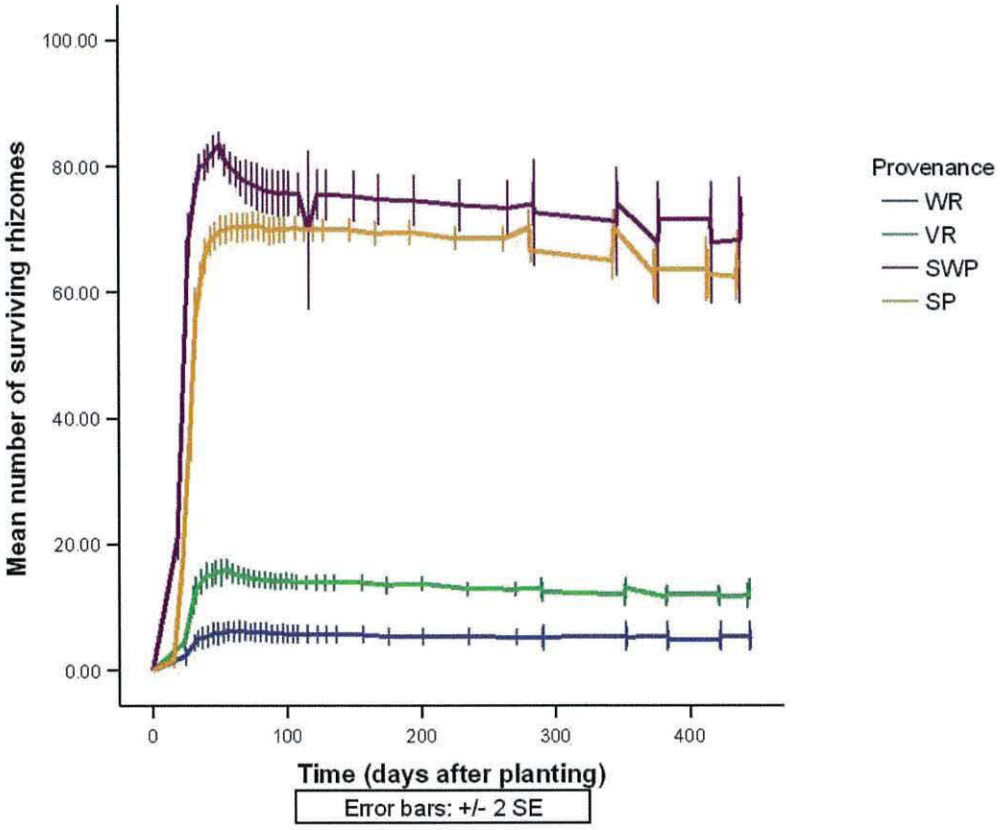

Figure 4.5 The mean number of surviving rhizomes of four provenances of *Thaumatococcus daniellii* in a trial at Missellele experimental site, based on production of petioles.

Rhizome sections were considered to have survived if they produced one or more petioles. South West Province (SWP) and South Province (SP) material survived significantly better than Ghanaian material (F, 2527.884, $p<0.000$). The maximum number of surviving rhizomes was recorded between 40 and 64 days after planting,

and varied among provenances (Table 4.1). The time taken to reach the mean maximal number of emergent petioles (effectively maximum number of rhizomes that survived) was, 56 days for Western Region (WR), 55 days for Volta Region (VR), 50 days for SWP and 59 days for SP.

Table 4.1 Mean and maximum survival of rhizomes per planted section of rhizome per provenance.

Provenance	Maximum number of surviving rhizomes from a single plot	Days after planting	Mean ± SE maximum rhizome survival per provenance (n=185)	Mean days after planting
Western Region	11	64	5.8 ± 0.193^{a}	56
Volta Region	21	40	13.2 ± 0.264^{b}	55
South West Province	90	46	71.6 ± 1.245^{c}	50
South Province	77	51	60.94 ± 1.325^{d}	69

Means in the same column with different superscript letters are significantly different ($p< 0.000$)

New petioles emerged throughout the year from planted rhizome sections, sequentially and longitudinally from the first petiole that had emerged, indicating that rhizomes were growing continuously. Some rhizomes had produced up to seven petioles, with broadly the same pattern of petiole production in all provenances (Figures 4.6 to 4.9).

At the end of the monitoring period 51 WR rhizome sections were still viable from a peak of 61, just over 5 % of total planted material, as they were still producing new petioles. 13% of the total Volta Region rhizomes planted were still viable at the end of the first year. 72% of South West Province rhizomes and 61% of South Province rhizomes survived and were continuing to produce new petioles at the end of the monitoring period, May 2007.

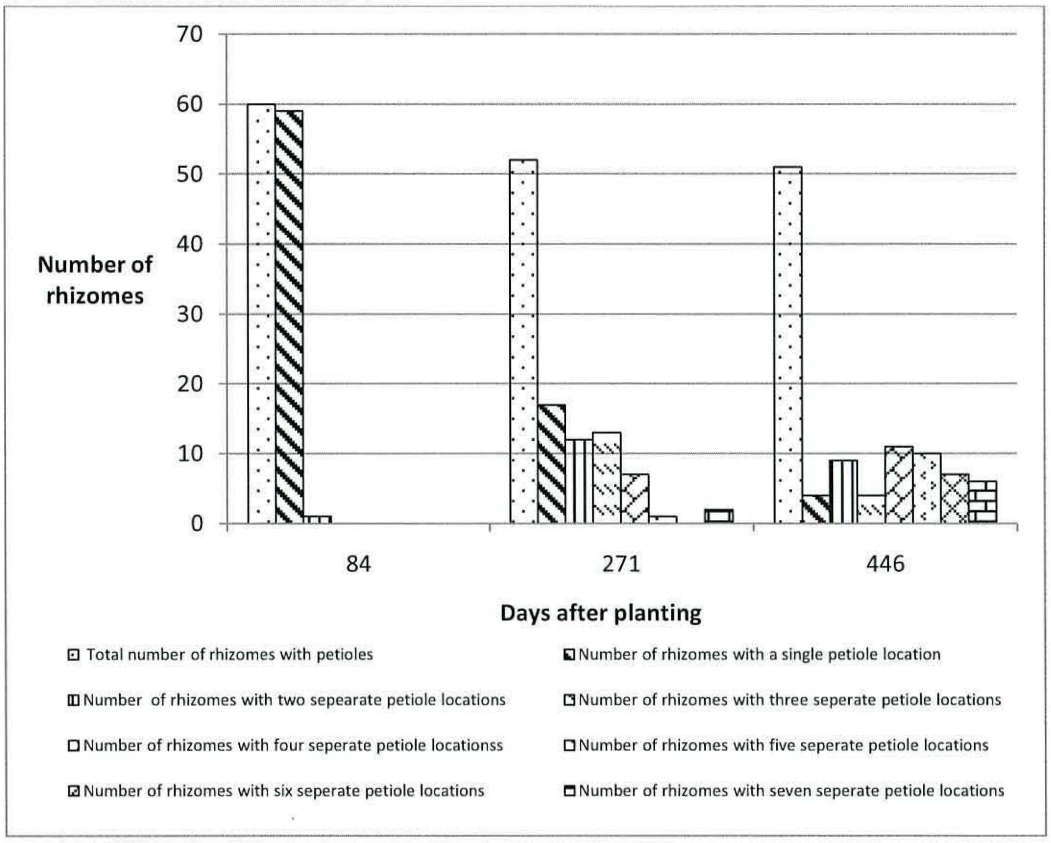

Figure 4.6: Western Region number of petioles per planted rhizome section.

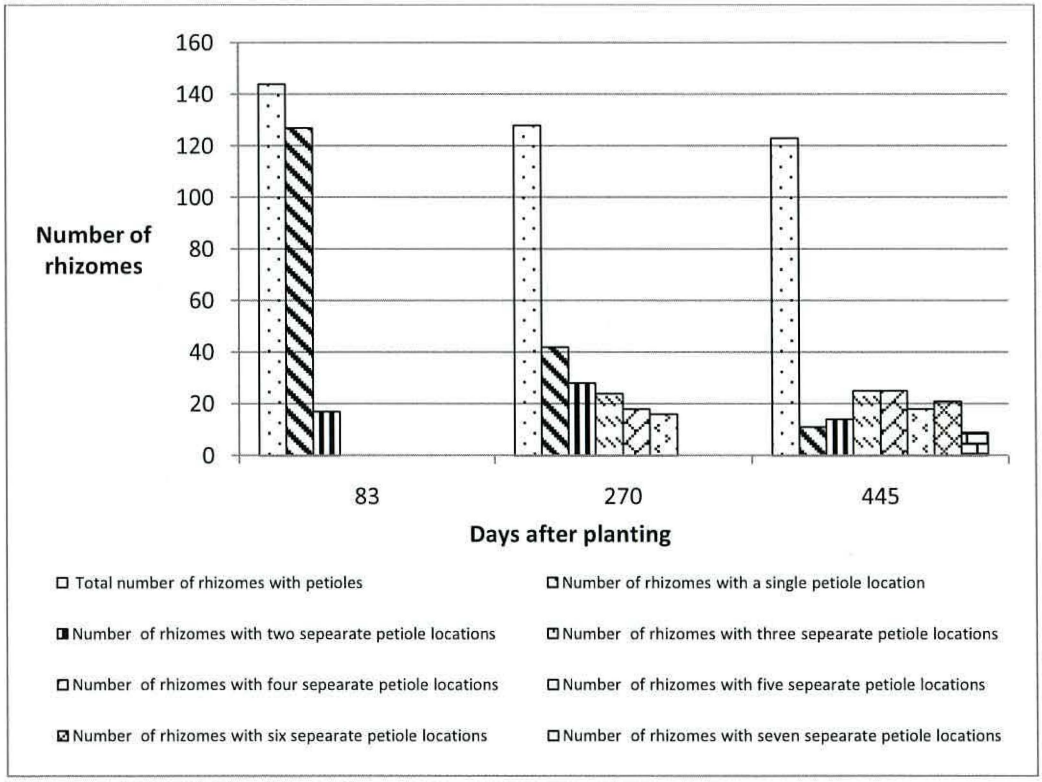

Figure 4.7: Volta Region provenance: number of petioles per planted rhizome section

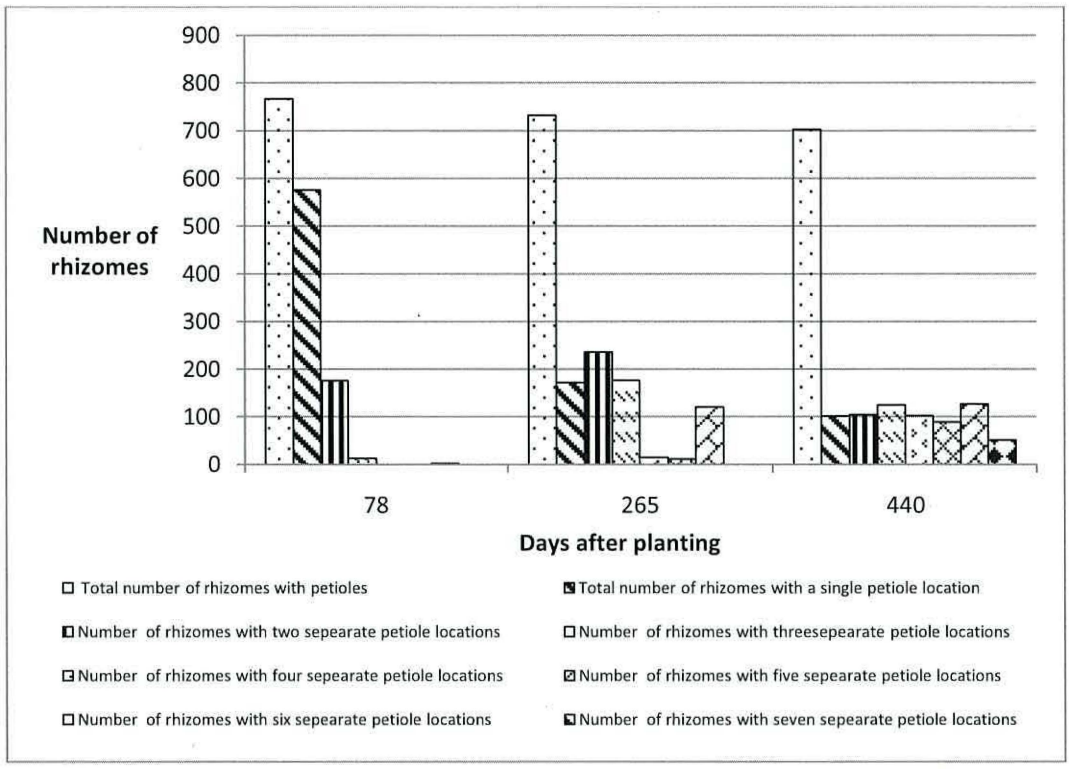

Figure 4. 8: South Western Province provenance: number of petioles per planted rhizome section

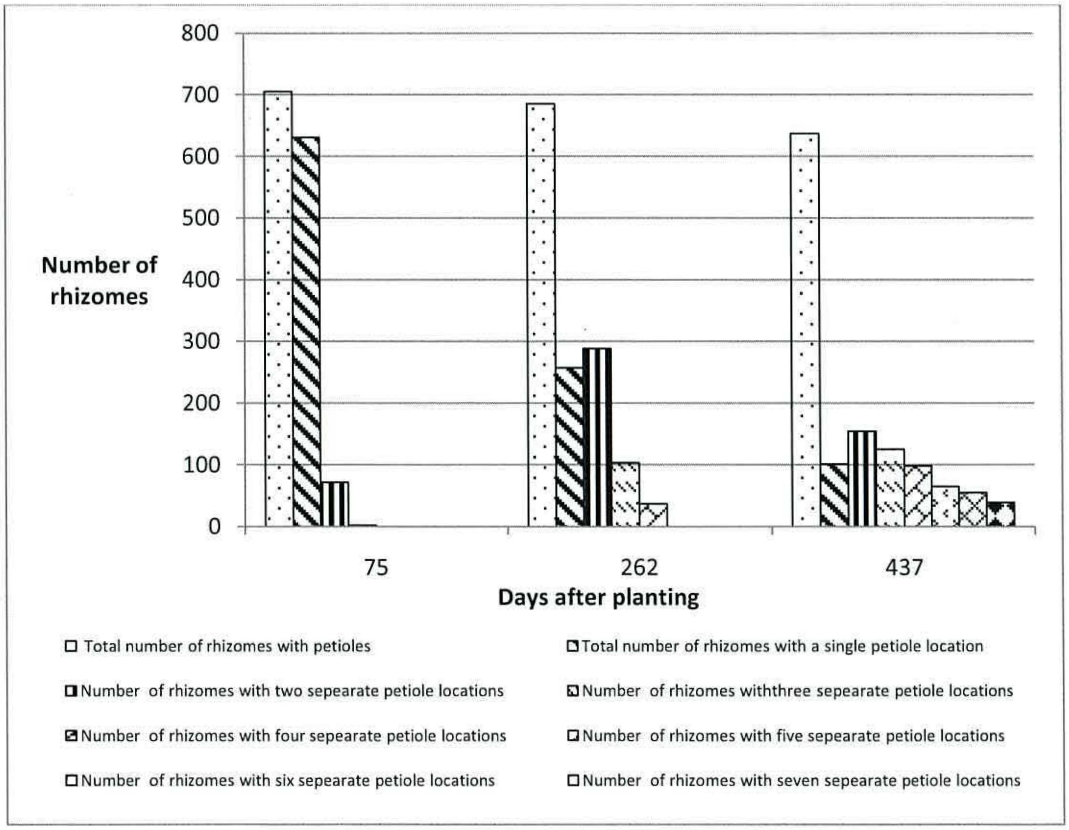

Figure 4. 9: South Province provenance: number of petioles per planted rhizome section

4.3.3 Leaf production

Due to the production of multiple petiole-lamina assemblages, i.e. multiple lamina emerging from a single location on a rhizome, the total number of leaves was greater than the total number of surviving rhizomes. The number of leaves increased during the main rainy season (April-September 2006), followed by a decrease in the number of leaves during the dry season and during the period of rubber tree senescence when tree branches fell on growing petioles (December to February/March) (Figure 4.10). At the end of the monitoring period there were significant differences in leaf production among provenances, with Cameroonian provenances producing many more leaves than Ghanaian provenances (Table 4.2).

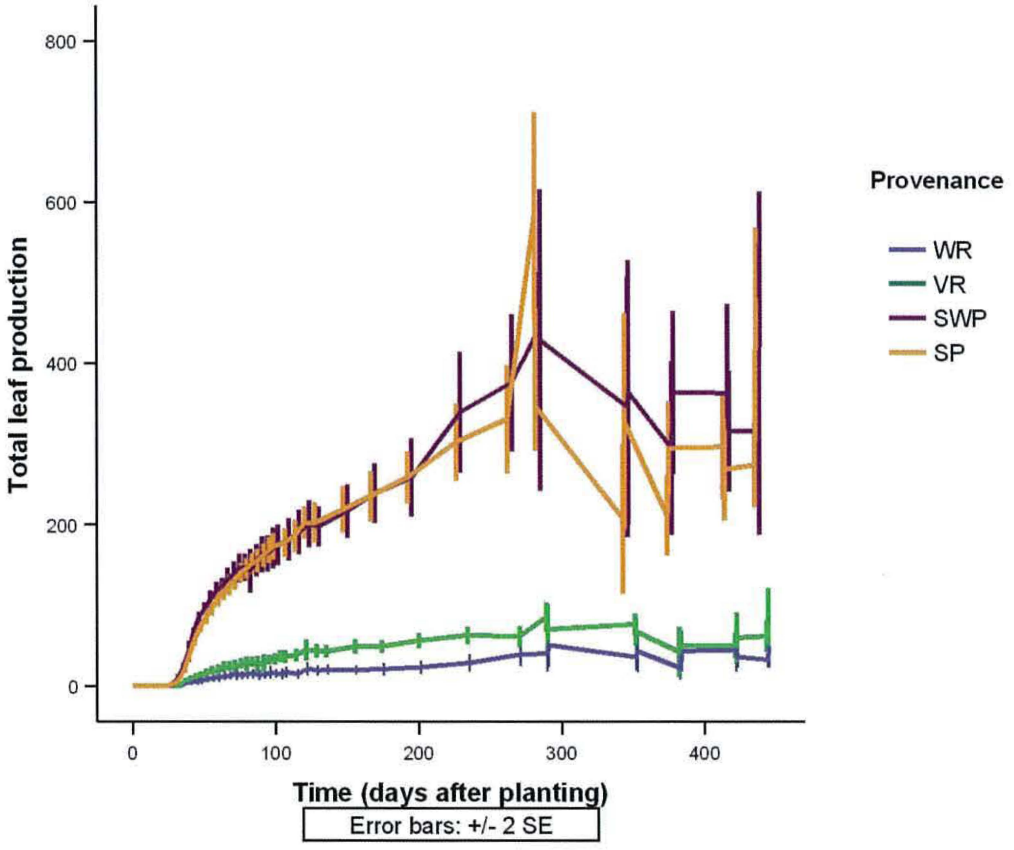

Figure 4.10 Total leaf production per plot per provenance, February 2006 – May 2007.

Table 4.2 Mean leaf production of four provenances of *Thaumatococcus daniellii* at the Missellele Experimental site in May 2007

Provenance	N	Mean (SE)
Western Region	10	40.4 ± 6.79[a]
Volta Region	10	72.2 ± 9.69[a]
South West Province	10	432.4 ± 50.95[b]
South Province	10	377.3 ± 41.59[b]
Total	40	230.58 ± 32.39

Means in the same column with different superscript letters are significantly different ($p < 0.000$)

4.3.4 Later growth – years 2-3

Once rhizomes had become established growth increased, noticeably during and following the rainy seasons. Plate 4.8 shows the development of *T. daniellii* from a South West Province provenance plot during three years.

It was common to see rhizomes growing above ground, producing small surface arches before returning to the soil. These were typically in the order of 15 cm in length and up to 10 cm above ground level (Plate 4.9).

Rhizomes spread tremendously over the two years, some of them producing in excess of 30 petioles (Plate 4.10). Plots became so dense that it was difficult to walk through them at the end of the experimental period to count and collect fruit (Plate 4.11). The canopy of *T. daniellii* was such that weeds that grew in areas surrounding the plots were absent from the plots themselves.

January 2007

March 2008

May 2009

Plate 4.8 Growth and development of *Thaumatococcus daniellii* in a single plot (block2, plot 11) of South Western Province material, January 2006 – May 2009 (Missellele Experimental Site). Photos: Mark Lyonga

Plate 4.9 Rhizomes arching and growing above ground (1st June 2009). Missellele experimental site.

Plate 4.10 A rhizome producing more than 30 emergent petioles in a Western Region plot (block2, plot 25), Missellele experimental site.

These petioles grew from a single rhizome section 20 cm in length (10th March 2008, 754 days after planting).

Plate 4.11 The author with Happy Daniel and Efosi Patience Njoh about to enter a dense plot of *Thaumatococcus daniellii* (18 May 2009, Missellele Experimental site) Photo: Mark Lyonga

4.3.4.1 Plant characteristics

There were no significant differences in plant characteristics between blocks (Table 4.3). No block term was included in further ANOVAs; instead each provenance was represented by ten plot means (five from each of the two blocks). The second site was established simply as insurance in case the first block failed (see Section 4.2.2), rather than to account for within-cite environmental variation.

Table 4. 3 Results of ANOVA for differences in plant characteristics between blocks.

Characteristic	May 2006	May 2007	May 2009
PH	F, 0.026, p = 0.873	F, 1.981, p = 0.167	F, 0.113, p= 0.739
LL	F, 0.311, p = 0.582	F, 0.984, p= 0.328	F, 0.063, p = 0.803
LW	F, 0.480, p = 0.494	F, 0.764, p = 0.388	F, .091, p = 0.765

PH: petiole height; LL: lamina length; LW: lamina width

For some of plots from May 2006, certain quadrats contained rhizome sections that had not survived (Section 4.3.2). In this instance data was excluded from subsequent analysis.

4.3.4.2 Petiole height (PH)

The majority of the variation in petiole height (75 % of the total variation) was accounted for by differences among provenances (Table 4.4), with the South West Province provenance (Plate 4.12) being significantly taller than other provenances (Table 4.5), $p<0.00$.

Table 4.4 Results of analysis of variance for differences in petiole height among four provenances of *Thaumatococcus daniellii* on three different dates

Date		Sum of Squares	df	Mean Square	F	Sig.	ω^2
May-06	Between Groups	448.903	3	149.63	7.924	0.001	0.42
	Within Groups	472.116	25	18.89			
	Total	921.020	28				
May-07	Between Groups	1760.05	3	586.68	12.46	0.00	0.46
	Within Groups	1695.71	36	47.10			
	Total	3455.76	39				
May-09	Between Groups	27806.81	3	9268.94	35.52	0.00	0.72
	Within Groups	9395.32	36	260.98			
	Total	37202.13	39				

Table 4.5 Plot mean ±SE petiole height (cm) of four provenances of *Thaumatococcus daniellii* on three different dates.

Provenance	May 2006	May 2007	May 2009
Western Region	$19.05 \pm 3.65^{a}{}_{(2)}$	$31.95\pm3.09^{a}{}_{(10)}$	$101.32\pm5.04^{a}{}_{(10)}$
Volta Region	$17.55 \pm 1.97^{a}{}_{(7)}$	$28.74\pm1.03^{a}{}_{(10)}$	$96.66\pm5.25^{a}{}_{(10)}$
South West Province	$27.28 \pm 1.37^{ab}{}_{(10)}$	$45.87\pm2.35^{a}{}_{(10)}$	$161.93\pm5.63^{a}{}_{(10)}$
South Province	$20.79 \pm 1.13^{b}{}_{(10)}$	$31.84\pm1.63^{b}{}_{(10)}$	$106.87\pm4.44^{b}{}_{(10)}$
Total	$22.13 \pm 1.07_{(29)}$	$34.6\pm1.49_{(40)}$	$116.7\pm4.88_{(40)}$

Means in the same columns with different superscript letters are significant ($p < 0.05$). Numbers in brackets refer to the number of samples.

Plate 4.12. The author standing next to a plot of *Thaumatococcus daniellii* grown from South Province material with taller plants from the South Western provenance in the background (Photo: Mark Lyonga, 7th May 2009).

4.3.4.3 Lamina length (LL)

Most of the variation in lamina length (75.4 %, May 2009) was explained by differences among provenances (Table 4.6), with significant differences between provenances. South West Province provenance material produced the longest lamina with the South Province material producing the shortest lamina, and both were significantly different from Ghanaian material (Table 4.7).

Table 4.6 Results of analysis of variance for differences in lamina length among four provenances of *Thaumatococcus daniellii* on three different dates

Date		Sum of Squares	df	Mean Square	F	Sig.	ω^2
May-06	Between Groups	97.981	3	32.660	11.56	0.00	0.52
	Within Groups	70.631	25	2.825			
	Total	168.612	28				
May-07	Between Groups	100.42	3	33.47	5.77	0.00	0.26
	Within Groups	208.82	36	5.80			
	Total	309.24	39				
May-09	Between Groups	565.85	3	188.62	36.96	0.00	0.73
	Within Groups	183.73	36	5.10			
	Total	749.57	39				

Table 4.7 Plot mean ±SE lamina length (cm) of four provenances of *Thaumatococcus daniellii* on three different dates.

Provenance	May-06	May-07	May-09
Western Region	$12.53 \pm 2.43^{a}{}_{(2)}$	$21.977 \pm 1.09^{a}{}_{(10)}$	$36.712 \pm 1.09_{a\,(10)}$
Volta Region	$17.23 \pm 0.39^{bc}{}_{(7)}$	$21.815 \pm 0.59^{a}{}_{(10)}$	$37.799 \pm 0.55^{a}{}_{(10)}$
South West Province	$19.22 \pm 0.64^{c}{}_{(10)}$	$24.983 \pm 0.62^{b}{}_{(10)}$	$43.059 \pm 0.49^{b}{}_{(10)}$
South Province	$16.02 \pm 0.42^{b}{}_{(10)}$	$20.712 \pm 0.63^{a}{}_{(10)}$	$32.503 \pm 0.55^{c}{}_{(10)}$
Total	$17.18 \pm 0.46_{(29)}$	$22.372 \pm 0.45_{(40)}$	$37.518 \pm 0.69_{(40)}$

Means in the same columns with different superscript letters are significant ($p < 0.05$). Numbers in brackets refer to the number of samples.

The low mean values seen for WR and VR in May 2006 are due to low numbers of plots producing vegetative growth.

4.3.4.4 Lamina width (LW):

As for petiole height and lamina length most of the variation in lamina width (72 %) was explained by differences among populations (Table 4.8). Lamina width was significantly narrower in South Province provenance material than other provenances (Table 4.9).

Table 4.8 Results of analysis of variance for differences in lamina width among four provenances of *Thaumatococcus daniellii* on three different dates

Date		Sum of Squares	df	Mean Square	F	Sig.	ω^2
May-06	Between Groups	43.538	3	14.513	8.659	0.00	0.44
	Within Groups	41.902	25	1.676			
	Total	85.440	28				
May-07	Between Groups	58.95	3	19.65	5.68	0.00	0.25
	Within Groups	124.51	36	3.46			
	Total	183.46	39				
May-09	Between Groups	292.67	3	97.56	31.82	0.00	0.69
	Within Groups	110.39	36	3.07			
	Total	403.06	39				

Table 4.9 Plot mean (±SE) lamina width by provenance from the Missellele experimental site.

Provenance	May-06	May-07	May-09
Western Region	10.02 ± 1.68^{ab} (2)	13.79 ± 0.841^{a}	25.03 ± 0.736^{a}
Volta Region	10.85 ± 0.34^{ab} (7)	13.16 ± 0.445^{ab}	25.5 ± 0.552^{ab}
South West Province	12.23 ± 0.49^{b} (10)	14.58 ± 0.52^{a}	27.49 ± 0.475^{b}
South Province	9.32 ± 0.33^{a} (10)	11.29 ± 0.456^{b}	20.13 ± 0.392^{c}
Total	10.74 ± 0.32 (29)	13.21 ± 0.343	24.54 ± 0.508

Means in the same columns with different superscript letters are significant ($p < 0.05$). Numbers in brackets refer to the number of samples.

4.3.5 Results from destructive sampling

4.3.5.1 Specific leaf area (SLA)

There were highly significant differences in SLA among provenances, $F(3,36) = 23.113$, $p < 0.000$, $\omega^2=0.62$, with the South Province provenance showing much higher values for mean SLA than the other three provenances (Table 4.10).

Table 4.10 Mean specific leaf area of four provenances of *Thaumatococcus daniellii*

Provenance	N	SLA (m^2kg^{-1})
Western Region	10	13.86 ± 0.352^{a}
Volta Region	10	14.01 ± 0.273^{a}
South West Province	10	14.12 ± 0.270^{a}
South Province	10	17.07 ± 0.374^{b}
Total	40	14.77 ± 0.264

Means in the same columns with different superscript letters are significant ($p < 0.05$).

4.3.5.2 Leaf thickness

The thinnest leaves came from the South Province provenance (Table 4.11), with a mean of 209.8 μm. This low value was significantly different from that of the other provenances, $F(3,36) = 38.292$, $p < 0.000$, $\omega^2 = 0.74$.

Table 4. 11 : Mean leaf thickness of four provenances of *Thaumatococcus daniellii*.

Provenance	N	Leaf thickness (μm)
Western Region	10	245.78 ± 2.969^{a}
Volta Region	10	246.39 ± 3.186^{a}
South West Province	10	250.11 ± 2.450^{a}
South Province	10	209.8 ± 3.518^{b}
Total	40	238.02 ± 3.005

Means in the same columns with different superscript letters are significant ($p < 0.05$).

4.3.5.3 Length to width ratio

Length to width ratios fell into two distinct groups. Ghanaian material had significantly lower values (indicating broader leaves) while Cameroonian material had narrower leaves $t(382) = -8.016$, $p < 0.000$, $\eta^2 = 0.14$. The broadest leaves came from the Western Region provenance (1.47 ± 0.010) and the narrowest leaves were from the South Province provenance (1.62 ± 0.116).

4.3.5.4 Correlations between plant characteristics

Plant characteristics (n=400) were all significantly correlated with each other, petiole height and lamina length ($R^2 = 0.360$, $p<0.01$); petiole height and lamina width ($R^2 = 0.176$, $p<0.01$); and lamina length and width ($R^2 = 0.632$, $p<0.01$).

4.3.6 Fruiting and fruit yield

Fruit were first seen in September 2008 in plots of the South Western Province provenance. At the time of fruit collection in May 2009 the number of flowers and buds was also noted (Table 4.12). Flowers were abundant throughout plots of the South Western Province provenance (Plate 4.13).

Table 4.12 Number of flowers and buds in four provenances of *Thaumatococcus daniellii* in May 2009 and fruit in May 2010.

Provenance	Flowers (May 2009)	Buds (May 2009)	Total flower/buds (May 2009)	Fruit (N) (May 2010)
Western Region	77	31	108	48
Volta Region	39	22	61	54
South West Province	795	744	1539	418
South Province	0	0	0	66
Total	911	797	1708	586

Plate 4.13 Abundant flowering of *Thaumatococcus daniellii*, South West Province provenance (7th May 2009).

Mature fruit from the South Western Province provenance were collected and sectioned according to methods detailed Section 2.3.6.3. Due to the low numbers of fruit produced by the other provenances they were left *in situ*. A total of 227 fruit were measured for all traits, length, width, mass, number of seeds, number of arils and total aril mass (Plate 4.14). Of these 23 were rejected: seven because seeds had started germinating (Plate 4.15); two because of damage during the cutting process; and the remainder because they were rotten. A further 98 fruit were weighed and cut, though measurements were not made of individual size or mass. A total of 3.76 kg of fruit was produced, from plots of the South West Province provenance in the first harvest from the experiment.

Plate 4.14 Fruit produced from plots of the South West Province provenance of *Thaumatococcus daniellii*, (24th May 2009).

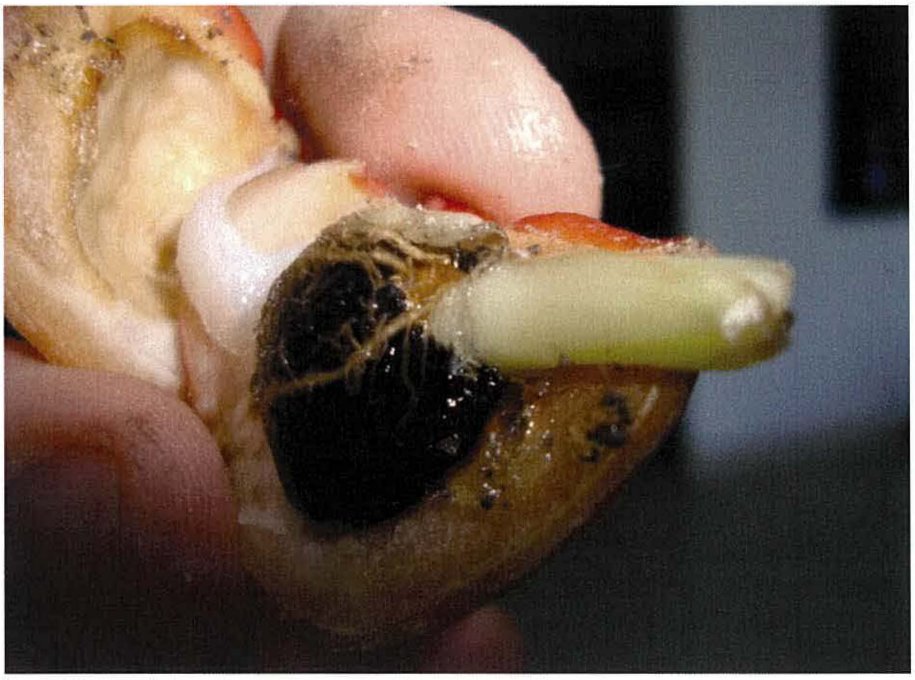

Plate 4.15 Germinating seed from collected fruit (24th May 2009).

A second harvest of fruit was made in March 2010. Fruit length, width and mass were measured but fruit were not sectioned and so aril mass was not determined.

This harvest produced a total of 7.24 kg of fruit from all provenances with the majority, 5.23 kg being produced by the South West Province provenance (Table 4.13).

Table 4.13 Fruit production of four provenances of *Thaumatococcus daniellii* in two years

Provenance	Year 1 harvest 2009 mean fruit mass (g)	Year 1 total fruit harvest (kg)	Year 2 harvest 2010 mean fruit mass (g)	Year 2 Total fruit harvested by provenance (kg)
Western Region			11.93±2.51^{a} (48)	0.560
Volta Region			11.67±3.46^{a} (54)	0.640
South West Province	12.79±0.31 (302)	3.76	12.56±1.67^{b} (418)	5.23
South Province			12.10±2.68^{a} (66)	0.798

Means in the same columns with different superscript letters are significantly different ($p< 0.05$). Numbers in brackets refer to the number of fruit.

Aril mass from the first harvest was 0.411 ± 0.22 g, and aril mass as a percentage of fruit mass 3.09 ± 0.11.

4.3.6.1 Fruit production per area (hectare)

The total mass of fruit produced in harvest two, by each provenance is shown in table (Table 4.14). A value for mass of fruit kg ha^{-1} has been calculated from this data. It can be seen that the local provenance, South West Provenance, has produced significantly more fruit than the other provenances.

Table 4.14 Fruit production in four provenances of *Thaumatococcus daniellii*

Provenance	N (fruit)	Mean (SE) plants per provenance per plot	Mean fruit production per plot per provenance (g .01ha^{-1})	Mean fruit production (kg ha^{-1})	Mean fruit production per plant (g $plant^{-1}$)
Western Region	48	6.1 ± 1.06	56.03±15.18^{a}	5.60	13.31 ± 4.88^{a}
Volta Region	54	12.4 ± 0.54	64.42±10.30^{a}	6.44	5.52 ± 1.03^{ab}
South West Province	418	69.9 ± 2.25	523.37±50.04^{b}	52.34	7.58 ± 0.73^{ab}
South Province	66	63.7 ± 1.55	79.87±17.99^{a}	7.99	1.26 ± 0.29^{b}
Total	586				6.92 ± 1.4

Fruit means in the same columns with different superscript letters are significantly different ($p< 0.05$).

4.3.6.2 Fruit yield prediction

The regression was highly significant (F, 25.155; p= 0.000, R^2 = 0.821), indicating that fruit yield could be predicted with confidence from plant characteristics. Petiole height (p = .042) and lamina length and width (p=0.000) were highly significant predictors with the other predictors, leaf area, provenance and block, not being significant (p>0.05).

A stepwise model suggested that all variables should be rejected from the regression model, as they did not significantly improve the regression model, except petiole height, which was highly significant (F, 94.119, p<0.000). The resulting equation for predicting fruit yield (R^2, 0.712) is

$$Total\ fruit\ yield_{(kg\ ha^{-1})} = 59.76\ petiole\ height\ (m) - 51.598$$

4.3.6.3 Percentage fruit set

Percentage fruit set was calculated as the total number of harvested fruit as of May 2010 divided by the total number of flowers and buds noted in May 2009 (Table 4.12). Fruit set for the Western Region provenance was 44%, for the Volta Region provenance, 88%, 27.16 % for the South Western Province provenance; a value for the South Province provenance could not be calculated as there were no visible buds or fruit in May 2009, though there were fruits in May 2010.

4.3.7 Colour of flower bud scales and flowers

Colour of flower bud scales and flowers varied among provenances. In the Western Region and Volta Region provenances flower bud scales were purple and yellow, and flowers were white with a hint of purple (Plates 4.16 and 4.17 respectively). Bud flower scales from South Western Province provenance were bright green and flowers were white (Plate 4.18). South Province provenance flower buds were green with a brown coloured edge (Plate 4.19).

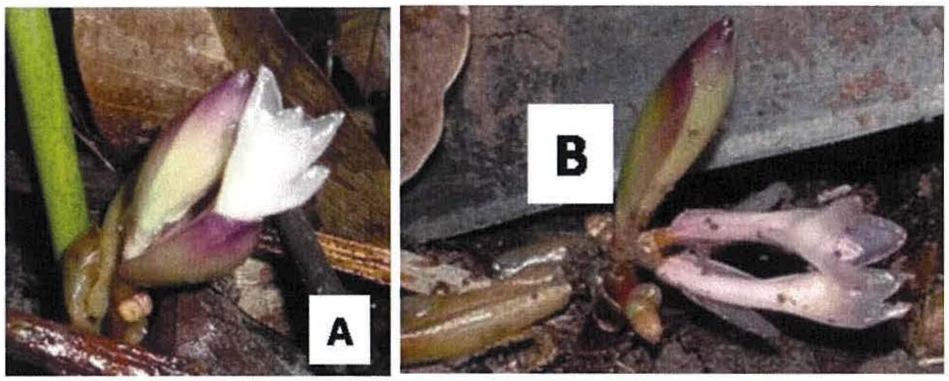

Plate 4. 16 Western Region provenance flowers and buds: A) experimental site; B) Mrebenini, Western Region, Ghana.

Plate 4. 17 Volta Region provenance flowers and buds: experimental site

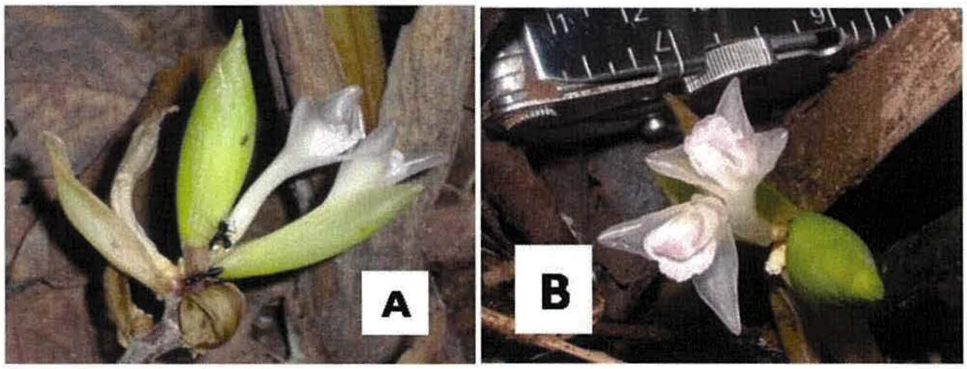

Plate 4. 18 South West Province provenance flowers and buds: A) experimental site b) Etome, South West Province, Cameroon.

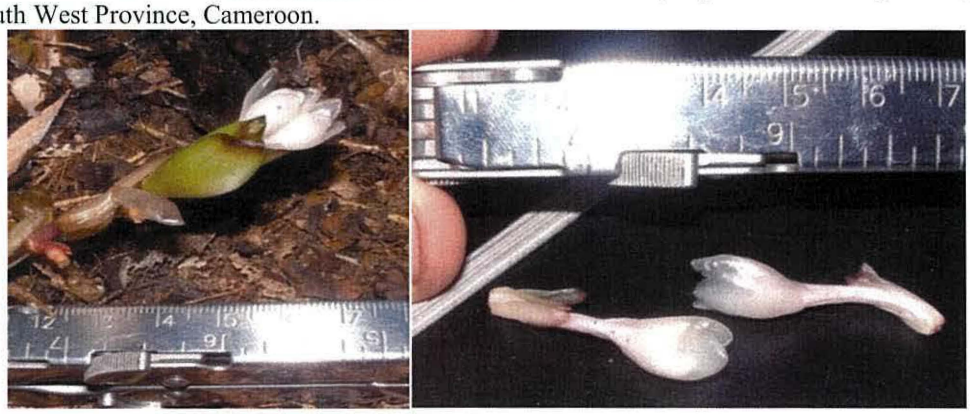

Plate 4. 19 South Province provenance flowers and buds: Mebanga, South Province, Cameroon.

4.3.8 Petiole pest and disease attack

Though very resilient to attack by leaf borers, snails, locusts and other beetles, lamina were affected by both these and (presumably) fungal disease . Perforated leaves indicated attack by leaf cutters, and holes within leaves indicated attack by leaf borers (Plates 4.20 and 4.21). Jagged cuts at the edges of leaves were probably due to beetles. A presumably fungal disease resulted in leaves that were translucent and mis-shaped (Plate 4.22). Ants were seen commonly at the base of petioles, more frequently when plants were mature and producing buds, flowers and fruit. Small frogs were also seen resting on leaves.

Plate 4.20 *Thaumatococcus daniellii* lamina blades cut and bored whilst still folded.

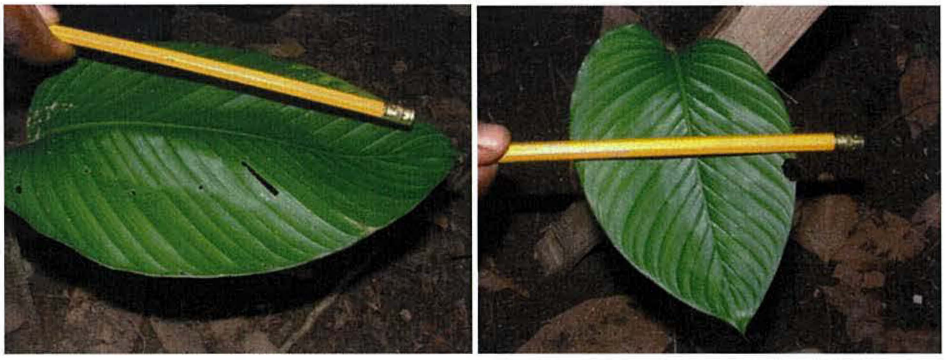

Plate 4.21 Attack of leaves by leaf borers and cutters

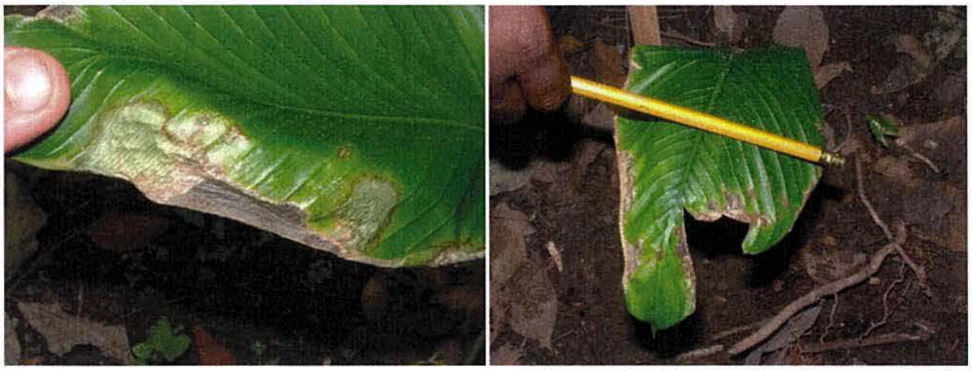

Plate 4.22 Possible fungal or bacterial infection of leaves

There was no evidence of disease or pests of rhizomes. There was one incident of mammals attacking and destroying some plants when pigs from a local village invaded some plots. The other main cause of petiole death was falling branches from the PR 107, rubber clone, which self prunes, and major impact by tree fall. Only once in the whole experiment was a plot damaged by accidental weeding by CDC personnel.

4.4 Discussion

4.4.1 Rhizome survival and growth of plants

There was great variation in the survival of planted sections of rhizome surviving planting. 5.8 and 13.2 % of Ghanaian material (Western Region and Volta Region respectively) survived compared with 71.6 and 60.9 % of Cameroonian material (South West Province and South Province respectively). Yeboah *et al.* (2003) found that 70 days after planting maximum survival was 25% under low shade, decreasing to 20 % and 10% in plots with medium and heavy shade. Attempts were made to keep rhizomes as fresh as possible after harvesting, by keeping them cool and moist during transit but it may be that the differences in survival were due to the length of time between harvesting and planting of rhizome sections. Ghanaian material was planted after 13 and nine days after harvesting, (Western Region and Volta Region respectively), possibly explaining its low survival; however, Cameroonian South Province material was planted one day after harvesting and had lower survival than South West Province material that was planted three days after harvesting. A trial to evaluate the effect of time from harvesting to planting on survival was not conducted, but would be worthwhile. Yeboah *et al.* (2003) planted their material the day after it was collected (pers. obs) at 0.5 m * 0.5 m spacing, which according to Most *et al.* (1978), would not have a deleterious effect on the survival of plants and would in the long term lead to more inflorescences per hectare. Rhizomes at the experimental site were planted at 1 m * 1 m spacing, following the suggestion of Onwueme *et al.* (1979), who planted undetermined lengths of rhizome sections with and without lamina and aerial stools (shoots). They found that within a few days attached lamina would died when planted, but if the rhizome had aerial shoots these continued to unfurl and produce lamina, while rhizomes without shoots took longer to produce

leaves (Onwueme *et al.,* 1979). For this reason and to reduce the bulk of material to be transported, all rhizomes had their petioles and aerial shoots removed during harvesting.

4.4.2 General growth from rhizomes

Though the number of plants surviving varied among provenances, all plants showed the same pattern of growth. Interestingly, rhizomes produced multiple petioles from the first emergent point. This has not been described before; the similarity to the growth form of *Sarcophrynium brachystachyum* var. *brachystachyum* (Benth.) K.Schum. is startling and there is also similarity to the multiple petioles that are produced by seedlings (pers. obs.). This may be an adaptation that enables the rhizome to maximise photosynthetic capability. It was not uncommon to see up to eight petioles arising from the first emergent point before new petioles were produced from other nodes. By the end of the first year the surviving rhizomes in the majority of plots had multiple petioles at several different nodes (Figures 4.13; 4.14; 4.15 and 4.16)

Juvenile lamina had microscopic hairs which could be felt with fingertips and lips. However, once mature the lamina lost the hairs and became glabrous, bringing in to question Dhetchuvi and Diafouka's (1993) assertion that one of the two defining characteristics of variety *puberulifolius* is hairs on the underside of lamina. The reason for juvenile leaves having hairs is unknown, though Esau (1977) quoted in (Moose *et al.,* 2004), suggests that juvenile leaves may act as physical and chemical deterrents to attack by insects.

Leaves showed a marked decline during the period November to February (Figure 4.10), corresponding to the dry, harmattan season in Cameroon. Rubber trees self prune during this period, resulting in a more open canopy. Falling branches destroyed some leaves, but leaf numbers declined still further during the dry season, supporting research conducted by Onwueme *et al.,*(1979). These authors describe a similar situation in Nigeria; the number of leaves per unit area declined during the dry season, with the decrease being less in shaded plots and with un-shaded plots showing generally stunted growth. Farmers in Ghana suggested that open canopies

led to an increase in soil temperature and a decrease in soil water content, which negatively impacted on the growth of *T. daniellii (Waliszewski et al., 2005)*. Local people in Ghana also suggested that there was an increase in predation of *T. daniellii* by rodents (*Thryonomys swinderianus* Temminck – grasscutter) looking for water in stems and leaves (Waliszewski *et al.,* 2005), though this was not noticed at the experimental site in Cameroon.

Rhizomes continued to grow throughout the second year after planting, producing high numbers of petioles from individual rhizome sections (Plate 4.10) and dense colonies of petioles (Plates 4.8, and 4.10), so that distinguishing petioles produced by different rhizomes became impossible without destructive sampling. The density of colonies was such that weeds could not establish themselves. Similar growth and weed exclusion was found in Nigeria (Onwueme *et al.,* 1979). On many occasions rhizomes were seen to grow out of the ground, producing small arches before returning to the ground (Plate 4.9). Why this occurs is not clear but was common across all planted material.

4.4.3 Flowering and fruiting: mean fruit mass and yield predictors

In the second year of growth plants began to produce fruit, with the first fruit noticed in September 2008, two years and seven months after planting. This is consistent with the findings of Onwueme *et al.* (Onwueme *et al.,* 1979), who recorded fruit being produced from plants aged between two years two months and two years seven months. It is earlier than observed fruiting on plants grown from planted rhizomes in Ghana, which began three and a quarter years after planting (Adansi and Holloway, 1977).

Plants continued to produce substantial amounts of fruit that were collected in May 2009. It was noted that fruit remained on the plant without abscission or detioration supporting, Onwueme *et al.'s* (1979) findings and confirming local collectors' knowledge of fruit set and time to harvest (Waliszewski *et al.,* 2005).

Based on Onwueme *et al.'s* (1979) finding that the time between flowering and fruit ripening is 13 weeks, it can be suggested that flowering at Missellele began

sometime in June. This was at the peak of the rainy season. The following year, from early May 2009 – June 2009, a profusion of flowers were seen in plots of the South West Province, with other provenances also producing flowers. The sheer number of flowers on the ground in May 2009 and comments from site staff suggest that flowering could have occurred earlier. It seems therefore that flowering at the site was related to the onset of the rainy season, strongly supporting similar reports in the literature (Enti, 1975; Onwueme *et al.,* 1979; Boy, 1994) and from collectors and cultivators in Ghana (Waliszewski *et al.,* 2005). It also seems that there is only one period of flowering and fruiting at the Missellele site, reflecting the one main season of rainfall. In Ghana some staff of Samartex Timber and Plywood Ltd. suggested that there may be two seasons of flowering due to the more bimodal rainfall pattern there (Figure 2.1). There is, however, disagreement about the time to first flowering: Onwueme *et al.* (1979) described flowering, within three months of planting rhizomes at their sites in Nigeria, whereas flowers were not seen before fruit at the Missellele site, suggesting that the earliest flowering occurred between 27 and 29 months after planting. Perhaps the explanation for Onwueme *et al.'s* (1979) early flowering result is that rhizomes already had flower buds at the time they were planted?

Ley (2008) described low fruit set in *T. daniellii* in Gabon; although higher fruit set was obtained by manually pollinating plants, there was only 7.73 % fruit set in control plants, supporting research conducted by Most *et al.* (1978) and Onwueme *et al.* (1979). Gnage *et al.* (Unpublished) described higher fruit set in experiments using pollinators, varying between 27.4 and 60.2 %. The mean fruit set at Missellele was 30.44 %, but, this varied among provenances from 88 % (Volta Region) to 27 % (South West Province). These findings must be treated with caution as they are based on only one observation of flowers and buds in May 2009, and one total fruit collection the following March. As has been suggested, May is at the start of the flowering season; one would expect the number of flowers and fruit to increase, giving a result between the limits of Ley (2008) and Gnage *et al.'s* (Unpublished) control results, 7.73 – 27.4 % fruit set.

Fruit mass differed significantly between the local provenance (South West Province) and the three other provenances. However, the variability of fruit mass collected from natural populations was much greater than that at the experimental site: sample mean ± SE of fruit mass collected from natural resource was 13.14±5.55 g compared with 12.07±0.374 g from the experimental site.

In the first year of fruiting, fruit was only produced from SWP plots. Percentages of aril per fruit were virtually identical to those of fruit collected from natural populations: for example, aril mass from the first harvest was 0.411 ± 0.22 g compared to aril mass from the natural population, 0.46 ± 0.01 g and aril mass as a percentage of fruit mass was 3.09 ± 0.11 compared to 3.55 ± 0.06g from the natural population. In the second year all plots produced fruit. There was no significant difference between blocks regarding the production of fruit (mass) F (1,27) = 1.875, p =.180. Onwueme *et al.* (1979) found that fruit yield was unpredictable; in some plots it declined while others showed increased fruit production. For example, 249.5 kg of fruit were produced from a 0.2 ha plot in the first year, declining to 96.7 kg and 1.75 kg in the second and third years (plot A); other plots produced between 3.7 kg and 32 kg of fruit over a two year fruiting period (Onwueme *et al.,* 1979). Yield varied between 57 330 and 715 fruits ha^{-1} yr^{-1}, equivalent to 1 247.5 and 8.75 kg ha^{-1} yr^{-1} respectively. Adansi and Holloway planted 0.1 hectares of rhizomes at 1 m* 1 m spacing (1000 sections) producing 68 fruits after three years and four months (Adansi and Holloway, 1977). Fruit production from Missellele falls within this range, with a maximum fruit yield of 52.54 kg ha^{-1} yr^{-1} from South Western Province plots and lowest production of 5.61 kg ha^{-1} yr^{-1} from Western Region plots. The maximum mass of fruit produced from a single 0.01 ha plot was 908.76 g, scaling up to 90.87 kg ha^{-1}: this was a SWP plot. Scaling the production of fruit from the most productive provenance plots would produce: 13. 81 kg ha^{-1} from Western Region provenance; 10.91 kg ha^{-1} from Volta Region provenance; and 16.9 kg ha^{-1} from the South Province. Interestingly, plants with the highest individual production were from Western Region plots; they produced 13.31 ± 4.88 g $plant^{-1}$, more than twice the mean for site (6.92 ±1.4 g $plant^{-1}$).

The variability in fruit production in Nigeria (Onwueme *et al.,* 1979) suggests that monitoring of fruit production will be required for many years to establish long term production patterns.

The model that can be used to predict fruit yield from mean petiole height works for plots at Missellele. Refinements to the model will be possible if fruit yield data becomes available from new plots that are established under rubber elsewhere.

4.4.4 Variation in plant characteristics

Mean values of petiole height, lamina length and lamina width at the experimental site were lower than mean values for natural populations, and standard errors were also lower (Table 4.15).

Table 4.15 Mean plant characteristics from natural populations compared with material grown at Missellele experimental site.

Characteristic	Mean values ± SE from natural populations (m)	Mean values±SE from the experimental site(m)
PH	1.437±0.221	1.167±0.152
LL	0.405±0.046	0.376±0.022
LW	0.268±0.032	0.245±0.016

Most of the variation in plant characteristics, between 75 and 72 % of the total, was explained by differences among provenances (Tables 4.4, 4.6 and 4.8). This clearly demonstrates that for the plant characteristics observed at the experimental site most of the variation was related to the origin of the plant material. However, with 28 to 25% of the total variation due to differences among plants within populations, there is scope to select individual plants for desired combinations of characteristics and propagate them vegetatively.

As with fruit mass, the local provenance (South West Province) was significantly larger than other provenances, with taller petioles and longer and broader lamina, this being evident after one year of growth (Tables 4.5, 4.7 and 4.9). Interestingly, phenotypic differences among natural populations were not evident in the common garden experiment for example; petiole heights of the Western Region, Volta Region and South Province provenances were not significantly different from each other.

Laminae of the South Province provenance were significantly smaller than those of all the other provenances, but this is the only case where patterns of variation in populations and in the common garden experiment were the same.

Results of the destructive sample supported the field observations that lamina of the South Province provenance differed from those of other provenances. It was noticed during field work that the form of feel of laminae from the South Province provenance were different to that of laminae from the other provenances, feeling more papery (pers. obs.). South Province lamina were thinner (Table 4.11) and had a significantly higher SLA (Table 4.10) than lamina of other provenances. This supported the finding that the leaves were thinner, as leaves with a high SLA are thinner than leaves with a low SLA (Dingkuhn *et al.,* 2000). The length to width ratios suggested that Cameroonian material had significantly narrower laminae than Ghanaian material. The South Province provenance had the narrowest leaves, and these appeared to be more ovate-oblong than the typical *Thaumatococcus* leaf, which can be classed as ovate-elliptic (Hutchinson and Dalziel, 1968).

Though monocot leaves are recognised as being 'tougher' than dicot leaves in lowland tropical rainforests (Dominy *et al.,* 2008), effectively and therefore more resilient to insect herbivory (Grubb *et al.,* 2008), there was evidence of insect attack. Leaf cutters and leaf borers did attack lamina (Figures 4.30 and 4.31), though leaves were quite resilient and continued to grow (pers. obs.). There was also damage to lamina by an un-classified fungal disease (Plate 4.22).

4.5 Conclusions and recommendations

It is has been demonstrated that *T. daniellii* will grow with mature rubber (clone PR107), and that fruit production begins approximately two and half years after planting of rhizome sections, though mean plant and fruit size are slightly depressed when compared with fruit found in natural populations. Fruit production under rubber at one experimental site can be predicted from petiole height. Variation among *T. daniellii* provenances is greater than variation within individual provenances, yet there is enough variation within provenances to begin identifying individuals with particular traits and propagating them vegetatively.

Even with the limited data from the experimental plots, on aril percentage per fruit, and an expected thaumatin yield from arils (Chapter 2), an attempt can be made to predict the amount of thaumatin that could be produced given different sized areas of rubber intercropped with *T. daniellii*. This information is vital for farmers and other stakeholders to determine the feasibility of establishing a new supply chain with fruit produced under rubber.

The first obvious recommendation is to establish experimental plots at each of the collection sites with material from these and other potential provenances and observe fruit production and plant growth to determine variation in growth among sites and the importance of provenance-site interactions. A second experiment to determine the effect of time-to-planting on the survival of rhizomal material would harvest rhizomes, section them and plant them at daily intervals to establish the survival of sections after different times between harvesting and planting.

An entomological study is needed to identify pests of *T. daniellii,* whether damage by pests is at an acceptable level that does not harm fruit production, and if not, whether methods to control them are cost effective. A similar study on the fungal pathogen would merit consideration.

As the site is now established at Missellele a year-long study could be conducted to establish the exact time of flowering, fruiting and time to maturation of fruit, and the time that fruit could be left on rachises before collection.

CHAPTER 5

THE EFFECT ON *HEVEA BRASILIENSIS* CUP LUMP RUBBER PRODUCTION OF INTERCROPPING WITH *THAUMATOCOCCUS DANIELLII*

This chapter examines the effect of growing *Thaumatococcus daniellii* as an intercrop with rubber (*Hevea brasiliensis).* The chapter starts with a brief introduction recapping crops that have been intercropped with rubber and the benefits of doing so. Plot selection and establishment, monitoring, data collection and analysis are detailed in the methods and materials section. The results section reports findings on the effect of *T. daniellii* on cup lump rubber production at plot level which is then scaled up to a practical working level. Discussion, conclusions and recommendations follow in sections four, five and six respectively.

5.1 INTRODUCTION

5.1.1 The effect of intercropping on rubber production

Section 4.1.2 detailed crops that have been intercropped with rubber to aid in the diversification of rubber smallholder incomes, making them less susceptible to fluctuations in rubber prices. Many authors suggest that the growth of intercrops during the immature rubber phase has a neutral or positive effect on rubber yield, in part due to husbandry of the cover crop, leading to better management of the rubber (Watson, 1989; Laosuwan, 1996; Rodrigo *et al.,* 1997; Punnoose *et al.,* 2000; Rodrigo *et al.,* 2005). Rodrigo *et al.,* (1997; 2005) suggested that shading immature rubber would reduce or alleviate light-induced depression of photosynthesis leading to an improvement of whole plant rubber photosynthesis. There are fewer crops intercropped with mature rubber compared with immature rubber but they include: cardamon, coffee, cocoa and some medicinal plants (RRISL, 1996; Punnoose *et al.,* 2000; Williams, 2000; Rodrigo, 2007).

Laosuwan (1996) stated that *Manihot esculenta* (cassava) was soil exhausting and should not be intercropped with rubber, supporting assertions made by the Rubber

Research Institute of Malaya (1972). Edgar (1958) suggested that *Gleichenia linearis* (Burm. f.) C. B. Clarke, grasses, (especially *Axonopus* spp. P. Beauv., and *Ischaemum muticum* L.) were highly competitive with rubber. Punnoose *et al.*, (2000) stated that intercrops should not be allowed to dominate the rubber intercrop association, and should be planted sufficiently away from the rubber to minimise competition. Laosuwan (1996) supports this stating that apart from the advantage in replacing weeds, intercrops should improve the growth of rubber or at least have no retarding effect on it. However, the benefit to smallholders of intercropping at the expense of productivity in the major crop (rubber) is a balance that they have to make in terms of reducing their reliance on one commodity thus becoming more risk averse (Ellis 1993), thus less susceptible to shocks.

To assess the impact of an intercrop on rubber one has to determine the effect of the intercrop on the growth or the yield of cup lump rubber or latex. If there is a decrease in rubber tree growth or production whether this decrease is outweighed by the benefits of the intercrop, is an important consideration – especially where smallholder farmers are concerned.

Impact has been variously estimated by calculating land equivalent ratios (LER), relative yields (RY), or crop performance ratios (CPR) and using either total production, total economic value, or biologically measurable factors such as dry matter and leaf area index (LAI) (Reddy and Willey, 1981; Willey, 1985; Azam-Ali *et al.,* 1990), and specifically for rubber by (Rodrigo *et al.,* 1997; Rajasekharan and Veeraputhran, 2002; Herath and Takeya, 2003; and Rodrigo *et al.,* 2005). Other factors also have to be considered when assessing the impact of intercrops, including economic, sociological and agronomic factors, the better use of available resources, and reduction in damage caused by pests (Vandermeer 1989). Edgar (1958) describes the reduction of soil erosion in mature rubber stands, due to increased direct rainfall following the formation of canopy gaps, by having a cover crop present under mature rubber.

The Rubber Research Institute of Sri Lanka suggested that intercrops should: not be highly competitive with the main crop; be suitable for the climatic and soil

considerations of the area; be non labour intensive; suitable for rain fed cultivation; free from diseases that would affect rubber and be readily marketable (RRISL, 1996). Additionally Punnoose *et al.,* (2000) suggested that intercrops be separately and adequately manured and that the space for intercrop should not be completely dug up with minor soil disturbance during establishment.

5.1.2 AIMS OF THE CURRENT STUDY

The following study therefore aims to assess intercropping of rubber with *T. daniellii* with the following objectives:

- To determine the relative yield of rubber intercropped with *T. daniellii* compared to sole rubber crop production, and
- To determine if source of germplasm of *T. daniellii* has an effect on the production of cup lump rubber.

5.2 METHODS AND MATERIALS

This section will deal with the establishment of the rubber trial at the Missellele Experimental Site: information follows on trial design, source of material used, and data collection and analysis.

5.2.1 Experimental site selection.

The rubber trial site was situated at the Missellele Experimental site (Figure 4.2) to the west of the main provenance blocks – growth trials (Figure 4.4). The entire site for the rubber trial, 4.8 ha, was surveyed and plotted in order that contiguous sets of eight consecutive productive trees could be determined. Where this occurred, a plot was designated. From the site a total of 26 potential plots were designated of which 20 were sampled at random (Figure 5.1).

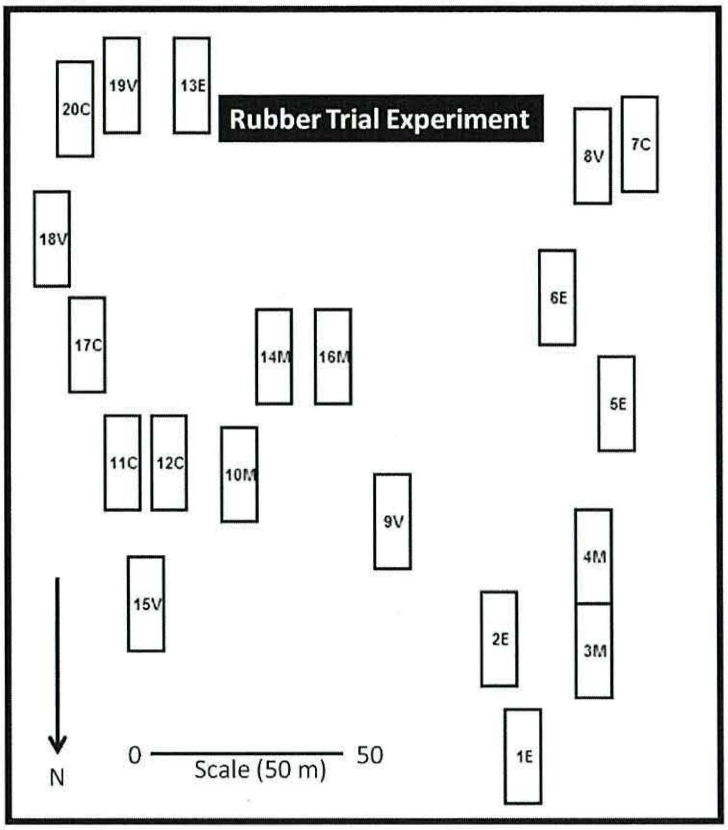

Figure 5.1 Experimental layout – rubber trials Missellele experimental site.

Letters correspond to the origin of material. E – Etome village,South West Province material, Cameroon; M – Missellele Rubber estate, Cameroon; V – Volta Region, Ghana; C – Control plots. Numbers indicate plot number used to distinguish plots. Each plot was 21.6 m x 10 m.

5.2.2 Experimental Design

The 20 plots were randomly assigned to one of four treatments, such that there were five replicates per treatment (Figure 5.1). The treatments were based on the origin of the planted material: SWP material from Etome, South West Province, Cameroon; VR material from Gbledi Gbogame, Volta Region, Ghana. VR material was selected as it was apparent that VR material had not established well at the provenance trial: in order to try and produce enough fruit for a future thaumatin analysis, new material was collected to augment already planted material, on a visit to Ghana in August 2006. On the basis that SWP material had established well in the provenance growth trial and that it was locally available, it was planted in this trial. Missellele material (MI) came from a local source (Missellele Rubber Estate: 30 year old PB 5/51, 800 m to the West of the experimental sites) discovered by one of the site personnel during weeding operations conducted for CDC. This was selected in order to reduce

costs and to see if germplasm adapted to rubber clone PB 5/51 would grow well with PR107 clonal rubber.

5.2.3 Preparation and establishment of experimental plots.

5.2.3.1 Plot preparation and maintenance

The preparation of each plot, staking of planting holes, planting method, maintenance, and establishment of quadrats to monitor the growth of *T. daniellii* was as described in Chapter 4 (Sections: 4.23, 4.26, 4.27) with the exception that plots were not watered as they had been planted during the rainy season and monitoring of the sites was conducted once per month following experience from the provenance growth trials. The site was prepared prior to the collection of *T. daniellii* rhizomes in August 2006.

5.2.3.2 Plot design

Plots consisted of eight productive rubber trees, one at each end acting as a buffer tree. Plots were planted with 200 rhizome sections at 1 x 1 m spacing. The first two rows of rhizomes being planted 0.5m from the rubber trees, as per the method in chapter 4, with the remaining rhizomes forming five lines of plantings either side of the central rubber trees: each plot covered approximately 0.0216 hectares (21.6 x 10m)

Each tree was painted with the treatment code and a number to assist tappers in distinguishing experimental plots so that they would not collect latex from the trees after tapping.

5.2.3.3 Collection of rhizomes and sectioning

VR rhizomes were collected on 17th August 2006 and were scheduled to arrive in Cameroon for planting on the 18th August 2006, however, due to transport disruption the rhizomes and researcher were stranded in Lagos, Nigeria for three days, before transit to Doula, Cameroon. Rhizomes were, however, placed in the shade and watered during the delay.

The rhizomes affected were finally planted on the 22nd August 2006, five days after harvesting. MI material was collected on the 23rd August 2006 and planted the next day, and SWP material was collected on the 29th August 2006 being planted the following day.

5.2.4 Tapping and collection of cup lump rubber

5.2.4.1 Tapping at Missellele Rubber Estate

Tapping at Missellele Rubber Estate was carried out every four days with individual tappers having their own sets of trees (400-600 in total) to tap. After tapping tappers would collect the latex, and the remaining latex that was still exuding from the tapping cut was collected in the tapping cup and left to coagulate to form cup lump rubber (CLR). Together with site overseers, CDC personnel responsible for an area of the rubber plantation, tappers were informed of the experiment and requested to leave latex in cups after tapping for coagulation to occur. At each subsequent tapping they were asked to place the cup lumps into plastic bags, attached to the rubber trees. This was found to be unsuitable by tappers and cup lumps were instead pierced onto the extruding wire following tappers' suggestions. Tapping was conducted throughout the year, except for the period of over-wintering when all tapping was suspended. This period varied year to year, but generally occurred between February and March, and lasted for 4-5 weeks. Tapping was postponed when hampered by severe rain.

5.2.4.2 Collection and monitoring of CLR

CLR from the six central trees of a plot was collected after every four tappings, henceforth termed a tapping episode (TE), on the day of the fifth tapping. Trays of bamboo tied with string were used to gather CLR (Plate 5.1). Each tree had a small wooden marker painted with its plot and tree number in order to identify production from individual trees. Collected cup lumps were taken to the site shed and weighed using an Adam Equipment ACB plus 1000 laboratory scale (Adam Equipment Co. Ltd. Milton Keynes, U.K.) (Plate 5.1). Once weighed, cup lumps were bagged in sacks according to the tapper's task, taken to the site rubber collection point and given to overseers at the site.

Plate 5.1 Collected cup lumps in bamboo trays waiting to be measured and the measurement of cup lump rubber in the site shed.

5.2.4.3 Problems of CLR data collection

Data collection efficiency was reduced by certain factors: tappers forgetfulness in leaving latex to coagulate (addressed by painting of trees in bold red paint to remind tappers not to collect latex); tapper absence requiring other tappers to complete the task and invariably leading to the collection of latex; theft of cup lumps; severe weather preventing tapping; staff changes with new tapper inexperience; illness of rubber trial personnel prolonging the time needed to collect and measure CLR.

Another issue was that the rubber trial site straddled many tasks such that at any one time there were between 3-5 tappers tapping in the trial. Eventually and with collaboration of site overseers and the Estate manager, a relatively consistent group of tappers was maintained at the site and where possible stable data collection achieved.

The production of CLR from plots also was affected by experience of tappers: at times latex would be seen to be flowing from the tapping cut, spilling down the side of the tree (Plate 5.2). Spillage of latex from cups and or periods of intense rain would cause a loss of latex.

Plate 5.2 Loss of latex from a poor tapping cut.

5.2.5 Data analysis

5.2.5.1 T. daniellii plant characteristics

Plant characteristics, petiole height (PH), lamina length (LL) and lamina width (LW) were recorded using the methods in Chapter 4. (4.2.8). Due to budgetary constraints total numbers of petioles emerging and total numbers of lamina per plot were not recorded and routine collection of all quadrat data terminated ten months after planting. Measurement of growth of *T. daniellii* was only conducted for the first 6 months of the rubber trial, from 8th December 2006 till 22nd June 2007.

5.2.5.2 Calculation of total mean cup lump production per plot (CLP kg plot $^{-1}$).

The sum of the CLR produced for one tree after each TE was calculated. CLR totals kg tree^{-1} TE^{-1}were added together and divided by the total number of trees per plot to obtain a CLP kg plot^{-1}TE^{-1}. During the experiment two plots lost production from a single tree: the first plot lost a tree due to wind damage, the second because the tree was determined by the overseers to have become a brown-bast tree, that is, one that had lost its productivity due to disease or poor tapping. In these two cases the total

mean CLP kg $plot^{-1}TE^{-1}$ was determined as the mean production from the five remaining productive trees.

5.2.5.3 Statistical analysis of treatment differences in CLP

An analysis of variance (ANOVA) was conducted using total mean CLP kg $plot^{-1}TE^{-1}$ from the data. Subsequent analysis involved independent samples t-test of mean total CLP kg $plot^{-1}TE^{-1}$ between control plots and treatment plots to test the effect of *T. daniellii* on rubber production. Values obtained for mean total CLP kg $plot^{-1}TE^{-1}$ were scaled up to provide useable values - CLP kg $ha^{-1}year^{-1}$. Relative rubber yield was determined as the total mean CLP from intercropped plots divided by the total mean CLP from sole cropped rubber plots (control plots) (Willey, 1985). Where values were significant an effect size was calculated as per Appendix I.

5.3 RESULTS

This section details the production of cup lump rubber from experimental plots by treatment and goes on to describe trade-offs between sole crop rubber production and rubber production intercropped with *T. daniellii*.

5.3.1 Cup lump rubber production at Missellele.

The overall plot-mean cup lump rubber production (CLP) mean was 0.364 kg TE^{-1} within a range of 0.288 kg to 0.460kg TE^{-1}. The mean total CLP $treatment^{-1}$ was highly variable between and within individual plots (Figure 5.2).

5.3.2 Effects of treatment on CLP

Production of CLP for each provenance over time is presented in Figure 5.2. Large error bars (CI - 95%) indicate the range of values of CLP from treatment replicate plots, and the random and erratic nature of the interweaving plotted treatment lines show that there was variation at different tapping periods in the total mean CLP between and within treatments. (Figure 5.2).

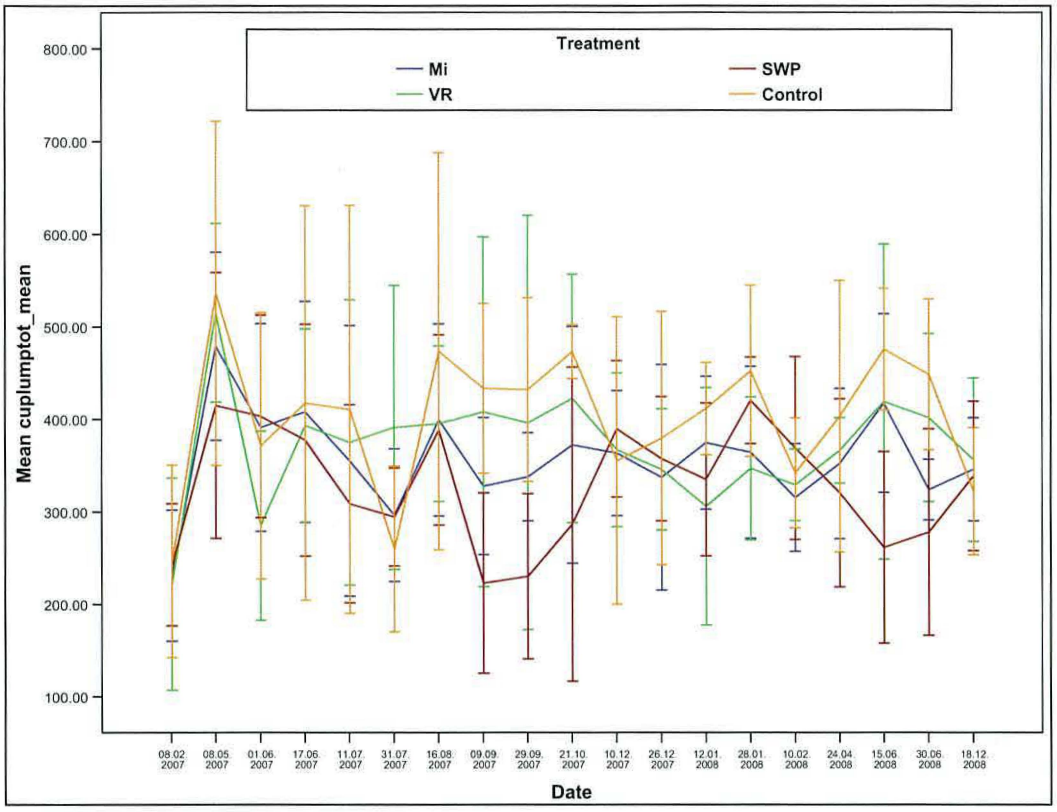

Figure 5. 2 Trend over time of mean cup-lump production per treatment with 95% CI error bars. Note: Date axis is not a linear scale but represent when tapping occurred.

CLP over the 19 episodes of tapping was significantly different between treatments. Control plots produced significantly more rubber than either SWP or MI plots (F, 8.521, $p<0.000$), but not significantly more than plots planted with VR material (Figure 5.3). VR material produced significantly more rubber than SWP plots, but not MI plots.

There is a small to medium effect size in the variation in total mean CLP between treatments due to intercropping with *T. daniellii* ($\omega^2 = 0.071$).

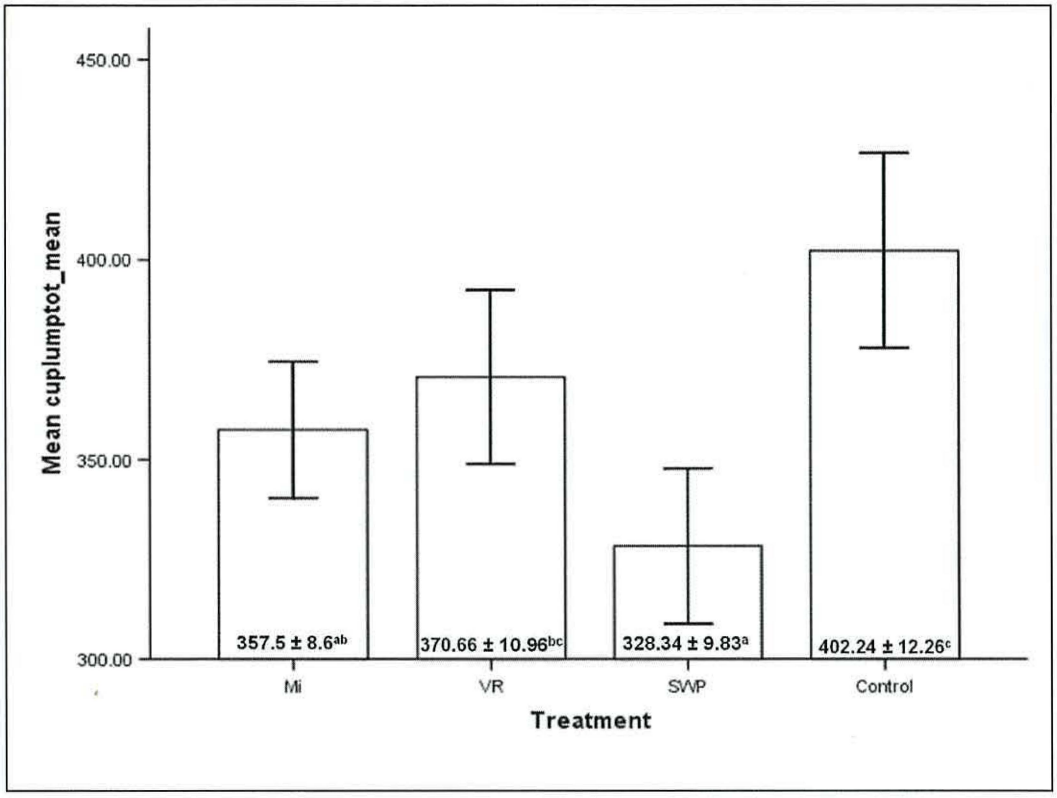

Figure 5.3 Total mean cup lump production treatment^{-1} from rubber trial, Missellele experimental site.

Treatment codes: Mi = Missellele, Cameroon; VR = Volta Region, Ghana; SWP = South West Province, Cameroon. Values on bars indicates means±SEM: superscript letters indicate significant differences at $p < 0.000$. n = 95 for each treatment.

5.3.3 Effect of *T. daniellii* on CLP compared with control plots.

To determine the gross variability between CLP between control plots and *T. daniellii* plots, a one way independent samples t-test was conducted. Control plots produced significantly more rubber 402.24 ± 12.26 g (n= 95) than *T. daniellii* plots 352.17 ± 5.76 (n = 285) (t, -3.696, $p < 0.000$, *df* 378), where there was a relative rubber yield of 0.876. In absolute terms, the actual difference between total mean CLP between control and *T. daniellii* plots was 50.07 g plot^{-1}TE^{-1}. This value equates to 12.51g plot^{-1}tapping $^{-1}$ or 2.085 g tree^{-1} tapping^{-1}. The magnitude of the difference between total CLP means between plots planted with *T. daniellii* and control plots was evaluated as having a small to medium effect ($\eta^2 = 0.035$).

5.3.4 Growth of *T. daniellii* in CLP trials.

Data regarding petiole height, lamina length and lamina width were collected and analysed using the same method as in Chapter 4, Section 4.2.8 Growth after 10 months suggested that the mean height from SWP plots was significantly higher than in the other two treatments ($F = 9.502$, $p = 0.003$) Table 5.2. Lamina length and width did not vary significantly ($p = 0.249$ and 0.331 respectively) between treatments. The means of the plant characteristics are broadly consistent with growth data from the experiment in Chapter 4.

Table 5.1 Mean plant characteristics of planted material in June 2009 (10 months after planting).

Mean plant characteristic	Treatment	N	Mean± SE
Mean height	Missellele	5	23.85 ± 0.63^{a}
	Volta Region	5	22.55 ± 0.49^{a}
	South West Province	5	27.74 ± 1.01^{b}
	Total	15	24.71 ± 0.48
Mean lamina length	Missellele	5	18.64 ± 2.75
	Volta Region	5	20.42 ± 0.76
	South West Province	5	20.33 ± 1.21
	Total	15	19.8 ± 1.86
Mean lamina width	Missellele	5	11.98 ± 2.12
	Volta Region	5	14.91 ± 5.55
	South West Province	5	11.92 ± 0.87
	Total	15	12.93 ± 3.52

Letters in italics indicate significant differences in the mean at p =0.003.

Although plant measurement was discontinued in June 2007, by June 2009 (2 years 9 months after planting) there were pronounced differences in the growth of *T. daniellii* between plots, with locally sourced material, from Missellele (MI), growing taller and more luxuriously than other planted material (Plate 5.3). Two plots did exceptionally well, MI 3 and 4, and merit further discussion (Plate 5.3, top two figures). There was no significant difference in total mean CLP $plot^{-1}$ between these two plots and control plots: 381.19 g ± 11.73 ($n = 38$) compared with 402.24 g ± 12.26 ($n = 95$) respectively, ($t = -1.012$, $p = 0.313$, *df*, 131). However, when the total mean CLP of MI plots is compared with control plots there is a significant difference ($t = -2.987$, $p = 0.003$, *df* 188) $\eta^2 = 0.046$, a small to moderate effect.

Plate 5.3 Variable growth of *Thaumatococcus daniellii* around rubber trees in rubber trials compared with control plots (Missellele Experimental site, 1st June 2009).

Missellele planted material plots 3 (top left) and 4 (top right); Volta Region plot number 19 (centre left); South West Province plot number 6 (centre right); Control plots 11 (bottom right) and plot 12 (bottom left).

5.3.5. Derived income from sole rubber production vs intercropped rubber

Scaled up data expressing per hectare yields shows a net decrease in total mean CPL rubber production of 89.63 kg $ha^{-1}yr^{-1}$ from intercropped plots relative to control plots (Table 5.2). This decrease equates to XFA 26 812 ha^{-1} at a rubber price of XFA 300 kg^{-1}. (GBP 1 ~ XFA 797.44, 25th June 2010: www.fx-ware.com/forex-currency). The value further decreases to XFA 13 406 as the price of cup lump rubber decreases

to XFA 150 kg^{-1} (Table 5.3). The prices for CLR were current as of June 2009, when discussions and farm visits were held with small holders.

Table 5.2 Total mean cup lump production (CPL kg) from the Missellele Rubber Estate rubber trials at different scales: per plot, per year and per hectare.

Treatment	Total mean cup lump production ± SE (CPL kg^{-1})		
	plot four tappings^{-1}	plot yr^{-1}	$ha^{-1}yr^{-1}$
Missellele $_{(95)}$	0.36±0.009	7.51±0.18	638.14±15.35
Volta Region $_{(95)}$	0.37±0.011	7.78±0.23	661.63±19.56
South West Province $_{(95)}$	0.33±0.010	6.90±0.21	586.09±17.55
$\bar{x}T.d_{(285)}$	0.35±0.005	7.40± 0.11	628.62±9.64
Control $_{(95)}$	0.4±0.0120	8.45±0.26	718.25±21.88
Total $_{(380)}$	0.36±0.005	7.66±0.11	650.95±9.64

$\bar{x}$ *T.d* = Total of all *T. daniellii* treatments. numbers in brackets indicate *(n)*.

Table 5.3 Annual income from total mean CPL kg-1 ha-1 yr-1 with associated incomes and net change in income through intercropping at two different CLR prices.

Treatment: germplasm material and control	Total mean cup lump production kg $ha^{-1}yr^{-1}$	Mean annual income based on total mean cup lump production kg $ha^{-1}yr^{-1}$at a cup lump rubber price of XFA 300 kg^{-1}	Net change in mean annual income per treatment, at a rubber price of XFA 300 kg^{-1}	Mean annual income based on total mean cup lump production kg $ha^{-1}yr^{-1}$at a cup lump rubber price of XFA 150 kg^{-1}	Net change in mean annual income per treatment, at a rubber price of XFA 150 kg^{-1}
MI $_{(95)}$	638.14±15.35	191441	-23958	95720	-11979
VR $_{(95)}$	661.63±19.56	198488	-16911	99244	-8455
SWP $_{(95)}$	586.09±17.55	175826	-39573	87913	-19786
$\bar{x}T.d_{(285)}$	628.62±9.64	188587	-26812	94293	-13406
Control $_{(95)}$	718.25±21.88	215399	0	107699	0
Total $_{(380)}$	650.95±9.64	195286	-20113	97643	-10056

Numbers in brackets indicate *(n)*. GBP 1 =XFA797.44 – 25th June 2010.

5.3.6 Small holder production costs

Discussions with small holders determined that the price paid to rubber tappers was XFA 100 kg^{-1} or XFA 2000 per 100 trees if not using family capital. Other costs in the management of rubber small holdings included weeding costs, given at different rates: either XFA 15 000 per 0.5 hectare, or XFA 1000 per four hours. Weeding was conducted once or twice per year. Other additional costs included formic acid to

increase the coagulation of latex, especially during the rainy season when there is a need to coagulate rubber quickly and Etreyo, a stimulant to help latex flow, used especially after wintering.

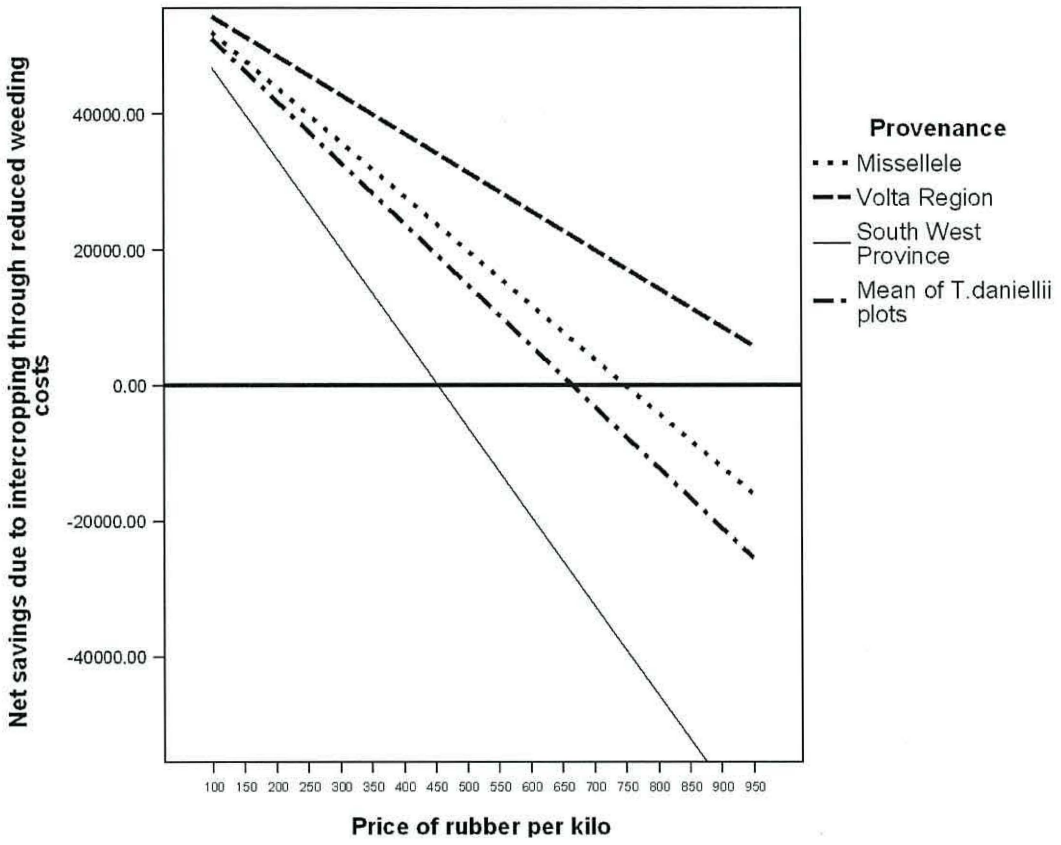

Figure 5.4. Potential net benefit from intercropping *Thaumatococcus daniellii* with rubber, due to reduced weeding per hectare, not including income or costs from fruit production, relative to non-intercropped rubber per hectare.

Values do not include the cost of production of rubber or income or costs associated with *T. daniellii* fruit production. Weeding effort assumed to be twice per year at 15 000 XFA per half hectare (60 000 XFA p.a.). Gross annual income from rubber per hectare per treatment, calculated as potential annual income from non-intercropped rubber per hectare minus potential annual income from rubber per hectare intercropped with *T. daniellii*.

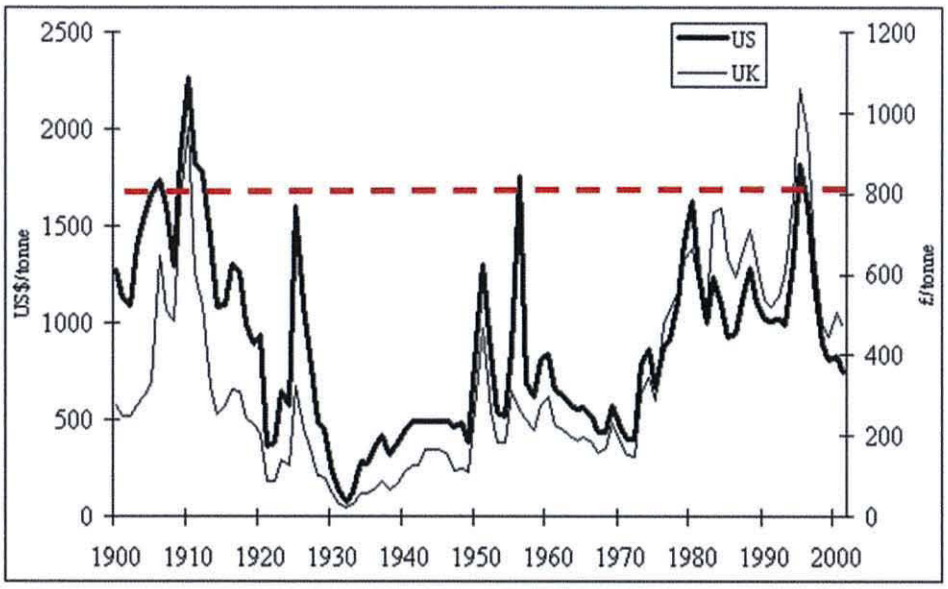

Figure 5. 5: Ribbed Smoked sheets (RSS1) prices in New York and London (ammended after Buddiman, 2003) and price of cup lump rubber XFA 650 kg^{-1}

The dashed red line indicates the price of unprocessed cup lump rubber XFA 650 kg^{-1}.

5.3.7 Price of *T. daniellii* fruit

The price paid to collectors of fruit in Ghana as of June 2009, was 45 pesewas kg^{-1} according to one trader who trades in the Western and Volta Regions of Ghana (Martin Laasen, pers. comm. 2009). Missellele growth trials showed that the fruit production varied from 5.61 kg ha^{-1} to 52.54 kg ha^{-1}. This would equate to, *cēterīs paribus*, income from fruit of between XFA 930 to 8721 $ha^{-1}harvest^{-1}$respectively. (GHS1 ~ XFA370.49 – 25th June 2010)

5.4 Discussion

5.4.1 Determining the impact of *T. daniellii* on productivity in rubber

T. daniellii has been shown to significantly affect the production of rubber ($t = -3.696$, $p < 0.000$, *df* 378), ($\eta^2 = 0.035$), a small to medium effect which can be interpreted as 3.5% of the variance in total mean CPL is explained by intercropping with *T. daniellii*. In practical terms the reduction in CPL of 86.93 kg $ha^{-1}yr^{-1}$ between control and intercropped plots equates to the loss of approximately 2.085 g CLR $tree^{-1}$ $tapping^{-1}$. A loss of 2.085 g of CLR $tree^{-1}$ $tapping^{-1}$ can be visualised and equated to a loss in latex due to poor quality tapping (Plate 5.2). There was a lot of variance in cup lump production between plots and between treatments during the course of

the study (Table 5.1; Figure 5.2). Whilst a significant proportion of this variance was due to intercropping with *T. daniellii* the high amount of variability puts into question the quality of the tapping and the lack of procedures to reduce loss during rainfall, such as a lack of covers for cups. The quality of the planting material for the rubber trees also has to be questioned with variability between plots with the same treatments varying by up to 48 g TE^{-1}. Due to the changes in tappers and not being at the site during each tapping, the effects of latex stimulation and the tapping procedures and quality cannot be addressed here.

Small holders, in Ghana, did not notice a decline in the cup lump rubber production at two demonstration plots established as part of this study with the co-operation of the Rubber Outgrowers Plantation Project (ROPP) managed by the Rubber Outgrower's Unit (ROU) of Ghana Rubber Esates Limited (GREL) (Robert Kofi Appia, pers. comm. 2009). Conversely, there have been studies that have suggested that there is no adverse effect of intercropping on the production of rubber (Chandrasekera, 1984; Laosuwan, 1996), with the latter suggesting that increases in growth of rubber were as a result of continuous activities performed by farmers in cultural practices, fertiliser application and weed control. In similar studies Rodrigo *et al.,* (2004) suggested an improvement in the performance of rubber with bananas (Yogarathnam, 1991; Rodrigo *et al.,* 1997); with sugarcane (Rodrigo *et al.,* 2000); with banana and pineapple (Jessy *et al.,* 1997); banana and seasonal crops (Keli *et al.,* 1997); and banana, capsicum and pineapple (Rosyid *et al.,* 1997). These studies focussed on immature rubber and the improvement in girth and analysis of dry matter content rather than the production of latex or cup lump rubber which was analysed in this study. Once the canopy of rubber develops, light interception is such that intercrops are removed and economically important crops cannot be grown (Laosuwan, 1996; Rodrigo *et al.,* 2004), unless the crops are shade tolerant (Laosuwan, 1996)(Nair, 2010).

Although *T. daniellii* competes with rubber, reducing cup lump production, its suitability as an intercrop still has to be evaluated: this is normally done by assessing the relative effects of intercropping versus the production from crops in monoculture (Willey, 1985). The land equivalent ratio (LER), crop performance ratio (CPR) and

relative yield ratio (RY) are used to assess the relative effects of intercropping versus the production from sole crops (Willey, 1985). For example, Rodrigo *et al.*, (1997) reported values of LER of 1.49 to 2.62 when banana was grown at different densities with rubber: these values exceeded unity for total crop dry matter yield and reflected a consistent advantage of intercropping. Calculating LER requires data from sole crop plantings and intercrop planting for both companion crops (Willey, 1985). In this study *T. daniellii* was only grown under rubber, not as a sole crop (*T. daniellii* has always been viewed as a companion crop and not one that would be grown as a sole crop) and without the use of fertilisers which is common with rubber intercrops (Watson, 1989; Rodrigo *et al.,* 1997; Punnoose *et al.,* 2000). The decision not to use fertilisers was taken following an assumption that the most vulnerable small holders would not be able or willing to invest in fertiliser for a companion crop.

Onwueme *et al.,* (1979) grew *T. daniellii* as a sole crop, in un-shaded plots where the mean mass of fruit produced was 23 kg $ha^{-1}yr^{-1}$. This is the only study to have done this. At the time of the current study fruit had not been produced in the rubber trials, but, if one were to assume similar production to that reported in the provenance trials (Chapter 4), a mean fruit production of 18.15 kg $ha^{-1}yr^{-1}$ could be expected. Using these values a LER could be calculated for *T. daniellii* intercropped with rubber with a resultant value of 1.65, well above unity. This could be interpreted as the combination of both crops yielding 65% more total output than both crops grown individually, or as an increase of 65% of the total land required to produce the benefits of intercropping if the crops were to be grown in monoculture (Willey, 1985).

However due to the fact that the earlier study was established in Nigeria with Nigerian *T. daniellii* germplasm (Onwueme *et al.,* 1979), whilst the current study was established under rubber with germplasm from three different sources and two different countries, the use of relative yield (RY) (Willey, 1985) may be more robust to determine the effect of *T. daniellii* on rubber production. A value of 0.88 RY suggests that *T. daniellii* exerted a competitive pressure on rubber resulting in a reduced cup lump yield of approximately 12.4 %. This RY value, below unity, suggests that although there is competition in the system, it is not such that *T.*

daniellii cannot grow and hence is out competed by rubber, with the result that both species can co-exist (Vandermeer 1989).

5.4.2 The cost of intercropping in terms of reduced rubber income

The concepts of LER, RY and CPR are theoretical constructs used by agronomists to determine the benefits and the disadvantages of growing crops together. For the smallholder, values in terms of economic gain or loss are more relevant as is the ability, through intercropping for example, to diversify income and increase resilience. Being reliant on one main crop leaves the farmer in a vulnerable situation to factors beyond their control: when the prices of commodities decrease, such as was seen during the course of fieldwork when the price of rubber plummeted and payments were delayed.

At a given price of rubber it may not be economically advantageous to the farmer to intercrop with *T. daniellii*. With increasing prices for CLR the advantage of intercropping reduces. This is because the loss of rubber production due to intercropping has potentially more impact on incomes when the price of rubber increases, as is shown in Table 5.4 where two rubber prices are compared, XFA 300 and XFA150 kg^{-1}. These were current at the time of the study[1]. The lower of the two prices was paid by intermediary traders who would offer immediate payment; the higher price was offered from the main buyer of cup lump rubber in the South West Province, Cameroon, the Cameroon Development Corporation, though it was not uncommon for smallholders to have to wait three or more months for payment.

The mean loss of income in terms of rubber production at the higher exchange rate was calculated to be XFA 26 812 (Table 5.4) reducing to half this value at the lower exchange rate. The only price available for *T. daniellii* fruit was the current Ghanaian market price for fruit, 45 pesewas kg^{-1}, (Martin Laasen, pers. comm. 2009). Though very crude and approximate, using a real price for fruit from an existing market has been done, simply, to provide an indication of the potential value of fruit

[1] The current price being paid to small holder farmers by CDC is XFA 886.11 kg^{-1} (April, 2010). The price of cup lump rubber has been increasing since December 2009, but it is not known for how long these high prices will continue (Odilous Mbuyeh, pers. comm. 2010).

produced through intercropping *T. daniellii* with rubber, assuming that smallholders producing fruit would be paid the same amount for fruit as collectors. It is clear that a market supply chain established in a new country, using a different paradigm for fruit supply, will experience different costs, economies of scale and hence lead to a change in the price of fruit, either decreasing or increasing. Therefore, a crude value for income from fruit produced based on the market price of Ghanaian fruit and with production data from the provenance trial would suggest a potential income of between 980 – 8721 XFA with a mean income of XFA 3012.

The loss of income from intercropping with *T. daniellii*, moderated by the potential income from fruit is therefore in the region of XFA 25832 to 18091 $ha^{-1}yr^{-1}$. This loss must be seen in a context where other monetary values not directly attributed to intercropping are taken into account.

Reducing money spent on weeding through intercropping may be of benefit to smallholders: assuming an annual weeding cost of 60 000 XFA, the price at which intercropping with *T. daniellii* becomes unattractive in terms of giving a negative net benefit, after the loss in income due to reduced productivity of rubber from intercropping, is when the price of rubber is more than 650 XFA $kilo^{-1}$ (Figure 5.4). It can be seen that the price of processed rubber has not been above 650XFA kg^{-1} (equivalent to ~ £850 $tonne^{-1}$) for the majority of the 20th Century, and thus the price paid at the factory for raw cup lump would be less than this value. Though a very crude model not taking into account external factors, it does provide a basis for future and more thorough economic sensitivity analyses. Any net income from fruit would shift the lines vertically upwards (Figure 5.4), increasing benefit to smallholders. As the price of cup lump rubber becomes cheaper it may be more advantageous to begin intercropping with *T. daniellii*.

Also, the fact that *T. daniellii* is at worst, a benign weed, in that does not snag, harm, cause injury to or impede tappers access to rubber trees must also be considered. Due to its versatility there are many ways to generate an income from *T. daniellii* (Abbiw 1990; Arowosoge and Popoola, 2006) thus increasing the potential for smallholders to diversify their income further. An added biological advantage of *T. daniellii* is the

fact that mature fruit can remain intact on the rachis for at least seven months providing an additional temporal resilience; in effect fruit does not have to be harvested immediately enabling farmers the potential to collect fruit during periods when they have excess available labour, for example during overwintering when tapping operations are suspended. Harvesting during overwintering, all other things being equal, would provide income when income is not being generated from rubber production.

5.4.3 Growth of *T. daniellii* in rubber trials

Early growth of *T. daniellii* mirrored growth seen in the main provenance trials, with SWP material being significantly taller ten months after planting. During the last visit to the rubber trials (June 2009) it was noticed that MI plots were generally growing better than other planted material (personal observation). Where *T. daniellii* grew particularly well, producing a dense and luxurious canopy, MI plots 3 and 4, (Plate 5.3) the total mean cup lump production was not significantly different from control plots. Is there a level of growth of *T. daniellii* beyond which it does not negatively affect the production of cup lump rubber?

5.4.4 The benefit of intercropping rubber to world rubber production

The volatility of the world rubber price and poor market prices together with increased urbanisation have led to a decline in rubber cultivation in the past (Rodrigo *et al.,* 2004). Rodrigo *et al.* (2004) warns that a short term decrease in the price of rubber should not allow the collapse of the (rubber) industry, the inference being that it is exactly these shocks that depress the price of rubber to a point where it is not economically viable for small holders to remain in rubber production (Buddiman, 2003).

The small holder sector produces most of the world's natural rubber production, accounting for between 75% and 80% of world production (Buddiman, 2003; Rodrigo *et al.,* 2005). Since 1995, the Rubber Outgrowers Plantation Project (ROPP) in Ghana, managed by the Rubber Outgrower's Unit (ROU) of Ghana Rubber Esates Limited (GREL) , has planted over 12 000 hectares of small holder rubber (GREL –

ROPP website) encouraging farmers to remain in the sector with technical assistance and access to good quality rubber seedlings.

Intercropping, therefore, plays an important role, especially in the juvenile phase, providing additional income to small holders and leading to improved husbandry of rubber trees (Rodrigo *et al.,* 2004) thus keeping small holders involved in rubber production rather than re-planting other crops (A. F. S. Buddiman, pers. comm. 2004).

5.5 Conclusion

Knowledge of potential incomes, losses, and supplementary benefits, are required by farmers in order to make informed decisions whether to establish *T. daniellii* under rubber together with establishment costs, logistical considerations such as access to germplasm, and information regarding access to a market for the fruit of *T. daniellii*.

This study has shown that *T. daniellii* can grow as an intercrop with rubber; competing with it and reducing the productivity of cup lump rubber by approximately 12 %. However, the relatively high estimated value of LER 1.65 suggests that there is an intercropping advantage to the *T. daniellii* – rubber system. For vulnerable small holder farmers it is likely that the advantages of reducing income spent on weeding activities, even when the price of rubber is high, plus an additional, if small, income from the sale of *T. daniellii* fruit at a time to suit the farmer, may make smallholders increasingly resilient to shocks in rubber prices, especially when the price of rubber plummets as was seen during the course of the study. With an established market chain for *T. daniellii* fruit in Ghana, it is envisaged that this intercropping system would be first established in Ghana and used as a model for Cameroonian smallholders.

5.6 Recommendations

It is recommended that the experimental sites continue to be maintained in order to gain a better long term understanding of the *T. daniellii*-rubber system and its affect on fruit and cup lump production. This should be augmented with monoculture plots

established of *T. daniellii* to obtain more relevant results for the calculation of LER and hence obtain a better, more precise and accurate value for the potential advantage of the intercropping system. Smallholder trials using rhizomal material from the experimental site should be established to assess the impact of *T. daniellii* on a range of rubber clones of different ages and planting densities. It would be sensible to investigate alternative strategies of planting rhizomal material, including alternate inter-row planting and different planting density. Alternative *T. daniellii* germplasm can be trialled under rubber to determine if germplasm is a key driver of CLP. An in-depth study of small holder farmer resilience to shocks in rubber prices and delay in payment should be conducted. Most importantly however is now that the preliminary study has been conducted and shows that *T. daniellii* can grow with rubber, and that the system, though competitive does indicate a positive advantage is possible, a thorough cost-benefit analysis of intercropping with rubber should be conducted in West Central Africa. Finally, an appraisal of the current market chain for *T. daniellii* fruit is required to see where and how the chain can be maximised for the benefit of smallholders.

CHAPTER 6

GROWTH AND MORPHOLOGICAL RESPONSES OF *THAUMATOCOCCUS DANIELLII* TO SHADE

This chapter continues and complements the previous two chapters on the theme of intercropping rubber (*Hevea brasiliensis*) with *Thaumatococcus daniellii* by investigating its response to varying shade cast by the rubber canopy. The first section puts into context the light environment under which *T. daniellii* exists outside the context of intercropping and discusses briefly the differences between shade avoiding and shade tolerant plants in respect of physiological and morphological responses to different light environments, and the aims and objectives of the chapter. The second section details the location of the experiment, how it was set up and describes how equipment was used to measure the light environment and the data were analysed. The results section follows with a discussion and conclusions to end the chapter.

6.1 INTRODUCTION

6.1.1 The natural light environment for *T. daniellii*

The habitat of *T. daniellii* was very succinctly described in 1855 by the army surgeon after whom the plant is named, as

> *"[T. daniellii] ...evidently delighted in humid, shady and marshy localities beneath the foliage of trees, or sheltered coverts of brushwood"* (Daniell, 1855).

Many subsequent authors have supported the notion that *T. daniellii* favours shade (Inglett and May, 1968; Adesina, 1994) in forests (Letouzey, 1986), both virgin (Raponda-Walker and Sillans 1961) and secondary (Enti, 1975; Nicol, 1976; Isawumi, 1981; Burkhill, 1985).

Enti (1975) and Isawumi (1981) both specified that it preferred heavy shade while Nicol (1976) reported its growth under a wide variety of crops including cassava,

coffee, banana, plantains, cocoa, rubber and oil palm. However, open settings such as old clearings, abandoned farms, waste areas, secondary scrub, canopy gaps in closed forest, and roadsides have also been recorded as sites for its growth (Enti, 1975; Nicol, 1976; Burkhill, 1985; Waliszewski *et al.,* 2005). Letouzey describes Marantaceae herbs as growing in rain forest, but also in abandoned cultivated fields and open forest undergrowth (Letouzey, 1986).

Hawthorne (1996) defined *T. daniellii* and *Megaphrynium macrostachyum* as cryptic pioneers. A cryptic pioneer being defined as a plant whose pioneer nature is easily overlooked because it is typically 'hidden away' in the understorey, where it persists after germinating and establishing outside of the 'twilight zone', a relaxed, un-thicketed, un-strangled understorey within a mature, multilayered structure (Hawthorne, 1996).

Waliszewski *et al.* (2005) reported local knowledge that *T. daniellii* had a wide margin of light tolerance, but that extremes of light and shade were detrimental to its growth, with constant direct light having negative effects on growth, through drying of the soil.

There is little research into the effect of light on *T. daniellii.* Yeboah *et al.* (2003) conducted a study on the growth and establishment of *T. daniellii* under artificial shade in a rubber plantation, finding that juvenile growth, up to 10 weeks after planting, under very dense shade, was significantly less ($p \leq 0.05$) than growth in open conditions and under shade provided by natural rubber. Brncic (2002) studied the response of four Marantaceae herbs, including *M. macrostachyum* but not *T. daniellii,* to different light environments produced in different sized gaps in the forest understorey in Ndakan, Central African Republic (CAR). She found that *M. macrostachyum* was more competitive than the three other species due to its greater maximum height and ability to inhibit tree regeneration, but that unlike the other three species, which were defined as light-flexible species taking advantages of gaps in the canopy and accelerating their reproduction when required, especially *Ataenidia conferta* (Benth.) Milne-Redh., *M. macrostachyum* was likely to invest more in

rhizomal spread as patch formation in via seed germination was due to large-scale disturbance in the canopy (Brncic, 2002)

6.1.2 Effect of light quality and quantity on plants

Light plays a major role in regulating survival and growth in tropical forests (Capers and Chazdon, 2004), with the amount of photosynthetically active radiation (PAR) affecting the growth and survival of tree seedlings (Beaudet and Messier, 2002). PAR, measured in μmols $m^{-2}s^{-1}$, is the photosynthetic photon flux density (PPFD), essentially light energy, measured in μmols $m^{-2}s^{-1}$ between the wavelengths of 400-700nm. There is a close linkage between the amount of radiation received by a crop and its growth (Bonhomme, 2000) with light quantity affecting plants directly by regulating productivity, while light quality regulates a number of physiological processes (Capers and Chazdon, 2004).

Light quality is recognised by three types of photoreceptors (Franklin and Whitelam, 2005): 1) the red (R) and far-red (FR) light-absorbing phytochromes (Smith, 1986; Smith, 2000), Pr and Pfr respectively; 2) blue/UV-A light absorbing cryptochromes (Senger and Schmidt, 1986; Ballaré, 1999; Franklin and Whitelam, 2005); 3) phototropins (Franklin, 2008), including flavoproteins and carotenoids (Senger and Schmidt, 1986).

These all play a role in the mechanism of shade avoidance by sun loving plants and understorey plants that are usually shaded. The ratio of red to far-red light (R:FR), that between 655 and 665 nm and 725 and 735 nm respectively (Franklin and Whitelam, 2005), affects the relative levels of Pr and Pfr and this, autonomously and with other photoreceptors triggers the shade avoidance syndrome (Smith and Whitelam, 1997). This is a group of strategies that is used to increase the ability of plants to obtain access to light, including: rapid elongation of stem and leaves, upward re-orientation of leaves (termed hyponasty) to maximise light harvesting and reduce over capping by neighbouring plants (Capers and Chazdon, 2004); reduced leaf chlorophyll content, apical dominance and, in situations where low R:FR continues, accelerated flowering leading to faster seed set and an increased chance of reproductive success and escape from shaded areas (Donohue *et al.,* 2001; Halliday

et al., 2003; Franklin, 2008). As a consequence of earlier flowering and fruit set, there is a reduction in biomass production, notably leaf production (Keiller and Smith, 1989).

Shade tolerant plants tend to have thinner leaves, higher chlorophyll content and lens-shaped epidermal cells to focus light within mesophyll tissue, together with an increased level of chlorophyll b over chlorophyll a (Boardman, 1977). Shade leaves of *Morus bombycis* Koidzumi exhibit higher net photosynthetic rates at low irradiance levels than sun leaves of the same species, and vice versa (Tateno and Taneda, 2007).

6.1.3 Measurement of light

There are two main methods to measure understorey light conditions, the first is hemispherical photography (Anderson, 1964; Chazdon and Field, 1987; Rich *et al.,* 1993; Capers and Chazdon, 2004) with the subsequent analysis of gap fraction with algorithms to ascertain various characteristics of the canopy (Rich *et al.,* 1999), and the second is the simultaneous comparison of understorey and open-site light with quantum sensors (Messier *et al.,* 1989; Montgomery and Chazdon, 2001; Montgomery and Chazdon, 2002; Capers and Chazdon, 2004).

6.1.4 Aims and objectives

The research reported in this chapter aims to assess the effect of pruning rubber on the understorey light environment and the response of *T. daniellii* to different light environments under rubber, in terms of both quality and quantity of light. Hemishperical photographs and direct measurements of PAR and R:FR with light sensors are compared.

6.2 MATERIALS AND METHODS

This section deals with the establishment of the experimental light plots, the measurement of *T. daniellii* within the plots and the measurement of the light environment in terms of the red to far-red ratio (R:FR), photosynthetically active radiation (PAR) and hemispherical photographs.

6.2.1 Experimental site location

Following consultation with the Cameroon Development Corporation (CDC), the Missellele estate manager and the General Rubber Manager (GRM) on the number of trees that could be included in this experiment and the degree to which each could be pruned, in conjunction with CDC personnel, two areas were selected that were uniformly similar at the Missellele experimental site (Figure 4.2) for the establishment of the light experiment blocks. Two blocks were situated either side of the provenance trial blocks (Section 4.2.3, Figure 4.4), 10 rows (70 m) equidistant from the provenance block trials, in order that any treatments imposed on plots would not affect the provenance trials.

6.2.2 Experimental design

Each block (63 x 67.5 m ~ 0.425 ha) consisted of nine plots assigned to one of three pruning treatments using a latin square design. Each plot was bounded on each longitudinal side by three rubber trees resulting in plots measuring 8.1 m x 7 m. Plots were separated from each other by a gap of three rubber rows (21 m) in an East-West plane, and by eight rubber trees, at 2.7 m, orthogonal to this, such that each plot was approximately 21 m distant from any other plot in either the E-W or N-S plane: this was done to minimise the effect of interaction between treatments.

6.2.3 Preparation, establishment and maintenance of experimental blocks

Each plot was cleared, prepared, staked and planted with 30 rhizome sections of *T. daniellii* at 1 x 1 m spacing as per earlier methods (Section 4.2.3, 4.2.6 and 5.2.3.1). All rhizomes came from the Etome village collection site, South West Province, Cameroon (Section 2.2). These were collected and sectioned according to methods set out in Section 4.2.5, on 2nd March 2006, and were planted the next day. Sites were maintained and managed as per provenance trial plots (Section 4.2.9)

6.2.3.1 Establishment of quadrats

Five quadrats per plot were established to monitor the growth of *T. daniellii*. These were established as per methods described in Section 4.2.7.

6.2.4 Monitoring and data collection of *T. daniellii* in plots and quadrats

The monitoring mirrored the frequency of monitoring and data collection conducted in the provenance trial (Chapter 4), however the days on which monitoring was conducted was changed, and conducted a day later in order to accomplish data collection from the same experiment in the same day. Six months after establishment it was decided to measure the plots once per month, with data collection on consecutive days for each block as it was not possible to complete all measurements for both blocks in one day. Fruit produced were counted in June 2009.

6.2.5 Pruning treatments

Treatments were allocated according to a Latin square design with three pruning treatments, 50 %, 25 % and a 0% (control) pruning, replicated three times per block. Pruning was done on the 10th March 2006 with a technical advisor from the CDC GRM's office. Pruning treatments entailed a reduction in the number of main branches for each tree bounding a plot by the appropriate amount, either 25% or 50% or no pruning. The number of branches on each tree was counted, assessed and then branches cut following a discussion with the technical advisor, by hand, using machetes (Plate 6.1). Trees were painted with plot identifier codes.

Plate 6.1 Before and after picture showing the effect of pruning rubber trees by 25%. Missellele experimental site, 10th March 2006.

6.2.6 Measurement and assessment of the light environment

The light environment and canopy openness were assessed using a range of methods. The ratio of R: FR radiation was measured since this was expected to be related to morphological responses in plants. Absolute values of photosynthetically active radiation (PAR) were measured as this would be expected to affect understorey productivity. Photographic images of canopy coverage provide a tool for objectively describing the understorey light environment (Anderson, 1964; Chazdon and Fetcher, 1984; Brandeis *et al.,* 2001; Beaudet and Messier, 2002) and so canopy openness was also assessed through the use of hemispherical photography.

The measurement of PAR and hemispherical photography was done during visits to Cameroon in January 2007, October 2007 and March 2008. Measurement of PAR and photography were completed on separate days as it was not possible to do both on the same day. Salimen *et al.*, (1983) noted that it was almost impossible to take photographs in the same position as other measurements of light. In order to reduce errors associated with variation in position of instruments, photographs and readings were taken using a grid positioning scheme, and results were aggregated to give plot values for PAR, R:FR and values from hemispherical photography. R:FR was measured at the start of the experiment, immediately after pruning in March 2006.

6.2.6.1 R:FR measurement and analysis

An SKR 110 Red/Far-Red sensor with a display meter (Skye Instruments Ltd, Llandrindod Wells, UK) was used to measure the ratio of red (660 nm) to far-red (730 nm) radiation.

There were three sets of R:FR measurements taken at the experimental site during each period of measurement: in the morning, at midday and in the afternoon. Open site measurements were done prior to measurement in the blocks and between each set of measurements in the blocks, and finally at the end of the last afternoon block measurements.

The only open area near to the experimental site was approximately 1km away at Missellele Market. R:FR readings made at the open site were taken to enable a

comparison between an open canopy position and measurements taken under the rubber canopy. Ten measurements were taken every 30 seconds during each period of measurement at the open site. The sensor was kept level during measurement with the use of a circular bubble spirit level.

Following the initial measurement at the open site, the researcher and assistant returned to the light experiments as quickly as possible, by motorbike, to start taking measurements in light plots. Each plot was visited in sequence. Firstly, block 1 plots were measured in plot order, one to nine followed by readings being taken in block 2 using the same method. Finally, the open site was revisited to conclude recording data for one set.

Ten R:FR readings were taken per plot. Each reading taken at a specific grid point, each point corresponding to a grid reference based on an inter-rhizome grid (Figure 6.1). Grid references were generated randomly for x and y axes using the random number generator in Microsoft Excel (Microsoft corporation). The sensor was placed above the grid reference point, kept level as above, and the reading on the display meter recorded.

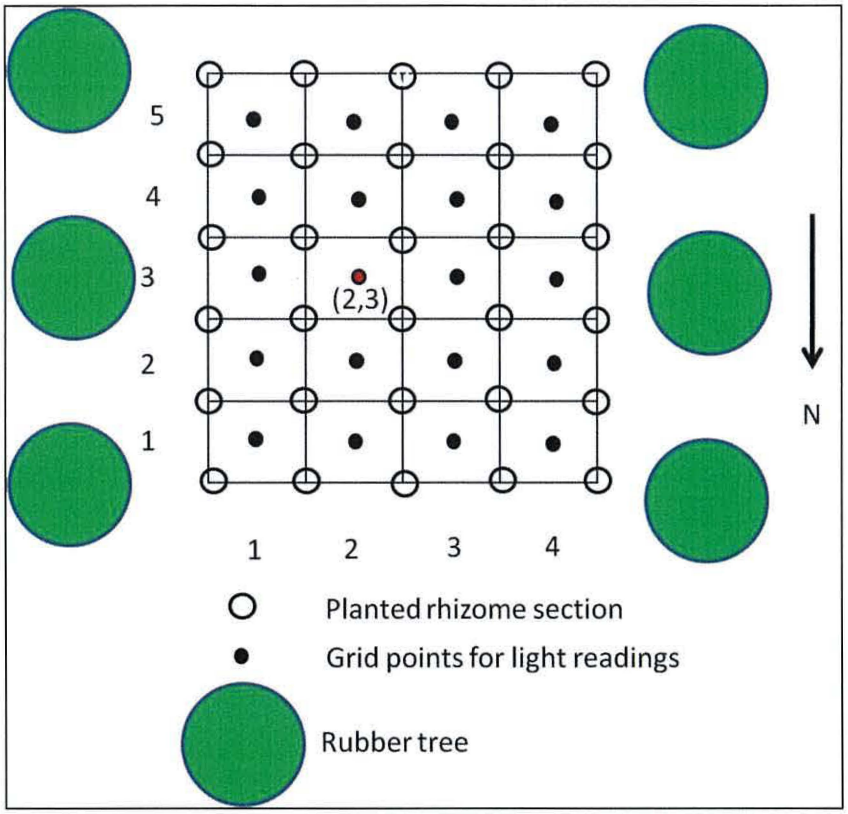

Figure 6. 1 Example of a light plot overlain with the grid system used to allocate positions for light measurements.

The red point shows the grid reference at that point.

6.2.6.2 – PAR transmittance

An AccuPar Linear PAR/LAI ceptometer (Model PAR-80,Decagon Devices, Inc., Washington, USA) was used to measure photosynthetically active radiation under the rubber canopy (Plate 6.2). The ceptometer consisted of 80 independent photodiodes, spaced 1 cm apart, and attached to a micro-controller, which displays values of PAR. The micro-controller has a spirit level integrated into the body to facilitate keeping the ceptometer level when taking readings. Each photodiode measures PPFD, in the 400-700 nm waveband, displaying PAR in units of μmols $m^{-2}s^{-1}$ and in a range of 0 to 2500 μmols $m^{-2}s^{-1}$. The ceptometer was set to measure PAR continuously using the whole probe meaning that the photodiodes gave an integrated measurement of PAR.

To measure PAR simultaneously at an open site a Skye Instruments SKP 215 PAR Quantum sensor, attached to a display meter (Skye Instruments Ltd, Llandrindod Wells, UK), was set up at Missellele Market. Staff were shown how to use the device and given sheets to record PAR. The sensor was kept level, and staff sat as far away as possible from the sensor so as not to shade the sensor. Readings were synchronised with those taken under the canopy at the start of data recording, and made every 30 seconds in order to complement readings taken under the canopy (Plate 6.2).

Plate 6.2 Taking measurements of photosynthetically active radiation (PAR) in the open canopy at Missellele Market and under a rubber canopy at Missellele experimental site January 2007 (Photos: Mark Lyonga).

PAR readings under the rubber canopy.

The same grid was used to assign points for taking readings in light experiment plots as used for R:FR measurements. A new set of grid references was produced together with an independent randomly generated direction point based on a 360° compass reading. To measure PAR in plots the specified grid location was found, the ceptometer was orientated in the compass direction specified, and was kept level, above the canopy of *T. daniellii,* using the integrated spirit level bubble: a reading of PAR was taken and noted down by an assistant. Ten readings of PAR were taken per plot. Readings were taken every 30 seconds, however if a time slot was missed, the reading would be taken at the next 30 second interval. The order of taking measurements in blocks was the same as for R:FR measurements.

Calibration of the Accupar PAR 80 ceptometer with the SKP 215 Skye Instruments quantum sensor.

An open space was found where measurements of PAR could be taken using both sets of equipment during a six hour period from midday to 6 p.m on 1st February 2007. A final reading was taken where both sensors were completely covered to check if they both read 0 when there was no incident light on the sensors. These data were used to calibrate both sets of equipment. Ceptometer readings were adjusted to the Skye 215 PAR quantum sensor using the regression equation,

$$y = 1.263x \ (R^2\ 0.998).$$

Percentage PAR transmittance

Percentage PAR transmittance (% PAR) was calculated using the following equation

$$\%\ PAR = \left\{1 - \left(\frac{Io - Ic}{Io}\right)\right\} x\ \frac{100}{1}$$

Where Io is the PAR reading in the open canopy in μmol $m^{-2}s^{-1}$ and Ic is the PAR reading taken simultaneously under the rubber canopy, also in μmol $m^{-2}\ s^{-1}$.

6.2.6.3 – Hemispherical photography

In principle hemispherical photography offers a more efficient means to measure the light environment under rubber and so these were compared with direct measurements using sensors. Hemispherical photographs were taken on days when the sky condition was uniform and overcast, though this proved difficult at the site during the times when photographs could be taken as the sky conditions were changeable throughout the day.

Equipment and method to take hemispherical photographs

Hemispherical photographs were taken in January and October 2007 and March 2008. A Nikon Coolpix 4500 digital camera (Nikon Corporation, Japan) was used with the self levelling mount (SLM4, Delta T-Devices Ltd, Cambridge, U.K) attached to a monopod. The technical set up of the equipment, attachment of the fish eye lens,

orientation of the camera in the gimbal plate, levelling of the gimbal plate and attachment to a monopod followed instructions in the user manual for the SLM4 mount (Wood and Potter 2003)

New grid references were produced using Microsoft Excel and positions for taking photographs were assigned using the grid system described above. Ten photographs per plot were taken above the canopy of *T. daniellii* (Plate 6.3). Care was taken to first orientate the equipment North-South using the embedded compass in the gimbal plate and to ensure that the gimbal was level using the embedded spirit level. A timer delay of two seconds was found to reduce camera shake, rather than using a longer shutter delay: immediately prior to taking a photograph the photographer would hold his breath when pressing the shutter. At times the gimbal would become not level. When this occurred it was immediately adjusted before anymore photographs were taken. A note was made of the photograph number after the photograph was taken in order to ensure that each photograph was correctly recorded to the position it was taken.

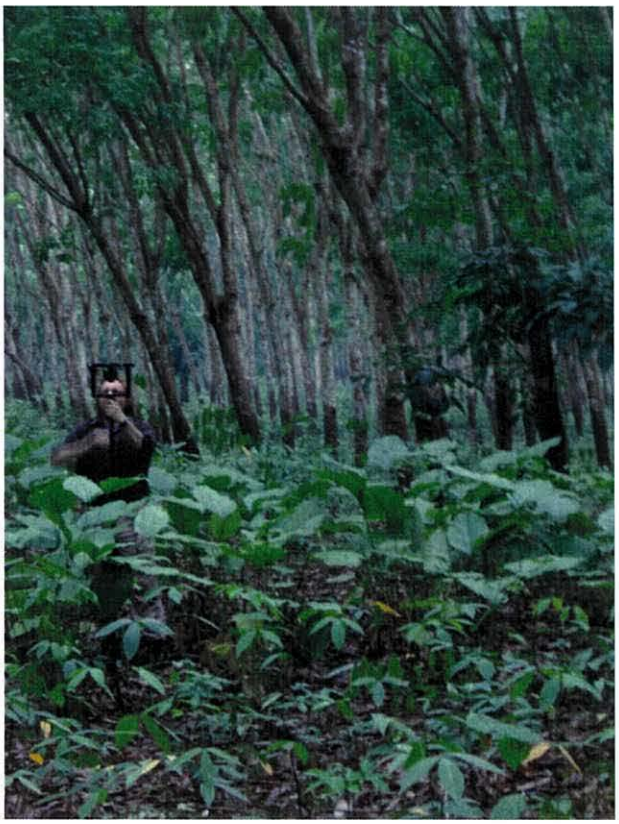

Plate 6.3 Taking hemispherical photographs at Missellele experimental site. March 2008 (Photo: Mark Lyonga, 2008)

Photographs were taken in the same order as readings for PAR and R:FR readings. After photographs were taken they were transferred to a laptop computer for later analysis.

Analysis of hemispherical photographs

Each individual photograph was first visually assessed to determine if it could be used for analysis. Photographs with sun burst, halos, obvious camera shake or where the gimbal was not level were rejected.

Remaining photographs were analysed using Hemiview 2.1 Canopy Analysis Software (Delta-T Devices LTd., Cambridge, U.K.). Briefly, once the photograph was opened, data regarding the site were input into the program: namely, the latitude and longitude of the location where the photograph was taken (in all cases the latitude and longitude of the site shed was used as it was difficult to get readings of latitude and longitude under the canopy of rubber); the date of the photograph and the magnetic declination, worked out using an online declination calculator (National Oceanic And Atmospheric Administration, 2010). The photograph was orientated correctly using the software and the digitised image classified into either black (completely blocked by foliage) or white (clear sky) sections (Newton 2007) (Plate 6.4).

This process, called thresholding, is the most critical step in hemispherical analysis as changes in the threshold value affect the estimates of canopy closure (Breda, 2003; Newton 2007). The threshold for each photograph was initially done visually changing between a colour image and a black and white image using the software until the researcher was satisfied that it was a true representation of the canopy. The decision to use an automatic thresholding program, Sidelook 1.1.01 for Windows (Nobis and Hunziker, 2005) was taken to reduce systematic bias by the researcher in thresholding photographs and was a faster method: once photographs had been thresholded using Sidelook 1.1.01 for Windows they were input into HemiView 2.1 software.

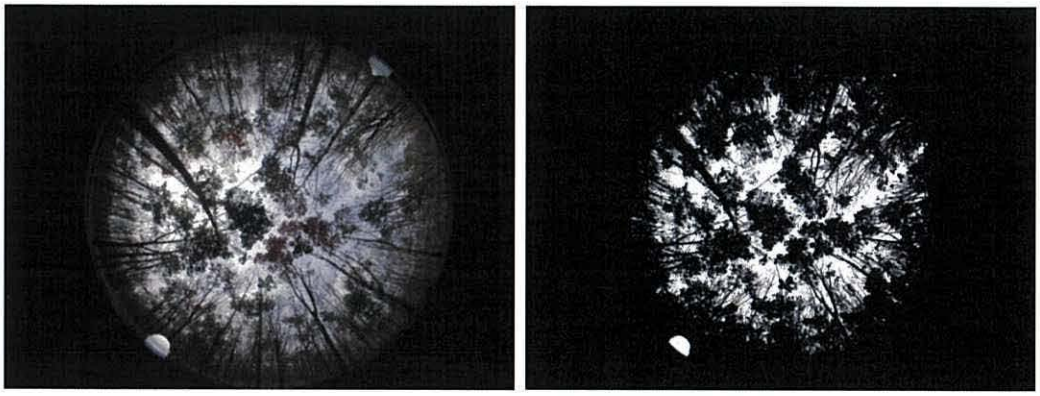

Plate 6.4 Setting the threshold for hemispherical photographs using Sidelook 1.1.01 for windows.

The left hand photograph was taken in January 2007. The right hand photograph shows the effect of an automatically thresholding the image using Sidelook 1.1.01 with the value of 128

HemiView 2.1 calculated a suite of sky measurements. Following discussion with Delta-T Devices (Edmund Potter, pers. comm. 2007) global site factor (GSF) was selected as the most parsimonious value to use to estimate canopy openness. GSF is defined as the proportion of global radiation under a plant canopy relative to that in the open and is typically calculated as direct (DSF) plus diffuse radiation (ISF), with reflected radiation ignored (Rich *et al.,* 1999)

DSF is the proportion of direct solar radiation reaching a given location, relative to that in a location with no sky obstructions and ISF is the proportion of diffuse solar radiation reaching a given location, relative to a location with no sky obstructions. Values for both indices range from 0 to 1, 0 indicating no direct radiation (DSF) or diffuse radiation (ISF) meaning complete sky obstruction, and 1 being the value for direct (DSF) or diffuse (ISF) radiation in an open location, meaning no sky obstruction (Rich *et al.,* 1999).

The mean of the ten GSF readings per plot was used as the GSF value used in analyses.

6.2.7 Analysis of data

6.2.7.1 Analysis of PH and LA

Values for petiole height (PH) and lamina area (LA) measured in quadrats were aggregated to produce quadrat means, which were in turn aggregated to produce plot mean values which were used in all analyses. Lamina area was calculated using a regression equation ($R^2 = 0.773$) derived when calculating SLA in Section 4.2.12.1, where lamina length and width are inputs.

$$LA_{(m^2)} = LL_{(m)} \; x \; LW_{(m)} \; x \; 0.5062$$

Where LA is the lamina area measured in m^2, and LL is lamina length and LW is lamina width, both measured in m.

Where necessary, for significant value effect sizes were calculated (Appendix I).

6.2.7.2 Pruning treatments relation effects on R:FR radiation

To see if pruning treatments had affected the R:FR ratio an analysis of variance (ANOVA) was done with treatment, block and block-treatment interaction input as dependent variables (factors). This was conducted separately for each time period where light measurements were taken.

6.2.7.3 The effect of R:FR on PH and LA

To determine if variability in R:FR effected a response in *T. daniellii* ANOVA was done based on monthly measurements of PH and LA.

6.2.7.4 Comparison of mean % PAR transmittance with mean GSF values

A regression analysis was conducted to see the relationship between mean % PAR transmittance and mean GSF per plot.

6.2.7.5 Pruning treatments relation to mean % PAR transmittance levels

To see if the imposition of pruning treatments had affected the PAR transmittance consistently according to treatment ANOVA was done with treatment, block and block-treatment interaction input as dependent variables (factors). This was conducted separately for each time period where light measurements were taken.

6.2.7.6 The effect of % PAR on T. daniellii plant characteristics

It was evident from ANOVA analysis of % mean PAR transmittance per plot, with treatment, block and treatment-block interactions as dependent variables, that pruning treatments were not successful in creating consistently different light levels, therefore, correlation and regression analysis between PAR transmittance and plant characteristics was the chosen method to analyse data rather than categorical comparison of treatments. Mean PH and LA measurements per plot recorded nearest to and prior to the date of light measurements were used for analysis. Plant data for January and October 2007 were not collected due to the necessity to conduct other work during the visit to Cameroon, such that data from December 2006 and September 2007 were used in the analysis. Plant data was collected just prior to light measurements in March 2008 and was used for the analysis.

Effect of % PAR transmittance on PH and LA March 2006 to January 2007.

Values of mean plot PH and LA for December 2006 were regressed with mean % PAR transmittance plot readings taken in January 2007 to ascertain the effect of % PAR transmittance on early growth of the plants in terms of PH and LA.

Effect of % PAR transmittance on ΔPH and ΔLA January 2007-March 2008

To determine the effect of % PAR transmittance values recorded in October 2007 and March 2008 on plant characteristics (PCH), namely, PH and LA, during the periods between December 2006 to September 2007 and September 2007 to March 2008 respectively, the following generic equation for a relative change in the plant characteristic (ΔPCH) was used:

$$\Delta PCH = \frac{(PCH_f - PCH_o)}{T_f - T_o}$$

modified from Brncic (2002)

Where PCH_f is the plot mean value for the plant characteristic (petiole height or lamina area) at the end of the period of measurement (T_f) and PCH_o is the value of the plant characteristic at the beginning of the period of measurement (T_o). The period between measurements was in days.

6.3 RESULTS

This section firstly details the relationship between the two methods of measuring the light environment, and then the relationship between % PAR transmittance and mean GSF. The results of pruning treatments on the light environment under the rubber canopy are reported next. Changes in morphology of *T. daniellii* in terms of growth in in petiole height (PH) and lamina area (LA) are then related to the light environment and the section concludes with fruit production data.

6.3.1 Effect of pruning treatment on plot mean R:FR

An analysis of variance (ANOVA) to determine whether pruning treatments had affected the Red:Far Red ratio, showed R:FR values were significantly higher in plots with more severe pruning treatments, immediately after pruning had been conducted, March 2006 as well as amongst blocks although there was no block x treatment interaction (Table 6.1). Subsequent measurements of mean R:FR per treatment were not significantly different from one another (Table 6.2) The greatest values of R:FR radiation at the open sites were recorded in March 2008, 1.15 ± 0.007, compared with the lowest value of 1.05 ± 0.02 recorded in March 2006.

Table 6. 1 Analysis of variance of block, treatment and block * treatment interaction on plot mean R:FR by period

Period	Dependent variable (factor)	F	Sig	ω^2
March 2006	Block	11.572	0.005	0.01
	Pruning treatment	31.508	0.000	0.95
	Block*pruning treatment	1.416	0.280	
January 2007	Block	0.665	0.431	
	Pruning treatment	0.089	0.916	
	Block*pruning treatment	0.608	0.561	
March 2008*				
	Pruning treatment	2.201	0.192	

* March 2008 data only for block 1 hence no block or block*treatment effect.

Table 6.2 Mean ± Standard deviation of mean R:FR per treatment by date of collection.

	Date of PAR collection		
Treatment	March 2006 (60)	January 2007 (60)	March 2008 (B1 only) (30)
0	0.28 ± 0.77[a]	0.995 ± 0.023	0.50 ± 0.25
25 %	0.44 ± 0.82[b]	0.994 ± 0.028	0.64 ± 0.12
50 %	0.63 ± 0.14[c]	0.986 ± 0.054	0.46 ± 0.13

Values in italics are significantly different from each other at P<0.05. Number in brackets are numbers of readings.

6.3.2 Effect of R:FR on PH and LA

ANOVA analyses were used to test the effect of treatment, effectively R:FR, (and block as there were significant differences in the R:FR ratio attributed to block and treatment (Table 6.1)) on mean plot PH and LA, monthly from March to December 2006: block-treatment interactions were not investigated as these did not significantly affect R:FR values. In all instances PH was not affected by treatment (~ R:FR) or block during March to December 2006 ($p > 0.05$) (Table 6.3 – only data for the first five months is shown). Lamina area was significantly larger in 50% pruned plots, i.e. high R:FR, than other pruning treatments during April 2006, F (2,12) = 4.083, p = .044. However during the preceding and following months there were no significant differences between the mean LA attributed to treatment or block ($p >$ 0.05).

Table 6. 3 *Thaumatococcus daniellii* mean petiole height (m) under different pruning treatments and the associated Analysis of variance (ANOVA) statistics for treatment and block effect.

Month 2006				ANOVA			
	Petiole height by treatment (m)			Treatment effect		Block effect	
	0 %	25 %	50 %	*F*	*p*	*F*	*p*
March	0.076	0.094	0.113	1.71	0.343	0.35	0.566
April	0.297	0.236	0.266	1.66	0.23	0.075	0.789
May	0.314	0.245	0.298	1.729	0.219	0.026	0.875
June	0.311	0.224	0.290	3.32	0.071	0.288	0.601
July	0.335	0.295	0.321	0.917	0.426	1.908	0.192
August	0.329	0.291	0.315	1.93	0.188	0.097	0.76

No further analysis of the effect of R:FR on PH or LA is shown as there was no significant difference in the R:FR ratio corresponding to treatment for readings taken during January 2007 and March 2008 (Table 6.1) and because there had been no

effect of R:FR on PH and LA related to treatment or to block effects during March to December 2006.

6.3.3 Correlation between PAR and GSF

Mean plot % PAR transmittance and mean plot GSF values were poorly correlated during all periods of measurement (Figure 6.2). Readings taken in October 2007 showed the highest correlation with % PAR transmittance per plot explaining 58% of the variance in plot mean GSF values ($R^2 = 0.583$).

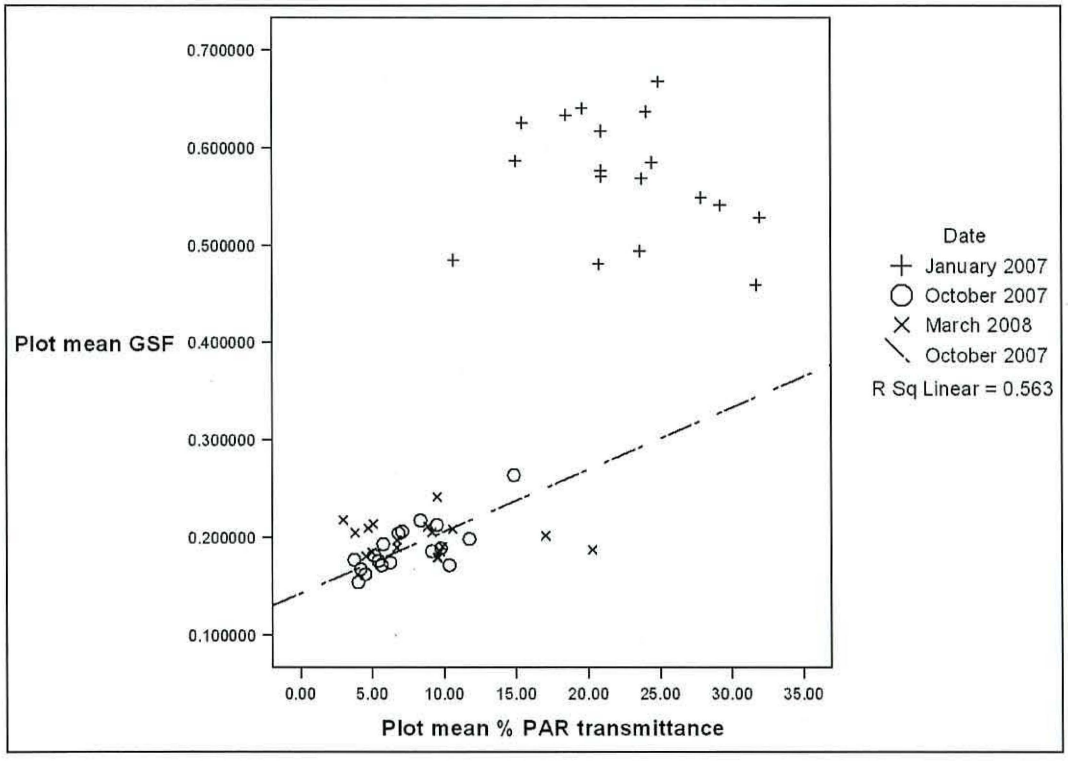

Figure 6.2 Correlation of plot mean % photosynthetically active radiation (PAR) transmittance with plot mean global site factor (GSF)

Due to the low correlation amongst methods (discussed later) % PAR transmittance was chosen for further analysis on the basis that it was a more robust measurement.

6.3.4 Absolute values of PAR

Absolute values for PAR transmittance varied greatly (Figure 6.3), between collection dates, and within open and closed canopy settings. Extremely low values of PAR were recorded under the rubber canopy (Table 6.4)

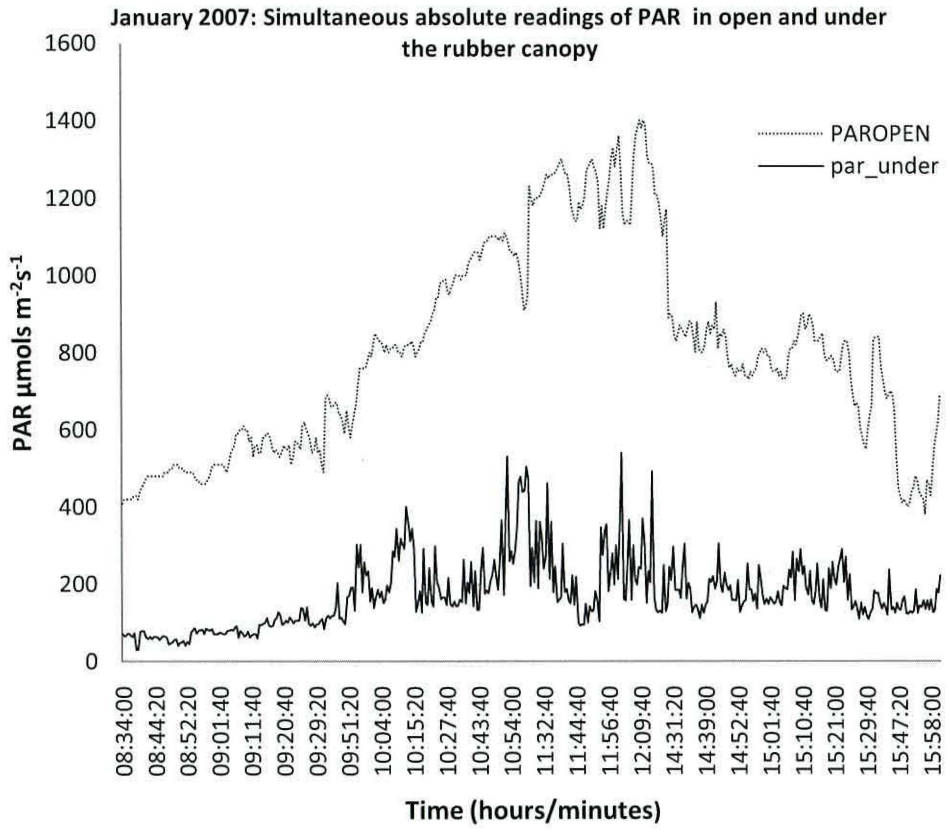

Figure 6.3 Example of variation in absolute photosynthetically active radiation (PAR) values recorded in open and closed canopy conditions. Data from January 2007 collection of PAR, Missellele experimental site.

Table 6.4 Absolute ranges of Photosynthetically active radiation (PAR) (µmols $m^{-2}s^{-1}$) recorded in open and under closed canopy conditions at Missellele experimental site during collection periods, based on instantaneous single measurements

Date of collection	PAR (µmols $m^{-2}s^{-1}$)			
	Open canopy reading		Closed / under canopy reading	
	Min	Max	Min	Max
January 2007	389	1403	30	524
October 2007	542	1850	17	335
March 2008	474	2092	29	404

6.3.5 Effect of pruning treatment on plot mean % PAR transmittance

The analysis of variance (ANOVA) to determine whether pruning treatments had affected the light environment showed that there was no significant difference between pruning treatments or the interaction between block and pruning treatments on % PAR transmittance during all the periods of light collection (Table 6.5) that began in January 2007, 11 months after the treatments had been applied. There were significant differences in % PAR transmittance between blocks, with block 1 having lower values of % PAR transmittance than block 2 during January and October 2007, though by March 2008 the effect of block on percentage PAR transmittance was no longer significant (Table 6.6):

Table 6.5 Analysis of variance of block, treatment and block * treatment interaction on plot mean % photosynthetically active radiation (PAR) transmission, by collection period

Period	Dependent variable (factor)	F	Sig	ω^2
January 2007	Block	6.085	0.030*	0.271
	Pruning treatment	0.811	0.467	
	Block*pruning treatment	1.355	0.295	
October 2007	Block	12.872	0.004*	0.406
	Pruning treatment	1.599	0.242	
	Block*pruning treatment	0.051	0.095	
March 2008	Block	1.244	0.287	
	Pruning treatment	0.432	0.659	
	Block*pruning treatment	2.277	0.145	

* Significant at the $p < 0.05$ level.

Table 6.6 Mean ± Standard deviation of mean % photosynthetically active radiation (PAR) transmittance per block by date of collection.

	Date of PAR collection		
Block	January 2007	October 2007	March 2008
1 (n =9)	19.59 ± 5.09 ^{a1}	5.36 ± 1.49 ^{a2}	7.42 ± 4.32
2 (n =9)	25.32 ± 4.86 ^{b1}	9.34 ± 2.89 ^{b2}	9.65 ± 4.52

Superscript values indicate significant differences at $P<0.05$. Superscript numbers refer to the period of collection. Values in brackets indicate number.

6.3.6 Regression analysis of plot mean percentage PAR with growth metrics

Due to block having a significant effect on percentage PAR transmission during the first two periods of light measurement, January and October 2007, regression analyses included block as an independent variable together with % PAR transmittance. The regression analysis done with March % PAR transmittance data did include block as independent variable.

6.3.6.1 Regression of January 2007 mean plot % PAR vs petiole height and lamina area.

Percentage PAR transmittance did not significantly affect the growth of lamina area (LA) ANOVA (F (2.15) = 0.399, p = 0.678, R^2 = .0506) or petiole height (PH) ANOVA (F (2,15) = 1.683, p = 0.219, R^2 =.183) during the first nine months of growth, from March 2006 to December 2006. There was no significant block effect on growth of LA and PH, t = 0.607, p = 0.553, and t = 1.832, p= 0.087 respectively.

6.3.6.2 The effect of % PAR transmittance on the relative change in PH and LA during December 2006 to September 2007.

Percentage PAR transmittance did not significantly affect the change in size of PH from December to September 2007, ANOVA F (2.15) = 2.544, p = 0.112, R^2 = .253.

Percentage PAR transmittance and block are significant in determining the change in size of LA, F (2, 15), = 4.223, p = 0.035, the model explaining 36 % of the variability in change in LA during December to September 2007, R^2 = 0.360.

6.3.6.3 The effect of % PAR transmittance on the relative change in PH and LA during October 2007 to March 2008.

Percentage mean PAR transmittance was not significant in determining changes in size of PH or LA during October 2007 to March 2008. For PH, F (1,16) = 1.198, p=0.290, R^2 = .070 and for LA F (1,16) = 0.048, p = .829, R^2 = .003

6.3.7 Fruit, flower and bud production in the light experiment

By June 2009 the plants had been growing for 3 years 3 months. Fruit production was very sparse amongst and within plots such that there were not sufficiently consistent data to test relationships between light and fruiting (Table 6.7). There was however noticeably more fruit produced in block 2 than in block 1.

Table 6. 7 Fruit production by block and plot, Missellele experimental site as of June 2009

Plot	Block1			Block 2		
	Fruit	Flowers	Buds	Fruit	Flowers	Buds
1	0	1	0	27	52	25
2	0	1	0	14	48	25
3	1	0	0	0	1	0
4	0	0	0	7	37	21
5	0	9	1	0	20	11
6	0	0	0	30	74	48
7	4	12	2	0	1	0
8	0	0	0	1	1	0
9	1	6	1	1	0	0
Total	6	29	4	80	234	130

6.4 Discussion

6.4.1 Cautions and limitations

The size of plots in the experiment was necessarily small due to the restriction on the number of trees that CDC allowed to be pruned. Establishing a baseline quantity of PAR transmittance prior to and following pruning was not possible due to the necessary equipment not being available at that time. Timing of the first visit to the site after return to the UK coincided with the period of senescence of rubber leading to readings taken in very open canopy conditions. Visiting the site at this time also meant assessment of the canopy in terms of further pruning was not conducted as it was not possible to determine if further pruning was necessary. It was assumed that it was not and this decision was supported by the fact that it was very possible to damage *T. daniellii* growing in plots below with falling branches. A destructive sample of plots was not conducted as the experiment was handed over to the Institute of Agricultural Research for Development (IRAD) at Ekona, South West Province,

Cameroon, to continue the trials to determine future fruit production as fruit production was low, especially in block one at the end of June 2009.

6.4.2 The quality of light, R:FR, and response in *T. daniellii*

It is clear that immediately after pruning there was a significant effect on the quality of light reaching *T. daniellii*. The ratio of red to far-red (R:FR) was correspondingly lower in shaded plots increasing as the level of pruning was increased, $F(2, 12) = 31.508$, $p<0.001$, $\omega^2 = 0.95$, a huge size effect. This supports Capers and Chazdon, (2004) that the R:FR ratio is affected by the degree of canopy closure. This is because light that has passed through a canopy is rich in far-red but low in red and blue light: the former is predominantly transmitted and reflected (Vandenbussche *et al.,* 2005) to the understorey as the canopy is virtually transparent to far red radiation (Smith, 1986), whereas the red and blue light are absorbed by the chlorophyll in the canopy and hence depleted in the understorey. Light quantity is generally related to r:fr where leaf shading is causing reduction in light, so although not measured we can expect that there was also significantly less light in the more shaded treatments with lower r:fr ratios.

R:FR measurements under the rubber canopy were consistent with those in other studies: Wilson and Ludlow (unpublished data in (1990)) recorded values of R:FR under an immature rubber canopy of 1.17 and 0.62 for a mature canopy whilst full sun measurements were 1.20.

However, beyond nine months there is no evidence to suggest that the quality of light was different between pruning treatments. This is probably due to three reasons: 1) the senescence of rubber trees, in January 2007, leading to an open and hence homogenous canopy amongst treatments, with higher levels of R:FR; 2) the re-leafing of the rubber tree canopy by March 2009 explains the homogenous nature of R:FR measurements taken under a full canopy and the lowest values for the most highly pruned plots, and 3) that the initial pruning regime was not radical enough to ensure effects of pruning persisted past the first year of re-growth. Readings for March 2009 have to be regarded with caution as only values from block 1 were available. Values from block 2 were discarded as the sensor registered negative

values of R:FR. These were likely due to taking readings at low levels of photosynthetically active radiation (PAR): red and far-red wavelengths only account for a small proportion of total PAR, when light levels are extremely low it is possible for light levels to fall below the detection of the sensors (Capers and Chazdon, 2004), thus potentially providing spurious negative readings of R:FR radiation. Due to this reason negative readings recorded at low levels of PAR, taken in the early morning and late afternoon, were also disregarded: where possible readings were taken in overcast or cloudy conditions, as both red and far red increase in sunnier conditions (Capers and Chazdon, 2004).

The effect of the R:FR ratio, (effectively the pruning treatment), on the morphological characteristics of PH and LA showed no significant effect at each of the months where readings were taken, March to December 2006 ($p > 0.05$), except during the first month where LA was recorded, April, which was significantly different between treatments $F(2,12) = 4.083$, $p = .044$, $\omega^2 = 0.415$, a significant effect size. The increased LA in 50% pruned plots could be attributed to the plants early growth response to available light prior to re-growth of the rubber canopy and the subsequent shade environment. If *T. daniellii* were a shade avoiding species one would expect there to be significantly longer petioles and larger thinner leaves at lower R:FR levels (shaded plots) (Smith and Whitelam, 1997; Franklin and Whitelam, 2005; Franklin, 2008), though in a species adapted to shade, a weaker response to low R:FR would be expected (Morgan and Smith, 1979) consistent with the results reported here.

6.4.3 The quantity of light, % PAR transmittance and the effect on *T. daniellii*

The plot mean percentage PAR transmission was not well correlated with plot mean global site factor during readings taken in January 2007 and March 2008, though there was a weak correlation between readings taken in October 2008 ($R^2 = 0.563$). This contrasts with significant positive correlations between direct sensor measurements and indirect photographic estimates of PAR (Chazdon and Field, 1987; Rich *et al.,* 1993; Gendron *et al.,* 2001; Beaudet and Messier, 2002). The large spread of data points between PAR and GSF in January is most likely due to the open nature of canopy, due to senescence of rubber during this time. Conversely

when the canopy was full, as in October, there was a closer relationship between the measurements. Though hemispherical photography has many advantages, (Anderson, 1964; Chazdon and Fetcher, 1984; Chen *et al.,* 1997; Brandeis *et al.,* 2001) it is bounded by assumptions that all and any leaves completely block the passage of light and that the layer of leaves in a canopy are in a single layer (Roxburgh and Kelly, 1995). Chromatic aberration (halos) seen on hemispherical photographs taken with equipment used in this experiment are symptomatic of photographs taken in dense canopies, below 10% openness (Frazer *et al.,* 2001). Hale and Edwards (2002) suggest that at low levels of PAR, about 10% transmittance, hemispherical photography produces inaccurate values of transmittance, however taking photographs on overcast or cloudy days minimises these errors (Frazer *et al.,* 2001). Error can also manifest when thresholding images (Roxburgh and Kelly, 1995; Rich *et al.,* 1999), though in this experiment procedures were taken to minimize this (section 6.2.6.3) using available software (Nobis and Hunziker, 2005). Given that many images taken in October 2007 suffered from halos, that some images in March 2008 were rejected due to issues with focussing (these were removed from analysis), and that it was difficult to take a set of photographs during consistently cloudy or overcast conditions, it was decided to continue the analysis using measurements of PAR transmittance.

Incident PAR varied during the day, (Figure 6.3) with corresponding fluctuations in PAR in the understorey. Minimum values of absolute PAR under the canopy though very low, between 17 – 30 μmols m^{-2} s^{-1}, and of percentage PAR transmission, between 3% to 4% in March 2008 and October 2007, are not unusual for mature rubber canopies: Ng (1990) reported large reductions in % PAR transmittance under growing rubber from 53-19% and 90-50% in his trials as did Chong *et al.,* (1990) with reductions of % PAR transmittance from 89 to 62% in just eight months in three year old rubber and a value of 18% PAR transmittance in eight year old rubber, this increasing to 27% during senescence; Wilson and Ludlow note values as low as 10% PAR transmittance under rubber between the ages of 12 to 18, after which % PAR transmittance increases to 55% at 30 years (Wilson and Ludlow, 1990); and values as low as 2% PAR transmittance have been recorded under rubber in

Indonesia, equating to ≤ 20 μmols m^2s^{-1}, assuming full sun values of 2000 μmols m^2 s^{-1} at midday (Sanchez and Ibrahim, 1990).

The lack of a significant effect on percentage PAR transmittance due to pruning in the later measurements is most likely due to the re-growth of the rubber canopy following pruning at the start of the experiment. Subsequent pruning throughout the experiment might have maintained treatment differences but was not permitted by the site managers as previously explained. As the pruning regime was kept the same between treatments and blocks, an explanation for the significant increase in % PAR in block 2 compared to block 1 is that canopies in block one re-grew faster than those in block 2. Potential differences in soil type between the two blocks could be responsible for an increase in available nutrients, though the experiment was expressly set up in as similar and uniform site as possible. Block 1 is situated in the Pm1A – Missellele series, a sandy clay loam, with 0-2% slopes, it is deep, moderately well drained with yellowish brown heavy clays with poor levels of nutrients and high levels of exchangeable aluminium. Block 2 and most of the rest of the experimental site exists on part of the Tiko soils complex, Pt1A1 – yellowish brown, very gravelly heavy clays, underlain with a rigid impenetrable iron crust at a depth between 50 - 100 cm. Their surface is overlain with an iron-gravel. They are poor in nutrients and have high exchangeable aluminium indicating high weathering (Kips *et al.,* 1984).

Without equipment available at the start of the experiment to measure PAR, we only have indications from the r:fr measurements at this time as to whether percentage transmittance, immediately after pruning, was different under different treatments. Capers and Chazdon (2004) were able to estimate 97% of the variation in percent diffuse transmittance (%T) under cloudy skies from R:FR measurements using the following equation:

$$\%T = 0.548 + \exp\ (-2.451 + (5.6594\ R{:}FR)$$

However using this equation to estimate %T, followed by a regression analysis with known values of percent PAR transmittance, resulted in a non significant result for

each of the periods where data for both R:FR and PAR transmittance were available: F= 0.358, p = .558, R^2 .022, for January 2007 data and F = 0.501 p =.502 R^2 = .067 for March 2008 data. Therefore no attempt was made to estimate the percentage PAR transmittance immediately after pruning using the above equation.

Using PAR transmittance as a continuous variable to analyse the effect on the growth of *T. daniellii*, in terms of absolute growth in PH and LA during the first nine months of the study, was shown to be non significant, thus % PAR transmittance did not affect the growth in height of petioles or lamina area. Using a relative change in the height of petioles for the remainder of the study, there was no evidence to suggest that % PAR transmittance affected the change in height of petioles. Other than the periods between December 2006 to September 2007, where there was a significant, but weak effect of % PAR transmittance on the relative change in LA, F (2,12)= 4.223 p = 0.035, R^2 = 0.360, indicating that over 64% of the variation in lamina area was due to other factors, there was little evidence to suggest that changes in the size of lamina area were affected by % PAR transmittance.

Fruiting was variable between blocks, with block 1 producing far less fruit than block 2 (Table 6.7), however, there was no detectable effect of light on fruiting quantity. It is known, that shade avoiding plants in conditions where there is sustained low levels of R:FR radiation will produce fruit earlier as a strategy to 'escape' from the shaded environment by placing more energy and resources into reproductive organs, flowering and the production of fruit (Smith and Whitelam, 1997; Franklin and Whitelam, 2005). However, Hawthorne suggests that *T. daniellii,* similar to some other Marantaceae species are cryptic pioneers, often germinating and establishing in a light environment (outside of the 'twilight zone') and then persisting, perhaps for decades, as mature individuals in the shade (Hawthorne, 1996). Brncic (2002) supports this idea with some caveats; after studying four Marantaceae species, including *M. macrostachyum*, she suggested there were two mechanisms for the survival and growth of these species: the first, for three species, *Ataenidia conferta*, *Sarcophrynium schweinfurthianum* (Kuntze) Milne-Redhead and *Megaphrynium trichogynum* Koechlin, were light flexible understorey species that could persist in the understorey whilst taking advantages of gaps to grow and

reproduce at elevated rates, whilst *M. macrostachyum* was highly dependent on gaps for growth, reproduction and germination thus being dependent on large-scale disturbances. Given the conditions of the experimental trial, that pruning was not severe enough to effect the level of R:FR beyond nine months, it appears that *T. daniellii* is able to grow in both open and closed rubber canopies and can persist there, its response to elevated light conditions does not appear to be as immediate as seen in some of the other Marantaceae species (Brncic, 2002).

Local knowledge from Ghana suggests that in wild populations. lack of water availability caused by high soil temperatures under open canopies may be a limiting factor in the growth of *T. daniellii* (Waliszewski *et al.,* 2005). High intensity of solar irradiance can also induce depression of photosynthetic productivity in many crops (Senevirathna *et al.,* 2003) including neo tropical shade leaves (Barth *et al.,* 2001) thus reducing growth: it has also been suggested that shade leaves in high light environments can adapt by producing more UV-b absorbing pigments and reducing the chlorophyll a and b quantity thus increasing the chlorophyll a:b ratio seen in sun leaves (Krause *et al.,* 2004).

A personal observation made of plots of *T. daniellii* in the light experiment, does suggest a phototaxic response to light, where *T. daniellii* lamina were more horizontal in shaded and diffuse light but more vertical in less shaded direct light (Plate 6.5) consistent with higher R:FR, PAR and UV-b, (Bonhomme, 2000; Capers and Chazdon, 2004). Erect leaves may be less vulnerable from the damaging effects of UV-b by reducing the area of the lamina in direct light (Jansen *et al.,* 1998).

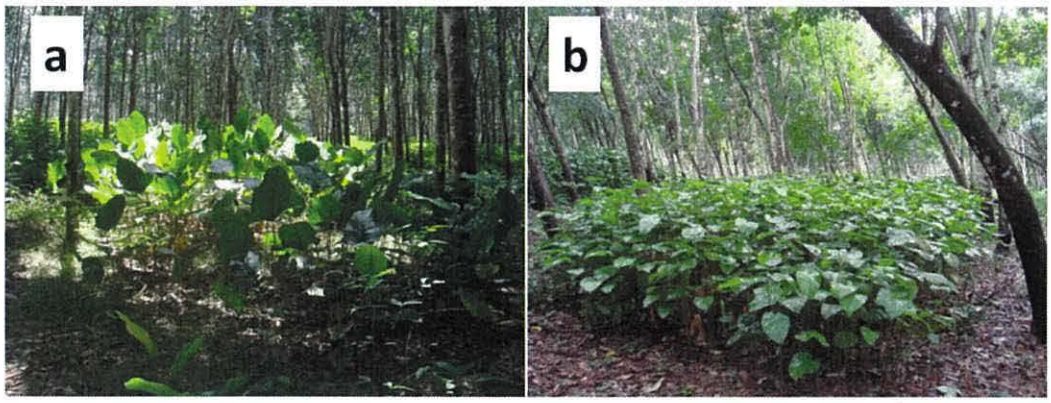

Plate 6.5 Differences in the orientation of *Thaumatococcus daniellii* lamina in a) direct and b) diffuse light under a rubber canopy rubber, Missellele experimental site (Photographs M. Lyonga and W.Waliszewski).

6.5 Conclusions

It is evident that the pruning regime imposed on the rubber canopy was not severe enough to ensure persistence of a sufficiently varied light environment in terms of both the ratio of red to far-red radiation and PAR transmittance to induce measurable responses in *T. daniellii*. Percentage PAR transmission measured using sensors did not correlate well with data from hemispherical photography, probably because light levels were too low for reliable photographic images to be obtained.

These results confirm that *T. daniellii* is a shade tolerant plant, able to persist in low light. There is no evidence to suggest that fruiting was affected by changes in shade and other factors are more likely to have limited growth and fruit induction. Perhaps, as suggested by local knowledge from Ghana (Waliszewski *et al.*, 2005), the key limiting factor was water availability in surface soil, or alternatively the nature of the sub-stratum soil conditions that varied within the site. The research suggests that *T. daniellii* is able to persist in a wide range of light conditions, from deep shade to fairly open conditions without detriment to its growth The plant is definitely able to grow under mature rubber with a dense canopy and it may also be possible to exploit its plasticity in light requirements to grow it under younger rubber.

6.6 RECOMMENDATIONS

It is clear that *T. daniellii* can grow in a range of light environments under rubber clone PR107 in a large plantation setting. It would now be useful to investigate whether *T. daniellii* can be established under younger rubber in the small holder environment. It is known that *T. daniellii* has grown under 35 year old mature PB217 (Odilous Mbuyeh, pers. comm. 2010), so it would be sensible to trial *T. daniellii* under various rubber clones and ages in the small holder setting. An experiment to determine the effect of open sun on *T. daniellii* should be set up to ascertain if this impacts negatively on the growth of *T. daniellii*, or whether it has the capacity to acclimate to these conditions. In order to determine the growth of *T. daniellii* in terms of biomass production related to PAR, new plots would need to be set up with a view to regular destructive sampling at given time periods, with increased frequency of measurements of PAR and R:FR, automating data collection through the use of sensors attached to data loggers. The experimental trials handed over to IRAD Ekona can be monitored for fruit production, or be destroyed and used as a source of germplasm for subsequent plantings in small holder trials: if destroyed, metrics should be taken of the size and length of rhizomes and inter-nodal lengths between petioles, characteristics that could be used to explore whether there has been a response to light conditions. If canopy openness under rubber in relation to the growth of *T. daniellii* is to be explored in the future, a review of the methodological approach conducted herein should be made, especially the extent and timing of pruning and measurements in relation to tree phenology.

CHAPTER 7

OVERALL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

In this chapter I set out a synthesis of what has been found across the other chapters that each deal with different aspects of the natural variation in plant traits and how *T. daniellii* performs as an intercrop with rubber.

7.1 GENERAL DISCUSSION

At present, the only efficient and pragmatic way to produce thaumatin is from arils collected from wild plants. Expression of thaumatin in bacteria, yeasts, fungi, and transgenic plants has shown that these are not viable methods (Higginbotham, 1979; Faus, 2000). Thaumatin is a highly competitive niche market that depends on the steady supply of arils from the collection of fruit from the natural resource. The expansion of the global market for natural sweeteners with the use of Reb A in soft-drinks (Jones, 2007) coupled with an increased demand for *T. daniellii* fruit, fuelled by the entry of a new thaumatin producer into the supply chain, Samartex Timber and Plywood Co., new sources of fruit supply are likely to be required to meet demand. Two methods currently exist to accomplish this: the first is an expansion of collection in areas where fruit is currently not being sourced; the second is the cultivation of *T. daniellii*. This thesis has focussed on variation in *T. daniellii* and its potential to be grown with rubber (*Hevea brasiliensis*) as an intercrop.

It is evident that *T. daniellii* can grow with at least three rubber clones, and can confidently be expected to have potential to be grown with others. The specific evidence relate to:

- 9 year old PR107, as in the experimental trials in Cameroon;
- 37 year old PB217, the location of *T. daniellii* found at the Missellele Rubber Estate and used in the rubber experiments;
- 10 and 12 year old GT1, as in the smallholder rubber demonstration trials in Ghana.

It is apparent that *T. daniellii* competes with rubber when intercropped, reducing the productivity per hectare of cup lump rubber by approximately 12% in the trial reported here, however, as has been shown the benefit in reduced weeding is advantageous at all but the highest prices of cup lump rubber: a mean loss of income at at a rubber price of XFA 300 kg^{-} would equate to XFA 26 812 ha^{-1} (not taking into account costs of rubber production) and would be offset by the production of 160 kg fruit ha^{-1} at a price of XFA 167 kg $fruit^{-1}$, equivalent to prices paid in Ghana in 2009 (Martin Laasen, pers. comm. 2005). This level of production was not seen at the experimental site: Onwueme *et al.* (1979) described a maximum production of 249.5 kg of fruit from 0.2ha plots, (although there was much variability in fruit production) which would more than offset rubber income losses as a result of intercropping with *T. daniellii*. Given the nature of the historical price of rubber (Figure 5.5) a return to sub XFA 650 kg^{-1} prices for cup lump is not unprecedented, at which point the benefit from intercropping would increase. This is of course based on many assumptions around the development of *T. daniellii*–rubber intercropping. The first being that a market chain exists for the purchase of produced fruit, the second, that fruit production can provide profit after all costs are taken into account, and the third is that smallholder perceptions may have to alter in order to engage with this type of intercropping.

Farmers engaged in rubber production already raised many questions regarding the establishment of *T. daniellii* with rubber during meetings held in Ghana and Cameroon in 2009. These included questions about the sale and purchase of *T. daniellii* fruit, likely income, market price of fruit, access to markets, grants and assistance to start planting. Specific questions regarding practical issues such as the recognition of the plant, its basic biology, type and quality of germplasm, and access to germplasm, plus methods of establishment and management of plants and fruiting frequency were raised in relation to propagation and establishment of *T. daniellii*. Specific questions pertaining to its affects on rubber production, which rubber clones it could be grown with and at what age of rubber it should be planted, were also raised by farmers.

What age rubber could be intercropped with *T. daniellii* was a key issue for farmers. The quality and amount of light provided to understorey plants in this experiment under a managed canopy of nine year old rubber (clone PR107) did not elicit major changes in *T. daniellii* growth or morphology, suggesting that they are shade tolerant and might also be successful in more open canopies. The study suggests that *T. daniellii* could be established in much younger stands of rubber. Other niches with an open canopy could also provide potential areas for the growth of *T. daniellii*, such as with oil palm (*Elaeis guineensis* Jacq.) where the spacing between palms is greater than for rubber and at the edges of cocoa farms (Plate 7.1A), conversely *T. daniellii* could be introduced into niches under increasingly densely shaded environments such as stands of teak (*Tectona grandis* L. F,) (Plate 7.1B) and under community woodlots more generally, as was discussed with the Bakingili Community Forestry Project (T. Njungo and A. Lyonga. pers. comm. 2006). Older stands of rubber with denser canopies than the nine year old trial in Cameroon may also be suitable.

Plate 7. 1 *Thaumatococcus daniellii* in different niches determined by light environment.

A: *T. daniellii* at the edge of a cocoa farm (Adam Village, Ghana. 2002). B: *T. daniellii* under *Tectona grandis* (Adwenaase Community Forest Reserve, Ghana, 2002).

With an increasingly open canopy there are factors which have to be considered. Less shade may increase bare soil evaporation and reduce water use efficiency of the understorey (Wallace and Batchelor, 1997), which is consistent with local knowledge from Ghana that *T. daniellii* does not grow so well in open conditions because of surface soil drying Waliszewski *et al.* (2005). The second factor is the potentially damaging effect of high direct PAR (UV-B radiation) levels on the lamina as suggested in Chapter 6. *T. daniellii* appears to have a phototaxic response to direct

PAR in which the plant reduces direct exposure through the use of the pulvinus that results in an almost vertical leaf position in high light conditions.

Not surprisingly, farmers were keenly interested in the quantity of fruit they could produce and how much this could be sold for. It was observed that fruiting under rubber was variable amongst the *T. daniellii* provenances grown at a single site, supporting Onwueme *et al.*'s position (1979). There were not enough fruit to determine whether thaumatin yield, which varied significantly amongst provenances *in situ*, also varied when it was sourced from fruit produced under experimental conditions at the same site. Fruit production from smallholder demonstration plots in Ghana (Plate 7.2) was also variable but the mean fruit production was much greater than that produced in Cameroon with a mean fruit production of 102 kg ha^{-1} as of June 2009: the source of germplasm, rhizome or seedling, apparently not giving any clear advantage in terms of fruit number or size of fruit produced.

Plate 7.2 Smallholder demonstration plots under GT1 rubber clone, near Akona Junction, Ghana. (Photo: Rob Kofi Appiah, Nov. 2009).

Note the mature (red) fruit at base of plants.

Measurements of plants at the collection sites *in situ*, identified that there were significant and large differences in morphological characteristics amongst provenances. This variation was suppressed when plants were grown at the same site as results from Missellele suggest: except for significant differences between locally adapted material and other provenances, phenotypes were broadly similar, suggesting that phenotype is determined principally by environmental factors, such as rainfall, lithography or local site conditions. Local knowledge from Ghana reported that gravelly and/or highly compacted soils were not good for the growth of *T. daniellii (Waliszewski et al., 2005)* yet, the largest plants with the largest fruit were found where the dominant lithography was leptosilic (FAO-UNESCO, 1977; Verelst, 2009). Rainfall throughout the collection sites was sufficient for the growth of *T. daniellii* (Figure 2.1), and it is likely that drying events in the past and current areas of low rainfall, limit the spread of *T. daniellii*.

The principal result from the molecular study and common garden experiments was that there was more variation between regions and populations than within populations. Low values of genetic diversity (He = 0.032-0.095) plus high SM coefficient values within provenances and low but significant values of inter-population variation (ΦPT 25%) support the low variation observed within-populations from the common garden experiments. Although variation within provenances is low, it is sufficient to support investigation into selective breeding for desired characteristics. Furthermore, the expression of phenotype seen *in situ* seems to be a response to environmental conditions rather than controlled solely by genotype, as evidenced by low levels of morphological variation described in common garden experiments.

The AFLP methodology used to elicit differences at a molecular level, has shown no evidence of speciation in the range studied, specifically using material close to the location of Bates 392 (BR, K) specimen, used in Dhetchuvi and Diafouka's work on var. *puberulifolius (Dhetchuvi and Diafouka, 1993; Dhetchuvi, 1996)*. The AFLP methodology is sufficiently robust to clarify the issue of Ley and Claßen-Bockhoff's *Thaumatococcus* sp. 1. nov. specimen (Ley and Claßen-Bockhoff, (in press); Ley and Claßen-Bockhoff, 2009) from the Monts de Cristal, area in Gabon, support for which

would extend the genus beyond monospecificty. It is not known whether this specimen's arils contain thaumatin, or if they do at what quantity and what yield could be expected from them? Interestingly, the Checklist of Gabonese vascular plants (Sosef, 2006) notes Ley's specimen, Ley 56 (LBV, WAG) det.: Ley, 2004, at the genus level. It is not known to what extent there is further variation in morphology and thaumatin content within the range, and whether if present, it is determined by environment, though it is clear that there is significant variation in thaumatin content with the range studied herein. Of interest would be to determine if there are populations of *T. daniellii* that produce yields greater than expected by industry, that is, greater than 2%.

Don's specimen from Sao Tomé (Plate 1.1) is so far from mainland West Africa that it raises the interesting question of whether the plant was introduced or whether it is indigenous to the island. Using molecular methods developed in this thesis together with a catalogue of *T. daniellii* molecular markers from specimens collected from the range, a similar approach to that of Hopkins (1983) who conducted a distribution study on *Parkia biglobosa* (Jacq.) R. Br. Ex G. Don using leaflet size to determine whether it was introduced to Sao Tomé, could be done to determine the origin of the material on the island.

The establishment of thaumatin production in West Africa has already been started with the establishment of cutting stations and thaumatin extraction facilities by Samartex Timber and Plywood Co. How this compares in terms of quality of thaumatin or market impact has yet to be established as has the benefit to the local and regional economy, in comparison to the established producers of thaumatin.

In terms of the benefits from intercropping of *T. daniellii* famers would hope to benefit from the sale of fruit, but also from the reduction in management costs associated with weeding and increased resilience to shocks due to fluctuations in rubber price. By diversifying income through using un-used land under the dense canopy of mature rubber benefits other than the principal economic ones of income from fruit and reduction in costs can also be envisaged, for example Vandermeer (1989) described the advantages of intercropping in terms of increased

productivity/yield advantage; better usage of available resources, such as, land, labour, time, water and nutrients; a reduction in the damage caused by disease, insects and weeds; and greater stability and increased human nutrition. (Vandermeer 1989). In terms of the rubber stand environment, though it competes with rubber, *T. daniellii* is a harmless weed, unlike others that snag and scratch and can trip tappers. Local markets already exist in West Africa for lamina and petioles of *T. daniellii* (Arowosoge and Popoola, 2006) and new research is being conducted in the region, focussing on the exploitation of extracts for taste and preservation properties (Okejale *et al.,* 2007; Adebayo and Kolawole, 2010), increasing the potential markets for *T. daniellii* grown under rubber.

7.2 Conclusion

With increased interest from consumers in natural products leading to manufactures investing in natural sweeteners (Jones, 2007) and an awareness that the 'obesity epidemic' is increasing worldwide (Kant, 2005), the use of thaumatin as a non-calorific sweetener, together with taste masking and flavour enhancement properties will likely lead to an expansion of the thaumatin market requiring supplementary sources of fruit supply. It has been demonstrated that there is clear variation in *T. daniellii* from the natural resource in terms of plant morphology and thaumatin content, though the latter is within industry standards. Most variation is between provenances with molecular studies and provenance experiments showing that that there is sufficient variation within populations to begin selective breeding for desired characteristics. *T. daniellii* is compatible with rubber as an intercrop and reductions in rubber productivity are potentially compensated by income from fruit and a reduction in management costs associated with weeding. *T. daniellii* has a large plasticity in its light requirement, surviving under mature rubber and in open canopy. It is rightly described as shade tolerant. *T. daniellii* has potential as a future intercrop of rubber in West Africa. Access to germplasm and establishment of, and access to, market chains are fundamentally important if the *T. daniellii*-rubber system is to become an accepted model in small holder rubber.

An integrated cross sector approach has the potential to drive this technology to diversify smallholder livelihoods and increase the long term sustainable supply of

fruit, benefiting existing actors, collectors, processors and end users, as well as new actors, as long as the market for thaumatin that currently exists does not decline.

7.3 Recommendations

7.3.1 Research recommendations

The present study has given rise to the following issues, which need further research attention. The study has shown that there is potential for *T. daniellii* to be incorporated with rubber as an intercrop but there is a need for socio-economic and agronomic research focussing on the following points.

- An economic sensitivity analysis should be conducted which would take into account the costs associated with its establishment and any yield loss in rubber together with production and income derived from *T. daniellii* fruit. Obviously the perception of *T. daniellii* as an intercrop across the broad spectrum of smallholders, from very marginal smallholders with a few hectares of land to those with larger smallholdings (40 ha) will have a great impact on uptake of the technology. By assessing smallholder incomes and livelihoods, reslience and level of risk aversion can be gauged and will help determine whether smallholders will benefit. The current market chains from Ghana and Ivory Coast could be used to ascertain and determine the transfer of wealth generated through them and if not equitable how to alter them to make them more so.
- Knowledge of the optimal period between harvesting and planting of rhizomes and the optimal lengths of rhizome for planting would be advantageous. Once a cutting station has been established there is an abundance of seedling material produced. Examining the time to fruiting and assessment of the advantages and disadvantages of propagation from seedling or rhizomes should be conducted, as should planting of germplasm from different parts of the range. As *T. daniellii* is seen as a potential intercrop of rubber determining the suitability of different clones would determine efficient use of germplasm material.

- The physiological response of newly growing material exposed to increased levels of PAR, and subsequent time to maturity should be studied in order to better inform the practice of planting *T. daniellii* with rubber.
- Taking plants from different provenances, including those outside of the range studied and planting them in different locations would be worthwhile to elicit factors that determine optimal growth characteristics, such as soil type and water deficit. An investigation into the variation of *T. daniellii* from other parts of the plant's range in terms of morphology, aril size and thaumatin content would be useful for finding suitable germplasm for selective breeding. A molecular analysis of type material, Ley 56, would help determine the status of the genus.

7.3.2 Promotion of the *T. daniellii*-rubber intercropping system

With the Common Fund for Commodities (CFC) already funding a multimillion dollar International Rubber Study Group project across West Africa "Enhancing Incomes of Smallholder Rubber Farmers in West and Central Africa" (CFC/IRSG/17) in a bid to retain smallholders in the rubber industry, through improving their knowledge, leading to best practice in an effort to increase their incomes, it seems they would be ideally situated to delegate the promotion of this intercropping system across Ivory Coast, Ghana and Cameroon to an appropriate body.

The technical co-ordinator for the CFC/IRSG/17 project in West Africa has suggested that a fast track project grant of US$100 000 be applied for from the CFC to promote basic research and promotion of this intercropping system (Bernard Nkouonkam, pers. comm. 2009).

Where market chains already exist, in Ghana and Ivory Coast, with cutting stations operating relatively close to areas where rubber smallholdings are situated it is thought that germplasm in the form of seedlings could be easily sourced. In-country organisations for promoting and utilising smallholder rubber, in Ghana, GREL/ROPP, in Cameroon, IRAD at Ekona, and in Ivory Coast, Centre National de Recherche Agronomique (CNRA) at Bimbresso, could support smallholders in the establishment of the *T. daniellii*-rubber intercropping system, with advice and

methods about planting and management based on findings in this thesis. Local academic institutions should be well placed to provide the academic research facilities to answer the research questions posed above. It is suggested that research is guided and co-ordinated by the international organisation responsible for research into agroforestry, the World Agroforestry Centre, based in Nairobi Kenya.

In Cameroon, where a market chain currently does not exist, models from Ivory Coast and Ghana can be studied to see if these models would work there. There is potential for cutting stations to be located at areas common to smallholders, for example rubber factories where the infrastructure is in place to develop these enterprises. It is assumed that initially arils would come from the natural resource: from national forests with the prior approval from the appropriate ministries or from the collection of this non timber product from local community or private land. An assessment of its range and abundance in the forests needs to be done to assess the impact of collection on the natural resource. It is envisaged that the first *T. daniellii* fruit produced from under rubber would begin to supplement natural collection three to four years after planting.

Discussion with stakeholders, including the end users of arils - those producing thaumatin, would determine the price of arils from Cameroon and lead to the development of a new aril processing industry there thus providing new sources of income to a wide range of new actors.

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Appendix I: Effect sizes: Eta squared, η^2, partial eta squared, partial $\eta^2$and omega squared (ω^2).

An effect size is simply an objective (and usually) standardised measure of the magnitude of observed effect. A highly significant result may have a very small effect, that is its impact or importance may be small. Field (2009)gives a succinct description of effect size measures and how to calculate them when using ANOVA and t-test analyses. Those used in this thesis are briefly explained below with reference to Field (2009), Brown (2008) and Thomas (Thomas, 2006).

Eta squared (η^2)

Eta squared, η^2 is an effect size calculated as the between-group effect sum of squares, SS_{effect}, (the sum of squares for whatever effect is on interest) divided by the total amount of variance in the data, sum of squares total, SS_{Total} (the total sum of squares for all effects, interactions, and errors in the ANVOA) . Essentially it is the proportion of total variance explained by the effect:

$$\eta^2 = \frac{SS_{effect}}{SS_{Total}}$$

Where t- tests are conducted the following equation is used to determine the effect size:

$$\eta^2 = \frac{t^2}{t^2 + (N1 + N2 - 2)}$$

Where t is the t-test statistic, N1is the number of the values in the first group, N2 is the number of values in the second group, where $\eta^2 = 0.01$ (1%) is a small effect, 0.06 (6%) is a moderate effect and 0.14 (14%) is a large effect (Thomas, 2006).

Partial eta squared ($\eta^2_{partial}$)

Partial Eta squared, $\eta^2_{partial}$, is similar to η^2 except that it explains the proportion of variance that a variable explains that is not explained by other variables in the anlaysis: it does this by including the error variability for the model,

$$\eta^2_{partial} = \frac{SS_{effect}}{SS_{Effect} + SS_{Residual}}$$

Where, $SS_{Residual}$, is the residual sum of squares, the error variability, for whatever effect is of interest.

Omega squared (ω^2)

However, a more robust measure of effect size is omega squared, ω^2, which uses η^2 together with the variance and error variance (the mean squared error ($MS_{Residual}$) explained by the model, (Field 2009), and is given by the following equation:

$$\omega^2 = \frac{SS_{effect} - (dfM)MS_{Residual}}{SS_{Total} + MS_{Residual}}$$

where dfM is the degrees of freedom.

Where interest in the interaction of factors is being investigated using ANOVA, a variance component and error for each factor plus the interaction of factors, has to be first computed. This is then simply divided by the total variance to give the size effect, ω^2.

Briefly variance is computed for each of the factors plus the total variance using the following four equations, given two effects and their interaction, in this case, (1) block, (2) treatment, the block*treatment interaction(3) and total variance (4):

$$\hat{\sigma}_{\alpha}^2 = \frac{(a-1)(MS_A - MS_R)}{nab} \quad (1)$$

$$\hat{\sigma}_{\beta}^2 = \frac{(a-1)(MS_B - MS_R)}{nab} \quad (2)$$

$$\hat{\sigma}_{\alpha\beta}^2 = \frac{(a-1)(MS_{A*B} - MS_R)}{nab} \quad (3)$$

$$\hat{\sigma}_{total}^2 = \hat{\sigma}_{\alpha}^2 + \hat{\sigma}_{\beta}^2 + \hat{\sigma}_{\alpha\beta}^2 + MS_R \quad (4)$$

where:
$\hat{\sigma}_{\alpha}^2$ = variance of factor 1 (block)
$\hat{\sigma}_{\beta}^2$ = variance of factor 2 (treatment)
$\hat{\sigma}_{\alpha\beta}^2$ = variance of the interaction (block * treatment)
MS_A = Mean square of factor 1 (block)
MS_B = Mean square of factor 2 (treatment)
MS_{A*B} = Mean sqaure of interaction (block * treatment)
MS_R = Mean sqaure of the error
a = number of levels of factor 1 (e. g. 2 blocks)
b = number of levels of factor 2 (e. g. 3 pruning treatments)
n = number of replicates per treatment (e. g. 3 replicates of treatment)

To find the effect size ω^2 divide the variance of the effect by the total variance, as shown below:

$$\omega^2_{effect} = \frac{\hat{\sigma}^2_{effect}}{\hat{\sigma}^2_{total}}$$

After Field (2009) and Brown (2008)

Effect sizes are only shown where a statistically significant difference is seen.

Appendix II: CTAB DNA extraction protocol, after Kerényi *et al.*, 1999, with notes

- 50 mg of dried tissue is taken in a safe lock tube (Eppendorf) along with two beads. The tube is suspended in liquid nitrogen for one minute, placed inside an adaptor set, loaded into the tissue lyser (Roche) and run for 60 seconds at 30 Hz twice. In between lysing of the material, place the tubes back into liquid nitrogen.

- Remove the safe lock tube from the adaptor set and immediately add 700 µl of pre warmed CTAB extraction buffer. The tube is vortexed to mix the contents thoroughly. The CTAB extraction buffer consists of:

    - 2 % CTAB: a cationic detergent used to destroy membranes, denature proteins and dissociate proteins from DNA (Weising et al, 2007).
    - 1.4 M NaCl: a high salt concentration that dissociates nuclear proteins from DNA (Aljanabi and Martinez, 1997) and keeps polysaccharides in solution during ethanol precipitation (Fang et al, 1992)
    - 20 mM EDTA: ethylenediaminetetraacetic acid. A chelating agent that capture bivalent metal ions
    - 100mM Tris: a buffer that maintains the pH in a range between 8 – 9, avoid thus reducing the activity of degrading enzymes. (Weising et al, 2007)
    - 1 % PVP: a polyphenol adsorbent, used to reduce the polyphenol and phenol quantity. Polyphenols and phenols can cause damage to DNA, and browning of DNA products through oxidation (Weising et al, 2007)
    - 1% β-mercaptoethanol: a reducing agent that inhibits oxidation processes caused by phenols, polyphenols, alkaloids and flavonoids (Weising et al, 2007).

- The tubes are then incubated for 3 hours at 65°C and gently swirled every 15 minutes.
- Following incubation the products are transferred to a fresh 1500 µl tube with a wide bore pipette (a normal pipette that has had its tip cut off will suffice). To this is added 500 µl Cholorform:Isoamyl alcohol (24:1). Cholorfom:Isoamyl alcohol is used to remove dissolved proteins. (Weising et al, 2007). The mixture is thoroughly mixed by inverting the tube continuously for 10 minutes.

- The tube and its contents are then centrifuged for 10 minutes at 8000 rpm. The supernatant is then transferred to a new 1500 µl tube with a wide bore pipette.

- To the supernatant add 0.5 volumes of 5M NaCl and then 0.4 volumes (of the supernatant and 5M NaCl mixture) of ice cold Isopropanol. The mixture is gently mixed by inverting the tube and then kept at -20° C for 1 hour. (This can be kept at this temperature overnight if needed).

- The tube is then centrifuged at 10 000 rpm for 10 minutes. (In case of no precipitation place the tube back on ice for 20 minutes and centrifuge again). After this process there should be a pale white pellet of DNA at the base of the tube.

- The supernatant is removed by pipetting. Add 1.0 ml 70 % ethanol. Gently swirl and let the DNA remain in the ethanol for 20 minutes. Remove the ethanol by pipetting and rewash with 70 % ethanol

- Dry the DNA pellet by inverting the tube onto a paper towel for 1 hour.

- Add 500 µl of TE (Tris EDTA) to re-suspend the DNA pellet and leave for 1 hour or overnight in a fridge at 4° C.

- Add RNaseA to degrade any RNA that may be present. RNaseA is added to a final concentration of 250 µg/ml. This is mixed and incubated for 1 hour at 37° C.

- To the resultant add 500 µl Choloroform:Isoamyl alcohol (24:1) and mix by inverting for 10 minutes. This removes degraded RNA and left over proteins, cleaning the DNA.

- Centrifuge the tube and its contents at 12 000 rpm for 1 minute. This separates the DNA in solution from the Chloroform:Isoamyl alcohol mixture containing the residues.

- Using a pipette the supernatant containing the DNA in solution is transferred to a new tube and an equal volume of ice cold Isopropanol is added to precipitate the DNA. This is kept at -20° C for 1 hour.

- The final centrifuging step takes place for 2 minutes at 12 000 rpm to precipitate the DNA. The supernatant is removed with a pipette, taking care not to dislodge the DNA pellet.

- The DNA pellet is washed in 70 % ethanol, dried by inverting the tube onto a paper towel for 20 minutes, and re-suspended in 50 µl TE. The DNA can then be stored in a fridge for immediate use or for long term at -20° C.

Appendix III: Quantification of DNA using the ND- 1000 Spectrophotometer (Thermo Fisher Scientific) with notes.

Equipment

DNA samples on ice

Buffer that DNA has been stored in, e.g. TE buffer.

PCR grade water

Micro-pipette tips (0.1 – 20µl), sufficient to quantify all your samples.

- Switch on the computer and log on as normally.

- Select the program ND-1000 from the desktop. Select DNA and RNA analysis.

- Intialise the Nanodrop:
- Lift the arm of the ND-1000, and swipe the lens on the main body and the receiving lens on the arm with a clean dry tissue. Select initialise on the computer screen, place a drop (0.2 µl) of ultra pure water on the lens of the main body of the ND-1000, and replace the arm gently above the drop of water. Press, to start initialisation.

- The arm of the ND-1000 will be pulled down by electromagnets to the main body of the machine and as such the drop will now be held between the arm and main body whilst the spectrophotometer takes a reading and initialises the machine.

- Once the ND-1000 has been initialised, clean the lenses using the tissues, then add a drop (0.2 µl) of the buffer that was used to store the DNA onto the main body lens. E.g. TE buffer. Again move the arm so that it is resting above the sample. Press Blank on the computer program. This again will move the arm down and take a reading.

- Once this has finished, clean the lenses again, type the name of the sample you are going to analyse into the computer program, add a 0.2 µl drop of the sample onto the lower lens, replace the arm, and select Test Sample. This will take a reading.

- Repeat for the rest of the samples. Save your information for export to Excel.

Appendix IV: Modified AFLP Protocol, (after Vos et al. 1995) plus additional notes

1. Preparation

Prepare working concentrations of primers and adaptors. See Appendix V

Quantify DNA and calculate volume that contains 0.5 ug (500 ng) DNA. (Appendix III). Appendix VI shows how to calculate the correct volume of DNA containing 500ng.

It is strongly suggested that a sample of DNA, of a different species to that being studied, with known size and concentration already used in an AFLP analysis is used as a template/guide and will help indicate whether your samples, primers, adaptors, and or enzymes are working/are of sufficient quality.

2. Restriction Digest – NOTE ADD MASTERMIX TO DNA SAMPLES!!!

Vol for 1 sample	Restriction Component
4 µl	10 x NEB Buffer
0.1 µl	5 units EcoRI (50U/ µl)
0.5 µl	5 units Mse I/Tru 9I (10U/ µl)
15.4 µl	PCR grade water
Total: 20 µl	
Plus	**DNA Component - DO NOT ADD TO MASTERMIX**
Total: 20 µl	DNA component containing 0.5 µg (500ng) DNA, made up to 20 µl with PCR grade water – see appendix for clarification and examples.
Total: 40 µl	Restriction component plus DNA component.

If there are multiple sample of DNA to restrict, make a batch of restriction digest component to the value of N + 4, where N is the number of samples are being restricted. Adding 4 more volumes of restriction component will ensure that pipetting loss will not result in insufficient restriction digest. If there are more than 10 samples produce an appropriate increased volume of master mix, perhaps N+6.

E.g: for **10 samples** make enough restriction digest for 14 samples:

Vol for 10 samples (i.e. volume for 14 samples)	**Restriction Component**
56 µl	10 x Buffer W
1.4 µl	5 units EcoRI (50U/ µl)
7 µl	5 units Mse I/Tru 9I (10U/ µl)
215.6 µl	PCR grade water
Total: 280 µl	**Pipette out 20 µl of this mastermix into each sample of DNA component.**
	DNA Component per sample
Total: 20 µl	0.5 µg (500ng) DNA component made up to 20 µl with PCR grade water – see appendix for clarification
Total: 40 µl per sample	Restriction component plus DNA component.

Pipette out 20 µl of the mastermix into each sample of DNA component.
Incubate for 1 hour at 37°C.

3. Ligation Step

Vol for 1 sample	**Ligation component**
1 µl	EcoR1 adaptor (5pMol)
1 µl	Mse1/Tru91 (50pMol)
1 µl	T4 DNA Ligase Buffer (10x)
0.3 µl	T4 DNA Ligase (1 unit)
6.7 µl	PCR grade water
Total: 10 µl	

Pipette 10 µl of the ligation component to the PCR tubes containing the result of the restriction digest.
This will make up a 50 µl solution.
Incubate tubes at 16°C overnight.

Note: follow the same procedure for producing larger batches of ligation component when restricting more than one DNA sample, as for the restriction component.

Vol for 10 sample (i.e volume for 14 samples)	**Ligation component**
14 µl	EcoR1 adaptor (5pMol)
14 µl	Mse1/Tru91 (50pMol)
14 µl	T4 DNA Ligase Buffer (10x)
4.2 µl	T4 DNA Ligase (1 unit)
93.8 µl	PCR grade water
Total: 140 µl	**Add 10 µl of this master mix to each sample from the restriction digest.**

4. Pre-Amplification PCR

Pre-Amp PCR Mix

5 µl **Pre-Amplification primer mix – the method to make the primer mix is given in the table below.**
10 µl Bioline Biomix ™ PCR Master Mix containing 2.0 mM $MgCl_2$
5 µl DNA from the R/L reaction

Pre-Amplification Primer Mix

Vol for 1 sample	Component	For 10 samples
0.3 µl	universal primer 50 ng/ µl (E00 for EcoR1)	
0.3 µl	universal primer 50 ng/ µl (M00 for Mse I)	
4.4 µl	PCR grade water	
Total : 5.0 µl		

Use thermal cycler (PCR machine) programmed as follows:
94°C 30 sec }
56°C 1 min } x 30 cycles
72°C 1 min }
72°C 10 mins

NB1: Quantities of primers have been reduced from 0.6 µl to 0.3 µl to reduce the effect of primer dimer and dimer/dimer peaks on the sequencing machine.

NB2: DO NOT USE master mix that has a dye, for example Bioline Biomix Red ™ as this can affect the peaks that the sequencer produces. Use a master mix without dye.

NB3: Use the same methods as previously to produce batches of master mix. N+4!

Check pre-amp product on 1% agarose gel for smear. If this does not show anything consider using a high resolution metaphor 3% agarose gel (see Appendix VII).

5. Selective amplification PCR

Dilute the pre-amp PCR product, from the previous step, in Sterile Distilled Water (SDW) or PCR Grade Water (try1:10, 1:30 or 1:50).

Note when diluting the PCR product do so as follows: 1:10 = 1 part PCR product to 9 parts SDW; 1:30 = 1 part PCR product to 29 parts SDW, and so on.

PCR the pre-amp PCR product using labelled primers for use on Beckman Coulter CEQ 8000:
Selective amplification PCR Mix

5 µl diluted pre- amp PCR product 'DNA' (from diluted pre-amp at 1:10; 1:30 or 1:50 or other concentration)
+
5 µl **Selective amplification primer mix** (See table below for production of Selective amplification primer mix)
+
10 µl Bioline Biomix ^{TM} containing 2.0 mM $MgCl_2$

Selective Amplification PRIMER Mix

Vol for 1 sample	Component	For 10 samples
0.25 µl	labelled primer (50ng/ µl)	
0.3 µl	unlabelled primer (50ng/ µl)	
4.45 µl	PCR grade water	
Total: 5.0 µl		

Use thermal cycler programmed as follows:
94°C 30 secs }
65°C 30 secs subtract 0.7°C per cycle} x13
72°C 1 min }

94°C 30 secs }
56°C 30 secs } x23
72°C 1 min }
10°C hold

NB4: Quantities of primers are reduced from 0.5 µl and 0.6 µl to 0.25 µl and 0.3 µl for labelled and unlabelled primers respectively in the Selective amplification Primer mix, to reduce the effect of primer dimer and dimer/dimer peaks on the sequencing machine.

6. Preperation of PCR products for running on Beckman Coulter CEQ 8000

You will need to optimise the dilution of your PCR product for fragment analysis. You must always run a complete row of 8 samples at once in a per sample plate. If you can't fill up a row, use 40 µl SLS (Sample Loading Solution – de-ionised formamide) in each empty well.

Defrosting of frozen aliquoted SLS is required; do this in a centrifuge, NOT A VORTEX!

Suggestion for initial CEQ run:

Volume	Component	Volume
40 µl	SLS (de-ionised formamide from Beckman Coulter)	
0.5 µl	size standard (from Beckman Coulter) per sample well	
0.5 µl	undiluted PCR product per sample well	

It is strongly suggested that you make a 'mastermix' is made of the SLS plus the size standard for the number of samples that are to be run. Remember to include more volumes for pipetting loss: the N+4 suggestion applies here too. It is suggested that this mastermix is pippetted into the wells in the sample plate tray, followed by the undiluted PCR product from the Selective amplification.

You can adjust this volume up or down according to signal intensity of size standards compared to PCR product peaks.

NB size standards are available in two forms
400 bp (standard fragments range from 60 bp to 400 bp)
600 bp (standard fragments range from 60 bp to 600 bp)

Appendix V: Preparation of working concentrations of primers and adapators

1. Re-suspension of adaptors:

Information received with adaptors will come in the following form

MseI – A1 (name of the adaptor)			
'GTAC GAT GAG TCC TGA G – 3' (oligo sequence)			
Amount Concentration (Volume 1M)		16pMol/µl	length 16 – mer
3.0 OD	Volume for 100pMol/µl	**165 µl**[1]	GC Content 56.3%
32µg	Molecular weight	4946g/Mol	Scale 0.01 µmol
16.5 nMol	Tm	51.7°C	Purification HPSF

[1]To obtain a 100pMol/µl solution of the oligo simply resuspend the dried powder into 165 µl of PCR grade water. **You will then have 165 µl of a 100pMol/µl solution of the adaptor.**

2. Production of adaptors:

The concentration of adaptor required determines the production of the adaptors. In this AFLP protocol two adaptors are used: EcoR1 adaptor and Mse1/Tru9 adaptor.

The requirements are as follows: EcoR1 at 5pMol concentration; Mse1/Tru9 adaptor at 50pMol concentration (See AFLP protocol)

EcoR1 adaptor production:

EcoR1 A1 = 5' CTC GTA GAC TGC GTA CC 3'
EcoR1 A2 = 3' CTG ACG CAT GGT TAA 5'

This produces an adaptor with an overhang of -TTAA

To produce 100 µl of EcoR1 adaptor at 5pMol concentration we need to ligate together both the A1 and A2 adaptors using the following quantities:

5 µl of 100pMol/µl EcoR1 A1 adaptor
5 µl of 100pMol/µl EcoR1 A2 adaptor
90 µl PCR grade water

Pipette these products into a PCR tube and place into a PCR thermocycler programmed to heat the products for 4 minutes at 98°C (Weising et al, 2005 pp155).

Mse1/Tru9 adaptor production:

Mse1 A1 = 5' GAC GAT GAG TCC TGA G 3'
Mse1 A2 = 3' TA CTC AGG ACT C AT 5'

This produces an overhang of - AT

To produce 100 µl of Mse1/Tru9 adaptor at 50pMol concentration we need to ligate together both the A1 and A2 adaptors using the following quantities:

50 µl of 100pMol/µl Mse1 A1 adaptor
50 µl of 100pMol/µl Mse1 A2 adaptor

Pipette these products into a PCR tube and place into a PCR thermo cycler (PCR machine) programmed to heat the products for 4 minutes at 98°C.

Hint:
Smaller quantities of adaptors can be produced using simple proportion to obtain the necessary quantities.

3. Re-suspension of primers:

Information received with primers is essentially the same as for adaptors, except that as units of ng/µl or µg/ml are used, different information has to be used.

M61 (name of the primer)			
'GAT GAG TCC TGA GTA ACT G' (oligo sequence)			
Amount Concentration (Volume 1M)		20pMol/µl	length 19 – mer
4.3 OD	Volume for 100pMol/µl	µl	GC Content 47.4%
101µg[2]	Molecular weight	5868g/Mol	
19.9 nMol	Tm	54.5°C	Purification HPSF

[2]Suspending the dry powder in 1ml of PCR grade water will produce a solution with a concentration of, in this case 101µg/ml, which is equivalent to 101ng/µl as can be seen below:

101µg/ml = 101000ng/1000 µl

Dividing by 1000 gives 101 ng/µl

(Remember 1 µg = 1000 ng and 1ml = 1000 µl) Therefore µg/ml ~ ng/µl

4. Production of primers:

This is a quick reference guide as it is clear that companies do not always provide primers in the same quantity/concentration. Thus, a simple method to produce new primers and understand the key information provided on yields from manufactures has been written.

Using the formula V1N1 = V2N2 where V1 and N1 are the volume and concentration for the stock solution and V2 and N2 are the volume and concentration for the working solution, we can work out the quantities of primers and PCR grade water that are needed to produce primers at working concentrations from stock solutions. These are produced from primers that come in dry powder form from manufacturers.

Note: The AFLP protocol tends to rely on the use of 50ng/µl for working concentrations, therefore it is sensible to have 50ng/µl as a minimum stock concentration.

Example:

Primer M00 in dry powder form had a yield of 117 µg. Adding this to 1ml of PCR grade water gave a **stock solution** with concentration of 117 µg/ ml ~ 117 ng/ µl. This is our **stock concentration**.

To get to a working concentration of 50ng/µl we need to use V1N1=V2N2. If we want to make 100µl of a 50ng/µl working concentration, let us first work out the amount needed to make 1ml working solution, then using simple proportion we can work out the quantity needed to make 100µl.

V1 (ml ?) * 117ng/µl (N1 – stock solution concentration) = 50ng/µl (N2) * 1ml (V2)

Is the same as:

V1 *117ng/µl = 50 (ng/µl / ml)
V1 = 50 / 117
V1 = 0.427ml

Therefore, to make a 1ml solution of 50ng/µl concentration working solution from a stock solution of 117ng/µl we need 0.427ml of the working concentration made up to a 1 ml quantity with 0.572 ml of PCR grade water.

0.427 ml+0.572 ml = 1 ml

This is equivalent to:

427 µl + 572 µl = 1000µl

As we require only 100 µl we can divide each side by 10 and get the values we require, in this case:

42.7 µl of stock solution + 57.2 µl of PCR grade water to give us 100µl of 50ng/µl working concentration of primer.

If The Yield Of The Primer Is Less Than 50µg We Can Still Make A Working Stock Solution Up To 50 Ng/µl Using The Following Formula.

Yield = Concentration * PCR grade water.

E.g: The yield of a primer, E15, was 49µg. To make a stock solution of 50 ng/µl do the following:
List the products you have:

- Concentration required = N (50ng/µl ~ 50µg/ml)
- Amount of PCR water primer needs to be diluted in to give the concentration required = W (?)
- Yield of product from suppliers in µg = Y (49 µg)

Then W = Y/N

W= 49 µg / 50 µg/ml

W = 0.980 ml ~ 980 µl

Therefore adding 0.98ml of PCR grade water to the primer in dry powder form will give you a stock concentration of 50ng/µl ~ 50µg/ml.

Appendix VI Method to calculate the necessary volume of DNA containing 500ng of DNA from quantification data.

For the restriction:ligation (R:L) reaction 500ng or 0.5 µg of DNA are required. This has to be suspended up to a volume of 20 µl using PCR grade H_20.

The total reaction volume for R is 40 µl made up of the following:

Restriction mastermix = 20 µl
DNA component = 20 µl

The DNA component:

Using V1N1 = V2N2 as previously, we can work out the necessary amount of DNA from our sample that has to be suspended in PCR grade H_20.

V1 (volume of DNA required from sample) *N1 (conc. of DNA) = V2 (1 µl) * 500ng/ µl (conc. of final DNA required).

V1 = 500/concentration of DNA from sample.

e.g. DNA concentration is 100ng/µl we will require:

V1 = 500/100
V1 = 5 µl

Then to work out the volume of PCR grade H_20 we simply subtract the volume of DNA required from our sample from 20 and the remainder is the amount of PCR grade H_20 required. These are mixed in an eppendorf and can be stored on ice during use.

Table Example of DNA concentration of sample and volume required for restriction reaction.

Sample name	DNA concentration (ng/µl)	Volume of DNA required (µl)	Volume of PCR grade H_20 (µl)
Mm 2.1	61.76	8.1	11.9
Mm 1.1	111.16	4.49	15.5
GBL 40	199.17	2.51	17.49
GBL 39	128.26	3.9	16.1

Appendix VII: Protocol for the preparation of high resolution (Metaphor) 3 % agarose gel

1. Set up gel casting tray with 20 deep well comb. Use the 80 ml volume tray.
2. Add 80ml chilled 1xTAE to a sealable bottle with a magnetic stirrer bar – make sure the bar is clean.
3. Sprinkle in 2.4 g SFR agarose while stirring with the magnetic stirrer
4. Leave stirrer on for 10 minutes – then remove the magnetic bead
5. Close lid then half open and heat in a microwave for the following period:
    a. 2 minutes at power level 7 then swirl gently
    b. 1.5 minutes at power level 7 then swirl gently
    c. 30s at power level 7 (2-3 times) stirring gently inbetween.
6. Allow to cool to 50-60°C (so that you can hold it in your hand)
7. Add SafeView™ 4µl to gel and pour immediately into the casting tray.
8. Allow to cool and set at room temperature, then chill in the fridge. At this stage you can cover with Saran wrap.

Appendix VIII: Running a Principal Component Analysis on molecular marker data (AFLP) using NTYSYpc.

Production of PCA plots; similarity and dissimilarity matrices; cluster analysis, to produce dendograms showing relationships between and in between species.

Introduction

This guide is meant as a quick reference when setting out to analyse molecular marker data (binary matrix of AFLP fragments from running an AFLP) principally using NTSYSpc.

Section 1: INPUT DATA INTO NTedit / NTSYSpc

1. Generate a binary data matrix in Excel so that the file looks like this:

1	8	5	9		
	Fuji	Cho	M3	M9	Ko
	0	0	0	0	1
	0	1	0	0	0
	1	0	0	1	0
	0	0	0	1	0
	1	0	1	0	1
	0	0	1	0	0
	0	0	0	0	1
	1	1	1	0	1

In the top row use the appropriate values for the data set, in the above example
8= number of data rows (these are the number of loci, fragments that are polymorphic)
5= number of data columns (effectively the number of samples you have)
9 = values for missing data

The first column must be empty except for the number1 in the top row: this 1 means that the matrix is a rectangular data matrix.

1. rectangular data matrix
2. symmetric dissimilarity matrix
3. symmetric similarity matrix
4. diagonal matrix
5. tree matrix for dissimilarity data
6. tree matrix for similarity data

7. graph for dissimilarity
8. graph for similarity

2. Save and shut the binary matrix in Excel. Make sure that it is the first worksheet in the Excel file.

3. Open this Excel file in NTedit, using **OPEN IN FILE GRID** , and save as **name.nts** (NB: when using NTSYSpc version 2.1, make sure that the Excel file is saved using Excel 97-2003, not the compatibility mode in Excel 2007.) Name.nts is your file name with the .nts extension.

4. Open NTSYSpc2.1 and select **SIMILARITY**, choose **INTERVAL DATA**. Change coefficient to **CORR**, click on input file and chose the correct path for the **name.nts** (file from above). Then name an output file, e.g. **nameout.nts**.
5. Finally press **COMPUTE**. (This produces a correlation matrix from your original binary data.) Other options here include DIST, EUCLD, VARCOS,COSINE – A screen displays the information for you. To move to the next step, just simply move the screen and click back onto the NTSYSpc screen.
6. Open NTSYSpc 2.1 and select **ORDINATION**, choose **EIGEN**. Use the filename **nameout.nts** as the input file and keep the number of dimensions as **4.** Name two additional new files: e.g. **namevec.nts** for the eigen vector file and **nameval.nts** as the eigen value file. Leave the other options set to default; ie. The vector scaling.
7. Finally press **COMPUTE**. Your output should look something like that below. You should have a screen with values and percentages. If the first three of these are quite large numbers then you have shown that the principal components of your analysis have explained most of the variation in your data. Which is good by the way! Save this screen as a text file, using the file menu.

eigen: NTSYSpc 2.10t, (C) 2000-2001, Applied Biostatistics Inc.

Date & time: 02/03/2010 19:54:16

Input parameters

Read input from file: D:\Work by year\2010\PhD 2010\Genetic Work\Fragment analysis\analysis 2_3_10\frag_anal_2_primer_out.NTS

Number of dimensions: 4

Save eigenvectors in output file: D:\Work by year\2010\PhD 2010\Genetic Work\Fragment analysis\analysis 2_3_10\frag_anal_2_primer_evec.NTS

Save eigenvalues in output file: D:\Work by year\2010\PhD 2010\Genetic Work\Fragment analysis\analysis 2_3_10\frag_anal_2_primer_eval.NTS
Scaling: SQRT(LAMBDA)

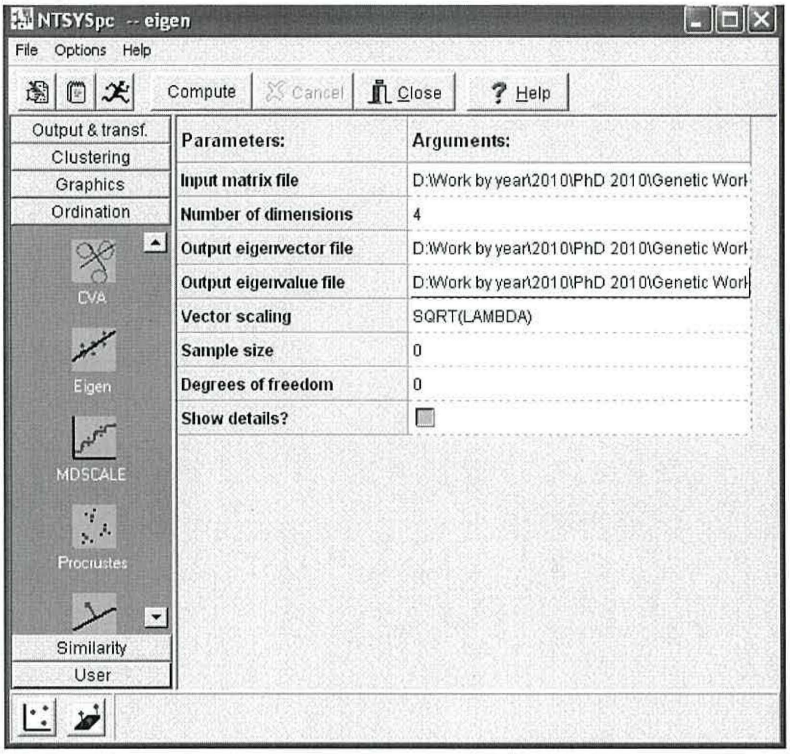
Comments:
SIMINT: input=D:\Work by year\2010\PhD 2010\Genetic Work\Fragment analysis\analysis 2_3_10\Frag_anal_2primer_2_3_10.NTS, coeff=CORR, direction=Cols
Matrix type = 3, size = 22 by 22, missing value code = "none" (similarity)

i	Eigenvalue	Percent	Cumulative
1	11.20119971	50.9145	50.9145
2	4.01805699	18.2639	69.1784
3	2.88153621	13.0979	82.2763
4	1.62677236	7.3944	89.6708
5	1.30084720	5.9129	95.5837
6	0.22386408	1.0176	96.6013
7	0.18644410	0.8475	97.4487
8	0.14098336	0.6408	98.0896
9	0.10767010	0.4894	98.5790
10	0.09343311	0.4247	99.0037
11	0.06768933	0.3077	99.3113
12	0.05681146	0.2582	99.5696
13	0.04750312	0.2159	99.7855
14	0.03347784	0.1522	99.9377
15	0.01371101	0.0623	100.0000
16	0.00000000	0.0000	100.0000
17	0.00000000	0.0000	100.0000
18	0.00000000	0.0000	100.0000
19	0.00000000	0.0000	100.0000
20	0.00000000	0.0000	100.0000
21	0.00000000	0.0000	100.0000
22	0.00000000	0.0000	100.0000

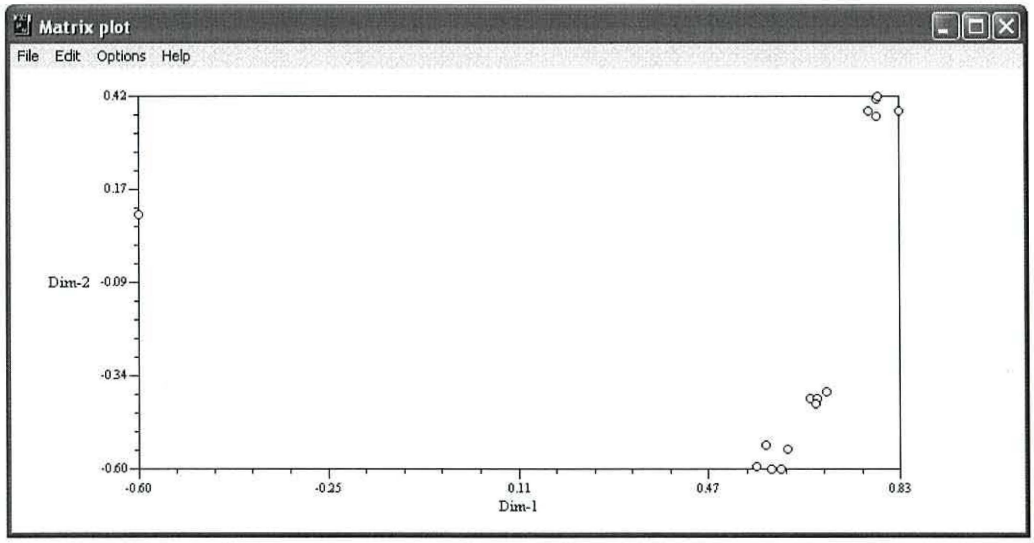
Sum of eigenvalues = 22.000000
Eigenvalues (4 by 4) saved in file: D:\Work by year\2010\PhD 2010\Genetic Work\Fragment analysis\analysis 2_3_10\frag_anal_2_primer_eval.NTS
Eigenvectors matrix (22 by 4) saved in file: D:\Work by year\2010\PhD 2010\Genetic Work\Fragment analysis\analysis 2_3_10\frag_anal_2_primer_evec.NTS

8. Click off the screen with all the data and click back onto the NTSYSpc screen. At the bottom left hand part of the screen there will be two icons from which

you will be able to produce some diagrams – click the left hand of these two diagrams.

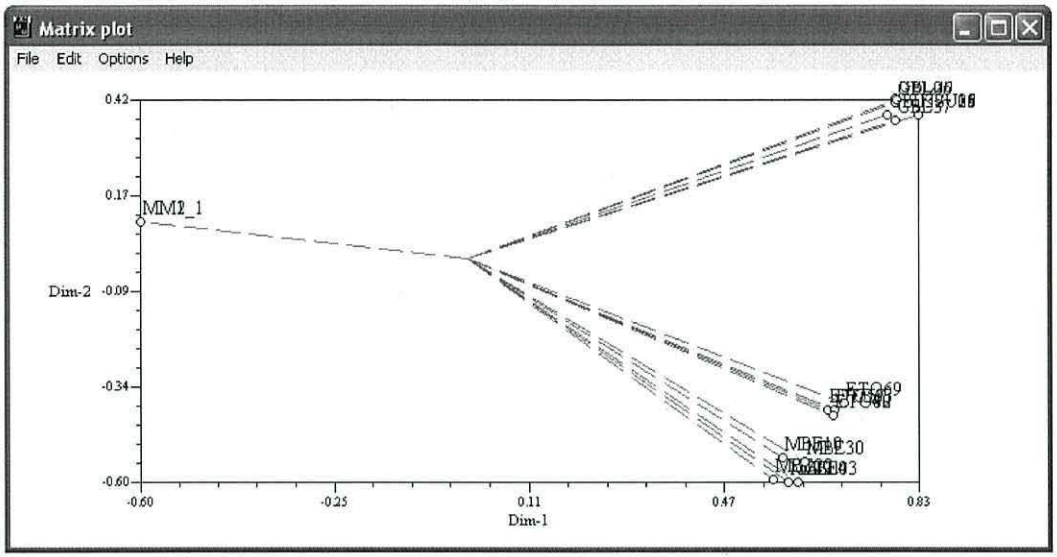


9. You should get a principle components analysis diagram like this.



10. This in itself is not too useful, so click onto the OPTIONS menu and select PLOT OPTIONS. A small box will appear; here you can select a title.

11. Select POINTS and select LABELS and click the show vectors check box: once selected click OK and continue. This will show you the correlations between samples and between populations, as below.



-
- **Lines to the right of centre indicate positive correlation between populations; lines to the left indicate a negative correlation. The angular distances between lines (i.e between samples) shows how much variation is found between the samples/clusters. In the diagram above it can be seen that there is little variation between clusters.**

12. This file can be saved as a metafile * .EMF e.g **name.emf**
    - **To look at this file again open it in NTedit.**

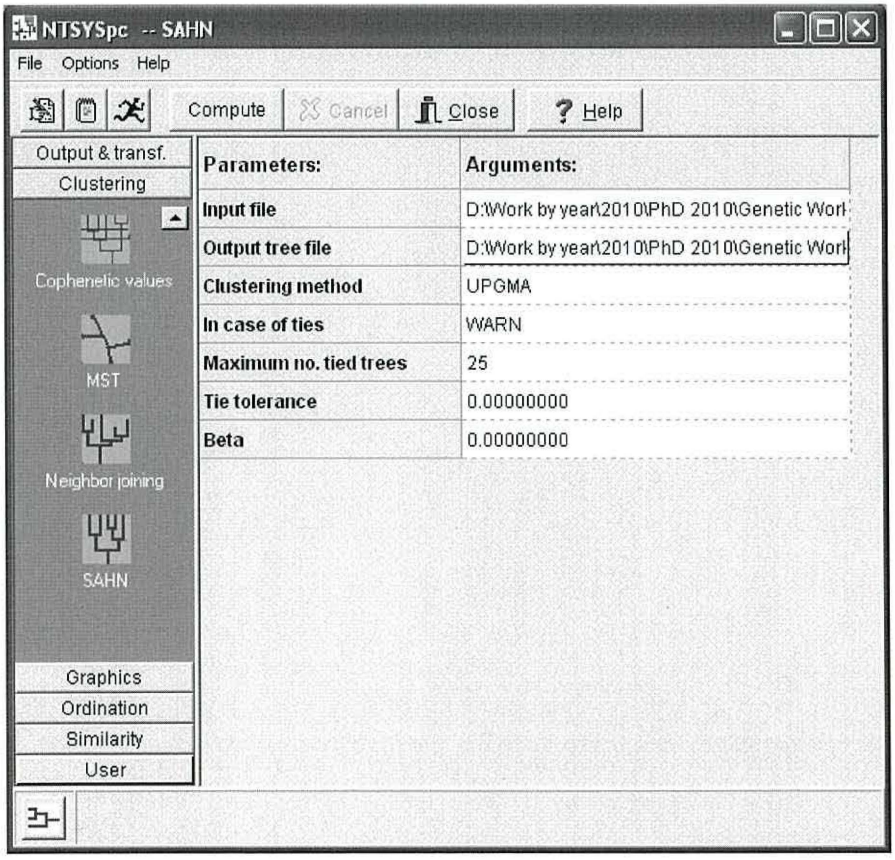
Section 2: Producing dendograms and tables of similarity coefficients.

(Jaccards; Nei and Li; and SM similarity coefficients.)

1. Select the **SIMILARITY** icon and then the **QUALITATIVE** icon.
2. Input the binary data file from Excel (rectangular data matrix)
3. Coefficient should be selected to **J** (Jacard, 1908) or **D** Dice (for a Nei and Li coefficient (1945)) or SM for Simple Matching coefficient.
4. Output: this will be the associated co-efficients file i.e: should be a similarity matrix describing the coefficient used e.g.when using the J (Jaccard's coefficient) use the file **name_jac.nts** for example.
5. Positive 1; negative 0.
6. Press Compute.

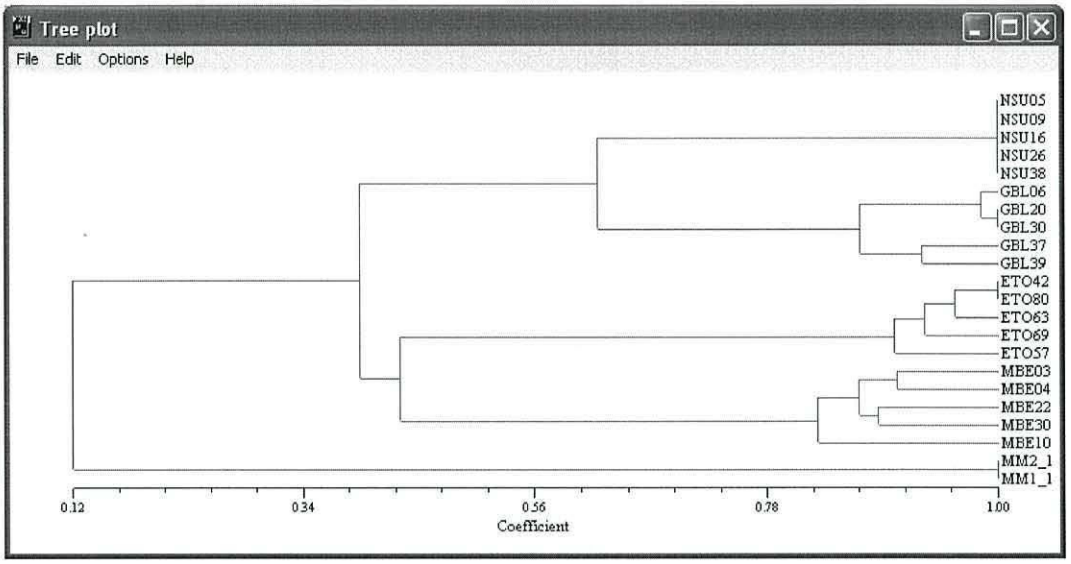
To produce the dendogram

1. Select **CLUSTERING** then SAHN – **this uses the UPGMA clustering algorithm.**
2. The input file should be the associated coefficients file from above. i.e. **name_jac.nts**
3. Output **namesahntreefile.nts**
4. Press COMPUTE



- **You may get a warning saying something like "At least one tie found that could change clustering. You may wish to use the FIND option." Ignore this for the time being.**

5. By clicking the dendogram icon at the left hand corner base of the screen you can get a dendogram of your samples produced.



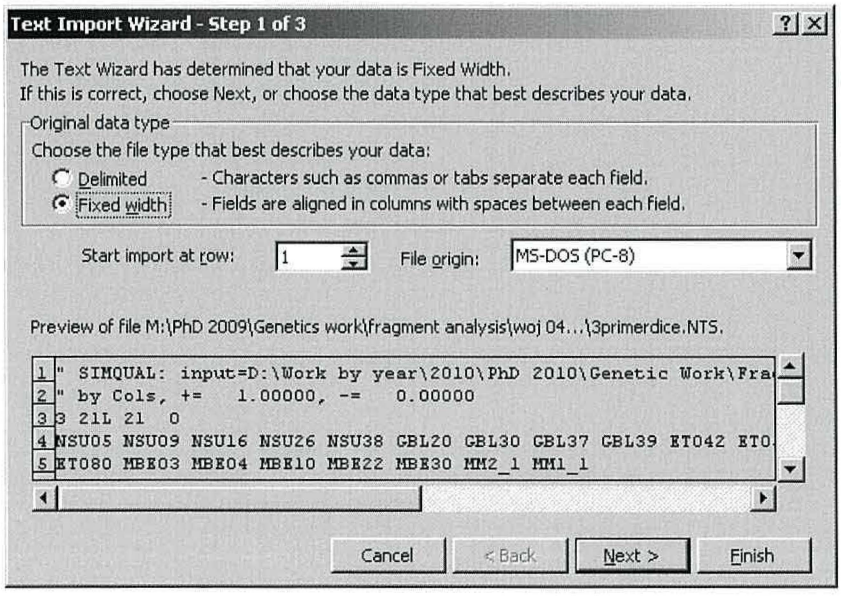
Section 3: The neighbour joining algorithm - production of similarity and dissimilarity matrices

To produce a dendodgram using the more advanced and robust clustering algorithm, Neighbour Joining (NJ) we firstly have to produce a dissimilarity matrix. This will be discussed after we have produced our tables of similarity using files produced in NTSYSpc using the Jaccard's, Nei and Li and /or SM coefficients (Section 2, point 3-4, above).

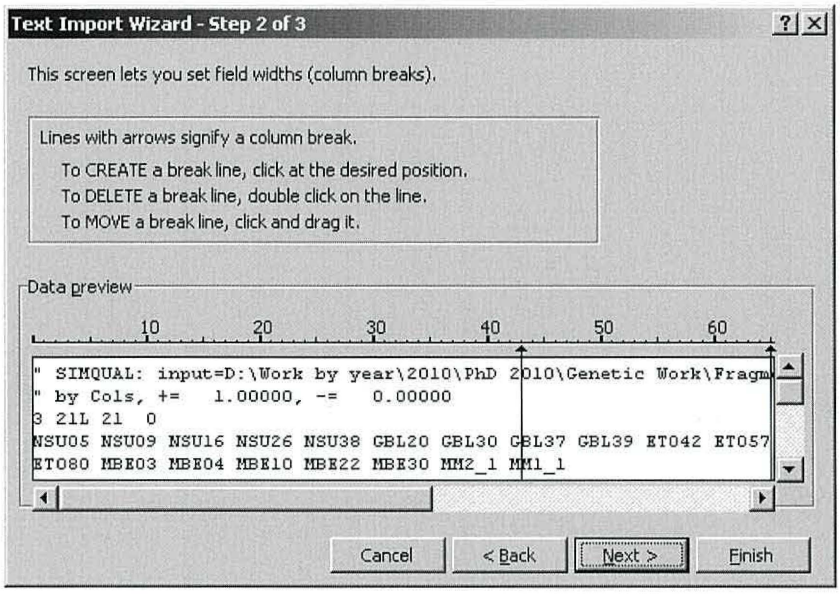
- **To do this we have to first get the similarity matrix from NTSYSpc into Excel in a format that we can read.**
- **Then we have to make a dissimilarity matrix, which is the complement of a similarity matrix.**
- **These are described below:**

To produce a Jacards / Nei and Li or SM table of similarity coefficients using a file from NTSYS pc in Excel.

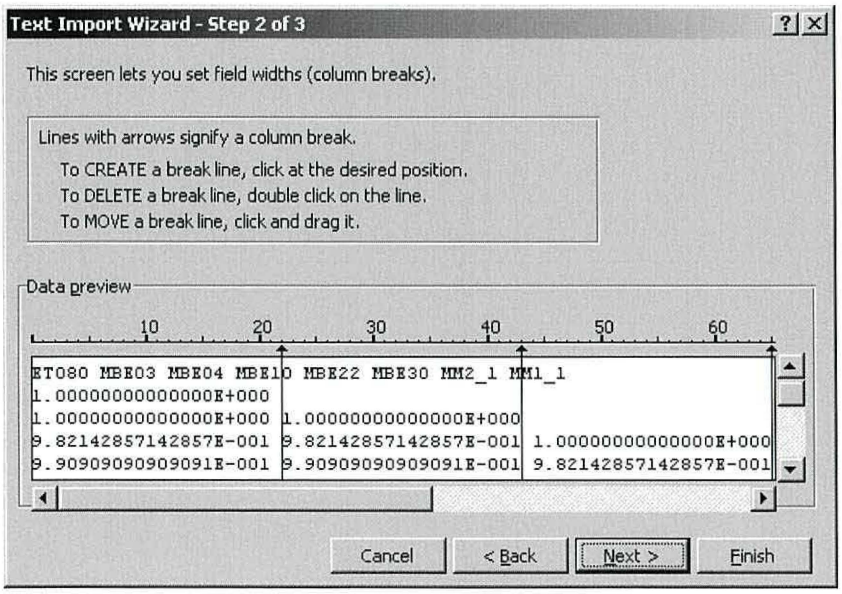
1. Open NTedit and open the file produced in Section 2 for the Jaccards similarity coefficient e.g. **name_jac.nts** using open file in grid.
2. Check that this file is correct and leave it open.
3. Open the file in Excel
4. When opening an **.nts** file into excel you will be given the following information:



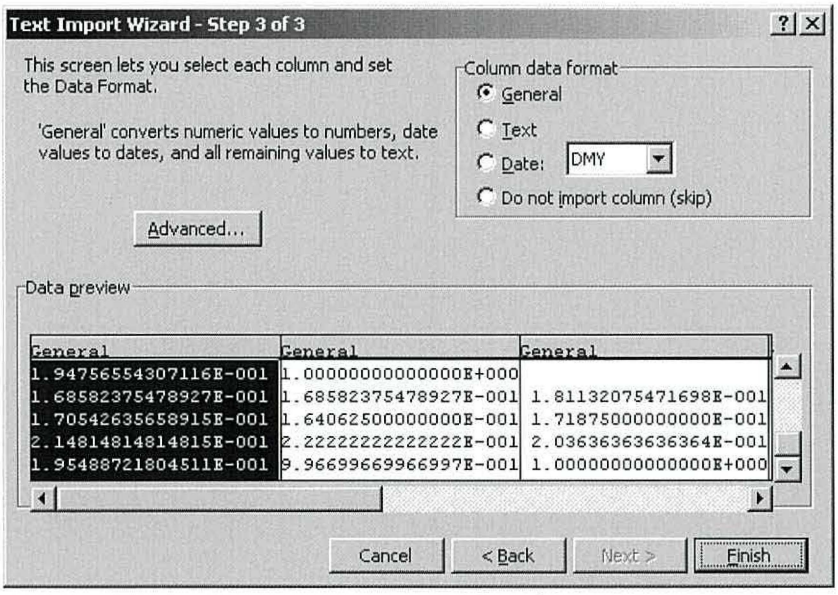
5. Click next and the following screen will show.
    a. Here you will be given the option to set "break lines". Scroll down using the scroll bar and select break lines at the ends of the data columns from the .nts file.



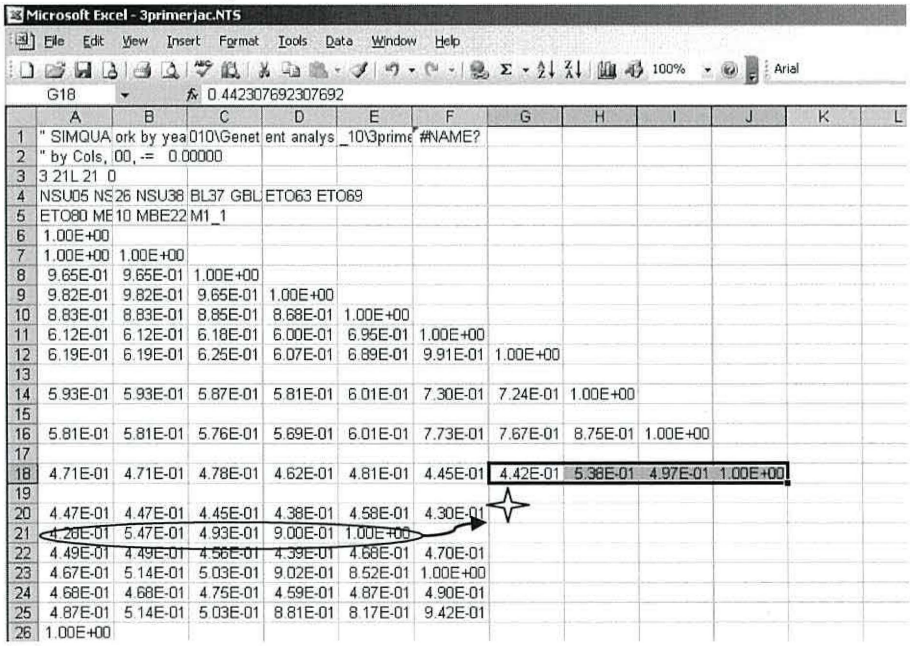
6. Once you have selected all the break lines (make sure that you have gone to the end of the file by scrolling down:



7. You will have to make enough break lines to break the .nts file into cells so that excel can read them.) click next.

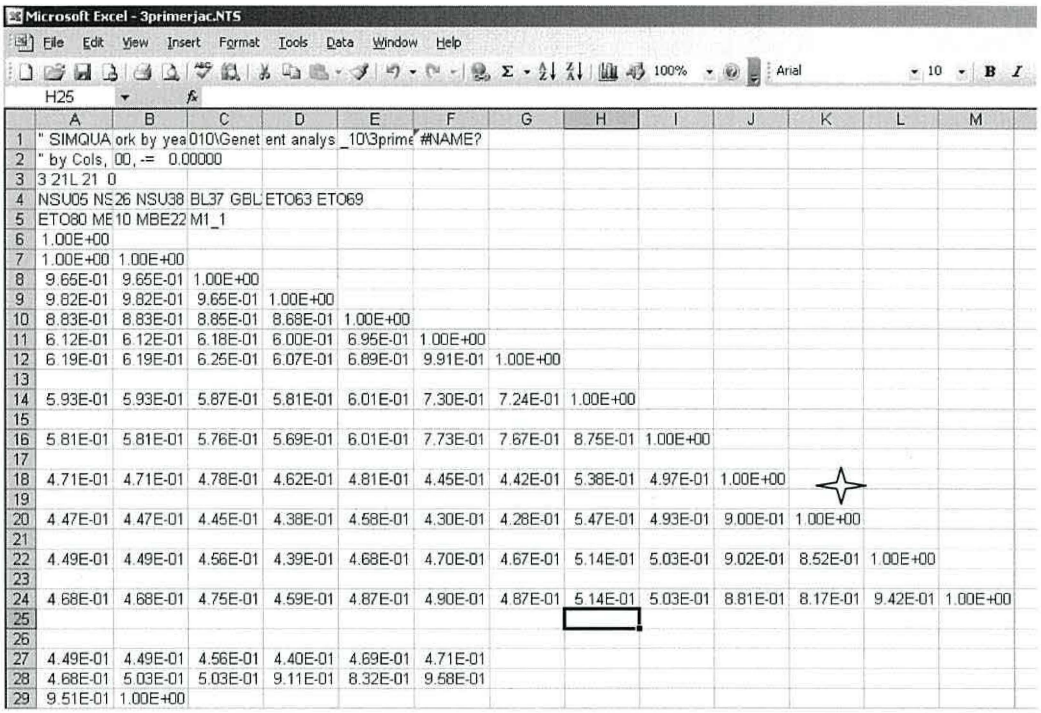


8. This will be the final screen. Here you select finish, and excel will display the results for you as below.



	A	B	C	D	E	F	G	H	I	J	K	L
1	" SIMQUA	ork by yea	010\Genet	ent analys	_10\3prime	#NAME?						
2	" by Cols,	00, -=	0.00000									
3	3 21L 21	0										
4	NSU05 NS	26 NSU38	BL37 GBL	ETO63 ETO69								
5	ETO80 ME	10 MBE22	M1_1									
6	1.00E+00											
7	1.00E+00	1.00E+00										
8	9.65E-01	9.65E-01	1.00E+00									
9	9.82E-01	9.82E-01	9.65E-01	1.00E+00								
10	8.83E-01	8.83E-01	8.85E-01	8.68E-01	1.00E+00							
11	6.12E-01	6.12E-01	6.18E-01	6.00E-01	6.95E-01	1.00E+00						
12	6.19E-01	6.19E-01	6.25E-01	6.07E-01	6.89E-01	9.91E-01	1.00E+00					
13												
14	5.93E-01	5.93E-01	5.87E-01	5.81E-01	6.01E-01	7.30E-01	7.24E-01	1.00E+00				
15												
16	5.81E-01	5.81E-01	5.76E-01	5.69E-01	6.01E-01	7.73E-01	7.67E-01	8.75E-01	1.00E+00			
17												
18	4.71E-01	4.71E-01	4.78E-01	4.62E-01	4.81E-01	4.45E-01	4.42E-01	5.38E-01	4.97E-01	1.00E+00		
19												
20	4.47E-01	4.47E-01	4.45E-01	4.38E-01	4.58E-01	4.30E-01						
21	4.26E-01	5.47E-01	4.93E-01	9.00E-01	1.00E+00							
22	4.49E-01	4.49E-01	4.56E-01	4.39E-01	4.68E-01	4.70E-01						
23	4.67E-01	5.14E-01	5.03E-01	9.02E-01	8.52E-01	1.00E+00						
24	4.68E-01	4.68E-01	4.75E-01	4.59E-01	4.87E-01	4.90E-01						
25	4.87E-01	5.14E-01	5.03E-01	8.81E-01	8.17E-01	9.42E-01						
26	1.00E+00											

9. You have to move the rows into the correct alignment so that you produce a triangular similarity matrix. Cells have to be cut from the row (below) in which they were in and added onto the row that they were supposed to be in (above). It is suggested that you have the coefficients file open in NTedit so that you can see which values have to be taken where.



	A	B	C	D	E	F	G	H	I	J	K	L	M
6	1.00E+00												
7	1.00E+00	1.00E+00											
8	9.65E-01	9.65E-01	1.00E+00										
9	9.82E-01	9.82E-01	9.65E-01	1.00E+00									
10	8.83E-01	8.83E-01	8.85E-01	8.68E-01	1.00E+00								
11	6.12E-01	6.12E-01	6.18E-01	6.00E-01	6.95E-01	1.00E+00							
12	6.19E-01	6.19E-01	6.25E-01	6.07E-01	6.89E-01	9.91E-01	1.00E+00						
13													
14	5.93E-01	5.93E-01	5.87E-01	5.81E-01	6.01E-01	7.30E-01	7.24E-01	1.00E+00					
15													
16	5.81E-01	5.81E-01	5.76E-01	5.69E-01	6.01E-01	7.73E-01	7.67E-01	8.75E-01	1.00E+00				
17													
18	4.71E-01	4.71E-01	4.78E-01	4.62E-01	4.81E-01	4.45E-01	4.42E-01	5.38E-01	4.97E-01	1.00E+00			
19													
20	4.47E-01	4.47E-01	4.45E-01	4.38E-01	4.58E-01	4.30E-01	4.28E-01	5.47E-01	4.93E-01	9.00E-01	1.00E+00		
21													
22	4.49E-01	4.49E-01	4.56E-01	4.39E-01	4.68E-01	4.70E-01	4.67E-01	5.14E-01	5.03E-01	9.02E-01	8.52E-01	1.00E+00	
23													
24	4.68E-01	4.68E-01	4.75E-01	4.59E-01	4.87E-01	4.90E-01	4.87E-01	5.14E-01	5.03E-01	8.81E-01	8.17E-01	9.42E-01	1.00E+00
25													
26													
27	4.49E-01	4.49E-01	4.56E-01	4.40E-01	4.69E-01	4.71E-01							
28	4.68E-01	5.03E-01	5.03E-01	9.11E-01	8.32E-01	9.68E-01							
29	9.51E-01	1.00E+00											

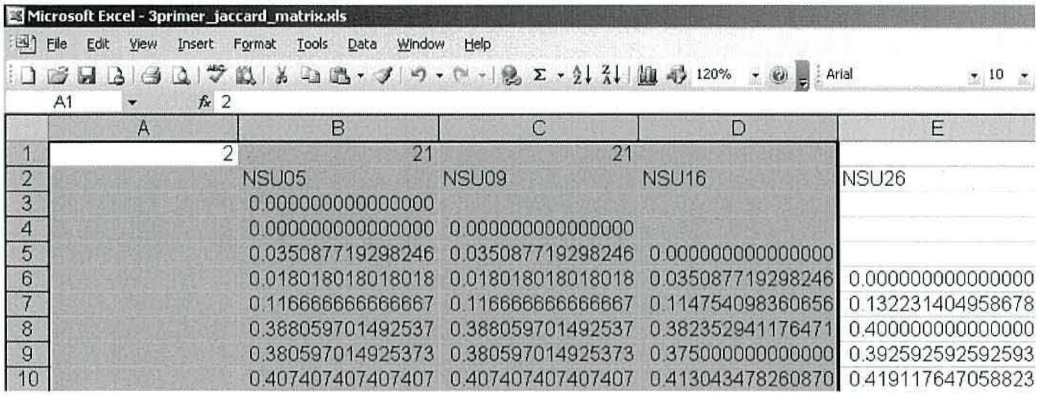
10. Once you have moved all the cells to their correct location you have to label the columns. Do this referring to the open file in NTedit. This can now be placed into your document or thesis.

As mentioned above, following the production of a similarity matrix a dissimilarity matrix can be produced, which will be needed if you wish to run the more robust clustering analysis using the **NEIGHBOUR JOINING** algorithm, rather than the **SAHN** clustering analysis using the **UMPGA** algorithm.

Production of a dissimilarity matrix:

1. Open a new worksheet in the file where you have the similarity matrix, and call it **name_Jac_diss_matrix**. The worksheet containing the similarity matrix should be called something appropriate, such as **name_Jacc_matrix**.
2. In the blank diss_matrix worksheet select the cell where the matrix starts in the simi_matrix worksheet and type **=1-** and then return to the original worksheet where the similarity matrix is and click on the cell containing the first value. Then press enter!
    a. **For example, if the first value of the similarity matrix is in cell A4, then you will want to click in cell A4 in the dissim_matrix worksheet and type =1-, then return to the similarity worksheet and click in cell A4, then press enter.**

3. This will return you to the **name_Jac_diss_matrix** worksheet and you should find a value in that cell location. Using the copy and drag feature in Excel you can quickly copy the formula into the adjacent cells and produce your dissimilarity matrix.
4. Once made, give the table column headings and make sure that the first value of the matrix is in position B3.
    a. This will allow you to fill in the information that NTedit requires for understanding the matrix, and will give you space for the column headings. An example is given below:



	A	B	C	D	E
1	2	21	21		
2		NSU05	NSU09	NSU16	NSU26
3		0.000000000000000			
4		0.000000000000000	0.000000000000000		
5		0.035087719298246	0.035087719298246	0.000000000000000	
6		0.018018018018018	0.018018018018018	0.035087719298246	0.000000000000000
7		0.116666666666667	0.116666666666667	0.114754098360656	0.132231404958678
8		0.388059701492537	0.388059701492537	0.382352941176471	0.400000000000000
9		0.380597014925373	0.380597014925373	0.375000000000000	0.392592592592593
10		0.407407407407407	0.407407407407407	0.413043478260870	0.419117647058823

- The number 2 in cell A1 ensures that NTedit will recognise the matrix as a dissimilarity matrix
- Cell B1 contains the number of rows
- Cell C1 contains the number of columns
- Cell D1, should not contain any number if there are no missing values. NTedit suggests putting in a value that is distinct, such as 99, however, do not do this. LEAVE THIS CELL BLANK OR NTEDIT WILL NOT BE ABLE TO READ THIS FILE.
- Row 2 can be used for column headings.

5. Once the dissimilarity matrix has been made in Excel, save it in a new excel file (97-2003 format only), **as the first worksheet in that file** and then close the file and give it a recognisable name, for example: **name_diss_jac_matrix.xls**

Running the NJ algorithm:

1. Open **NtEdit** and import the file using the Open file in grid option in the file menu. e.g. **name_diss_jac_matrix.xls**
2. Save the file with a new name e.g. **name_diss_jac_matrix.nts**

Now to produce the NJ cluster analysis

3. Select **CLUSTERING > NEIGHBOUR JOINING**

6. 2. Input file = this has to be a dissimilarity matrix (**name_diss_jac_matrix.nts**)
4. Output tree file = **name_nj_tree.nts**
5. Outpur graph file = **name_nj_graph.nts**
6. Leave all the other parameters the same.
7. Press compute.
8. At the bottom left of the NTSYSpc screen you will notice a new dendogram icon. Press on this to view the NJ dendogram.
9. Save this file in metafile format, the default format provided, with a suitable name, e.g. **name_nj_dendtree.nts**