AN INVESTIGATION INTO THE SAPSTAINING INTRINSIC PROPERTIES OF SELECTED RUBBERWOOD (*Hevea brasiliensis*) CLONES.

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed ..... Date: .....

(Prof. J. W. Palfreyman)

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LIST OF ABBREVIATIONS

ANOVA	ANALYSIS OF VARIANCE
C:N	CARBON NITROGEN RATIO
FDM	FURNITURE DESIGN AND MANUFACTURING
FRIM	FOREST RESEARCH INSTITUTE MALAYSIA
GT	GADANG TAPEN
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
LGM	LEMBAGA GETAH MALAYSIA
MDF	MEDIUM DENSITY FIBREBOARD
MEA	MALT EXTRACT AGAR
NaPCP	SODIUM PENTACHLOROPHENATE
PB	PERANG BESAR
PDA	POTATO DEXTROSE AGAR
RPM	REVOLUTION PER MINUTE
R	CORRELATION COEFFICIENT
R^2	COEFFICIENT OF DETERMINATION
RRIM	RUBBER RESEARCH INSTITUTE MALAYA (BEFORE 1970)
RRIM	RUBBER RESEARCH INSTITUTE MALAYSIA (AFTER 1970)
SEM	SCANNING ELECTRON MICROSCOPY
TAPPI	TECHNICAL ASSOCIATION OF PULP AND PAPER INDUSTRY
TEM	TRANSMISSION ELECTRON MICROSCOPY
UiTM	UNIVERSITI TEKNOLOGI MARA

ABSTRACT

ASHARI, A.J. AN INVESTIGATION INTO THE SAPSTAINING INTRINSIC PROPERTIES OF POTENTIAL RUBBERWOOD CLONES.

The general physical and chemical properties of rubberwood have been studied, as have the general relative levels of tolerance of sapstain fungi. New rubberwood clones have been recommended for planting since 1967. It is therefore timely to examine clonal differences in sapstaining properties of rubberwood clones. The purpose of this study was to investigate the intrinsic properties of 3 selected rubberwood clones vis-à-vis their relationship to susceptibility to sapstain fungi.

Two clones (PB217 and RRIM600) were obtained from the Bukit Pilah Estate, Negeri Sembilan, Malaysia and the third clone (GT1) came from the Tangkah Estate, Johor, Malaysia. The nitrogen contents were determined by the micro-Kjedahl method (Kjeldahl, 1883), the total organic carbon contents were determined by the Walkley-Black procedure (Walkley and Black, 1934), the sugar contents in the selected rubberwood clones were determined by High Performance Liquid Chromatography (HPLC), while starch content was determined by the method adapted from Humphreys and Kelly (1961). Quantitatively, biocontrol activity of several moulds and nonstaining fungi were examined using a range of agar and wood wafer techniques.

The results indicated that the nitrogen content of clone RRIM600 (1.06 mg/g) was significantly higher ($p<0.05$) than those of clones GT1 (0.73 mg/g), and clone PB217 (0.78 mg/g). After drying (45°C), clone PB217 contained significantly ($p<0.05$) higher amounts of fructose (5.55 mg/g), glucose (2.30 mg/g) and total sugars (13.15 mg/g) as compared to those of clone GT1 and RRIM600. Clone GT1 had the lowest fructose (0.23 mg/g), glucose (0.14 mg/g), sucrose (2.20 mg/g) and total free sugar (2.56 mg/g) contents. *B. theobromae* Pat. spreads significantly ($p<0.05$) faster on RRIM600 (4.3 days) than on PB217 (5.6 days) or on GT1 (5.8 days) which was associated with the overall higher nutrient availability in clone

RRIM600. Starch content was found to be highest in clone RRIM600 (5.81%), followed by GT1 (4.30%) and PB217 (3.63%)

A new tool attempted in the current investigation has been offered by the use of colorimeter, which has been used to conduct a quantitative assessment of the dark stain caused by the fungus *B. theobromae*. Results of the quantitative assessment of sapstain development (caused by *B. theobromae, Aureobasidium pullulans* and *Aspergillus niger*) in three rubberwood clones are reported here. Rubberwood blocks from three clones (GT1, PB217 and RRIM600) were inoculated with the test fungi and incubated for four weeks, after which the test samples were air dried and sanded (removing approximately 0.5 mm of the surface layer), and then quantitatively assessed for sapstain severity by spectrophotometry. Blue stain from *B. theobromae* was still present and pronounced after sanding, and was significantly ($p<0.05$) darker (absorbance: 56.5%) compared with wood infected by *A. niger* (38.24%) or *A. pullulans* (35.88%). Clone RRIM600 had significantly ($p<0.05$) higher mean percent darkness (41.85%) than clone PB217 (38.49%). The mean percent darkness of clone GT1 (39.67%) was not significantly different ($p>0.05$) from either clone RRIM600 or clone PB217. Single or mixed inocula *of A. pullulans* and *A. niger* showed biocontrol activities on clones RRIM600 and PB217.

CHAPTER 1

1. INTRODUCTION

1.1 IMPORTANCE OF RUBBERWOOD TO MALAYSIA

In the early nineteen seventies Malaysia was confronted with a sharp decline in the price of natural rubber and a downturn of the rubber industry. Fortunately, the decline of the rubber industry as a major export earner has been compensated by the growing importance of the rubber trees as a sustainable source of raw material for the wood-based industries.

The latest estimate is that there are about 2500 furniture manufacturers throughout the country, of which about 700–800 are totally export-oriented which accounted for more than 85% of the total export figures. Export of rubberwood-based furniture is still on upward trend (Yap, 1998). Export of the furniture industry has grown from a cottage industry of RM 120 million (US$ 31.6 million) in 1986 to more than RM 2.8 billion (US$ 737 million) in 1997. Latest export statistics show that from January to September 1999 export earning from wooden furniture totaled US$ 710.5 million, higher by 14.8% as compared to corresponding figure in 1998 (FDM, 2000).

Currently rubberwood is the most successful plantation species in Malaysia. The planting of the rubber tree for both latex and timber production during its economic life rotation of usually 25 years and for timber thereafter has been shown to be viable (Kadir, 1998). Indeed the rubber tree has an advantage over many other local timber species as it could be exploited for both timber and latex. Another advantage is that, since rubber trees are being grown in a sustainable and renewable plantation as such, rubberwood has recently been labelled as 'environmentally friendly'. This is an advantage in the marketing of rubberwood products especially in importing countries where the 'green movement' is very strong.

Apart from furniture, rubberwood is also sought after for the production of particleboard and medium density fibreboard (MDF). At present, there are 200

sawmills, 75 preservative treatment plants, 110 kiln drying plants, 3 plywood mills, 96 furniture manufacturers, 2 particleboard mills and 7 medium density fibreboard (MDF) mills using mainly rubberwood as raw material in Malaysia (Kadir, 1998). The total volume of MDF exported for the year 1999 was 749,717 m^3 amounting to US$ 166.9 million (FDM, 2000). Thus rubberwood will remain as the main raw material for the fast expanding wood-based industries in Malaysia for many years to come.

1.2 HISTORY OF RUBBERWOOD IN MALAYSIA

The rubber tree *(Hevea brasiliensis)* is indigenous to the Amazon forests of Brazil. When the demand for natural rubber soared after the discovery of the vulcanisation process in 1839, the need for adequate, continuous supply of this new industrial raw material provided the need to establish rubber-producing plantations. Henry Wickham was responsible for the introduction of the rubber tree to Malaysia (Oldfield, 1984).

In 1876 he shipped 70,000 rubber seeds from Brazil to Kew in the United Kingdom from which 2,700 were successfully germinated. From Kew about 1,900 seeds were shipped to Sri Lanka. Then in 1877 another 22 plants were shipped from Sri Lanka to the Singapore Botanical Gardens. Nine of these plants were planted in Kuala Kangsar, Malaysia. 20 years later after many seed multiplications and the development of the practical Ridley method of tapping hevea, the rubber producers in the Far East established the first commercial planting in 1890's. These rubber trees grown from Wickham's seeds initiated the rubber plantation industry in Malaysia until the 1970's (Barlow, 1978).

The eventual success of *Hevea* plantations (Plate 1-1) was in part due to fortunate events: a) the selection of *H. brasiliensis*, a good producer of high quality latex to seven other *Hevea* species b) the *H. brasiliesis* seed was free of the south American leaf blight fungus (Oldfield, 1984).

Plate 1-1. Picture showing a stand of 23-year old rubber trees in Tangkah Estate, Tangkak, Johore, Malaysia.

Rubber trees were originally planted for their latex and plantations were felled and replanted every 25-30 years when they were uneconomical for latex production. Initially sawing of rubberwood logs were carried out in existing sawmills using traditional bandsaws. Gradually mills are also set up specifically for sawing rubberwood. The bandsaw is the most common type of sawing system, other saws such as gang saws and mobile saws are also used. Boards from gang sawing do not have excessive spring (Ho and Roslan, 1994). Various types of mobile circular saw systems have been tested to cut rubberwood (Ho and Roslan, 1984). Mobile band saws are now the most common system used for cutting rubberwood in Malaysia in remote areas (Ho and Roslan 1984). A mobile saw such as a modification of the band saw or one with circular saw blades is usually employed on site.

In the past, rubberwood was of low economic value and used as firewood by the rural communities in addition to its use as a fuelwood in rubber processing and, brick making and, as a source of charcoal, for the steel industries. The major users of rubberwood then, were the fuel-wood consuming industry (e.g., drying and smoking of sheet-rubber, tobacco curing, brick making, etc.), the charcoal industry and the blockboard industry (Hong, 1994). The wood industry was initially reluctant to use rubberwood because of the abundant supply of forest logs (readily

available and inexpensive), and the non-durable nature of rubberwood. As such the commercial value of rubberwood timber three decades ago was almost negligible. However, with the decreasing forest area and decreasing supply of large diameter logs, coupled with increasing labour costs in the late nineteen seventies, rubberwood has now become much sought after (Hong, 1994).

The mature rubber tree in its native habitat, is about 25-30 m tall with an average girth greater than 1.0 m at breast height (Hong, 1994). The mature trees of the Malaysian rubber plantations are much smaller and have been bred primarily for the production of latex without taking into account the volume of wood produced (Hong, 1994). Realising the growing importance of rubberwood, effort was undertaken in the Amazon, Brazil to collect *Hevea* germplasm including those from nine other *Hevea* species (Kadir, 1998). The introduction of new genetic materials of *Hevea* from Brazil in 1951, 1966, 1981 and 1995 as well as through multi-lateral exchange of clones among rubber growing countries has resulted in an increased yield potential and other desirable secondary characteristics such as disease resistance and timber yield. In the current and future breeding and selection programme, parents with diverse genetic and geographic origin are now being used.

In the course of developing new cultivars (clones), a number of stages are involved: a) production of a large number of hand pollinated seedling progenies, b) early selection in the nursery, c) testing elite progenies in the field with proper experimental design, and finally d) recommending the new clone for planting. Presently it takes about 15 years (from a to d) to recommend a new clone for commercial planting for both the estate and smallholding sectors (LGM, 1998). The planting recommendation classifies the clones into two groups. Group I refers to clones or planting materials which have proven track record. Estate planters and smallholders are free to plant the clones from Group I without restriction. In Group II are the clones with limited information on yield and growth performance. For Group II the data on the performance of the clones in different climatic, soil and disease environment area are not available. With limited data the clones belonging to Group II are recommended for planting only under close supervision.

Repetition of the breeding and selection process over many generations led to the development of a number of high yielding hevea clones. Thus, although the

unselected trees first collected by Wickham produced only about 225 kg/ha/year of rubber, even using the best tapping methods available at that time, by the 1930's average Malaysian plantation yields were higher than 400 kg/ha/year (Oldfield, 1984). Since 1928, the Rubber Research Institute of Malaysian (RRIM) through breeding and selection was successful in increasing latex yield from 550 kg/ha/year to more than 2500 kg/ha/year (LGM, 1998). There are now more than 20 clones of rubber trees planted in Malaysia. At the end of 1996 a total of 1.672 million hectares of rubber plantations were found in Malaysia with the bulk of it (1.20 million hectares) in Peninsular Malaysia (Yahya, 1998). The rest were in the states of Sarawak and Sabah. Table 1-1 shows the percentage, mean latex yield, occurrence of wind damage, and growth vigour for thirteen rubberwood clones.

Table 1-1. Some important clones planted and recommended for planting in Peninsular Malaysia showing mean latex yield, wind damage and vigour ratings.

Clone	Parentage	Mean Yield (kg/ha/year)	Wind damage	Vigour
GT1	CPV SEEDLING	1180	Average	Average
RRIM937	PB5/51XRRIM703	2483	Below average	High
PB350	RRIM600XPB235	2428	Average	High
PB235	PB5/51XPBS/78	2273	Average	High
PB255	PB5/5XPB32/36	2255	Below average	Average
PB260	PB5/51XPB49	2168	Below average	High
RRIM936	GT1XPR107	2146	Below average	High
RRIM712	RRIM605XRRIM71	2126	Low	Below average
RRIM921	Pb5/51XFORD351	1393	Below average	High
RRIM600	TJIR 1XPR107	1990	Below average	Average
RRIM901	PB5/51XRRIM600	1971	Below average	Below average
RRIM940	PB5/51XRRIM701	1895	Average	Below average
PB217	PB5/51XPB6/9	1674	Below average	Above average

Source: LGM, (1995) and LGM, (1998)

The three rubberwood clones selected in this study were clones GT1, PB217 and RRIM600. Clone GT1 originated from Indonesia, was a primary clone developed from an ordinary seedling tree at the Godang Tapen Estate in Java in 1920. Clone GT1 was acquired by the Rubber Research Institute of Malaya in 1954 from the Centrale Proefstation Vereniging (CPV) under an agreement on the exchange of selected *Hevea* planting material between various rubber research institutes in the far East (RRIM, 1970). The clone was recommended by the Experimental Station in Java for large scale planting in Central and East Java in 1930, in west Java after the Second World War and in Sumatra in 1950 (RRIM, 1970). In Malaysia GT1 was recommended by RRIM for class II planting on a moderate scale in 1955 (RRIM,

1970). It was promoted to Class I for planting on a large scale in 1967 (RRIM, 1970).

Clones PB217 and RRIM600 are among the superior clones obtained from hand pollinated seedlings at the RRIM (Table 1-1). PB217 is a cross between clones PB5/51 and PB6/9. PB217 is quite susceptible to pink disease and black stripes (Ang and Sheppherd, 1979). Clone PB217 has a mean yield of 1674 kg/ha/year but is highly recommended for planting at Golden Hope plantation because of proven rising latex yield trend. This clone has been one of the top clones widely planted by Golden Hope plantation throughout Peninsular Malaysia.

RRIM600 is another clone developed by RRIM from hand pollinated seedlings, a cross between clone TJ1R and PR107 (Table 1-1). Clone RRIM600 is in the highest yield class with mean yield of 1990 kg/ha/yr. The clone is average or below average vigour during maturity, but has an exceptionally good girth increment of tapped trees. Bark thickness and bark renewal are good. The stem is straight, branching is high and crowns are narrow (RRIM, 1965). RRIM600 and GT1 are among the earliest recommended clones by RRIM, both have often been used as control in yield performance test and other secondary characteristics studies (LGM, 1998; Hong and Gan, 1995; RRIM, 1963; Najib Lotfi and Ramli, 1996).

Rubber trees being an important source of timber for the Malaysian wood-based industries are now highly demanded by furniture manufacturers. The current RRIM planting recommendations therefore give more weighting to selecting clones with high yields of timber and these have been classed as LTC (latex timber clones) (Ong *et al.,* 1994). Kadir (1998) reported that potential genotypes suitable for rubber plantations solely for timber production include those from the RRIM900, RRIM2000 and PB series. The top 20 genotypes of LTC as listed in Kadir (1998) were also found to have good growth vigour, and have clear bole height ranging from 6.27 m (MT/C/5-12/137) to 13.0m (ROIC19-231238). All these rubberwood clones are still under selection studies and are potential clones for the future.

1.3 REVIEW OF RUBBERWOOD RESEARCH AND FUTURE DIRECTIONS.

The properties and utilization of rubberwood have been studied since the 1950's. Grant (1952) reported the use of rubberwood for walls and floor-boards in some houses built for estate labourers. During the early-to-late 1950's, The Forest Research Institute (now FRIM) initiated research to determine the potential use of rubberwood timber (Thomas and Landon, 1953) and its applications in other products such as fibreboard (Peel, 1958) and wood pulp (Peel and Peh, 1960). These attempts showed the vast potentials of rubberwood, however the wood processing industry then was not receptive. This was because of the nondurable nature of rubberwood and because of an abundant supply of forest logs from other species that were readily available and inexpensive. However with decreasing supply of large diameter logs due to decreasing area of virgin natural forest coupled with the increasing of labour cost in the late 1970's, plantation grown rubberwood became a much sought after timber.

In promoting the utilisation of rubberwood trees in the early ninteen seventies research was focused on the processing characteristics and machining properties of rubberwood in general. The behavior of rubberwood was tested during sawing, drying, preservative treatment, gluing and machining for furniture manufacture. These initial studies found that rubberwood is ideal for making furniture. It is easy to saw and does not cause severe blunting of the saw teeth (Ho and Choo, 1982). In addition, rubberwood could be moulded with surfaces moderately smooth to smooth at cutting angles between 35° to 45° (Ho and Said Ahmad, 1990). The best cutting angle was found to be 35°, but the defect of wooly grain remains prominent on all the surfaces.

In Malaysia the tropical fungus *Botrydiplodia theobromae* is known to cause blue stain in rubberwood and light coloured (e.g. Jelutong and Ramin) sapwood timbers (Hong, 1976; Hong and Wong, 1994). Being naturally non-durable, rubberwood would not be economically usable without proper preservative treatment or immediate processing and drying after felling (Mohd Dahlan *et al.,* 1994; Hong *et al.,* 1982). The best preventive solution to the problem of sapstain infestation in

rubberwood is for the rubber logs to be processed and kiln dried within 2 days after felling. Research efforts were also undertaken to establish appropriate kiln-drying schedules and practical processing techniques for its proper utilization to minimise sapstain (Ho and Choo, 1982; Ho and Choo, 1983; Asano and Choo, 1990; Choo and Hashim, 1994). Such procedures are excellent for plantation material but best use of scattered smallholdings requires different logistics. The log holding time interval sometimes lengthen between felling and sapstain control treatments may lengthen to up to 2 weeks, resulting in serious sapstain losses in the market value of much of the otherwise stain-free processed material (Hong *et al.* 1994).

Sapstain (also referred to as blue-stain), has long been recognised as a major pathological problem in both freshly felled rubberwood and the other light hardwood and softwood timbers in Malaysia and the equatorial tropics (Hong and Wong, 1994; Wong *et al.,* 1999a). A wide range of formulations comprising insecticides (borax) and fungicides or mixtures of both have been tested in terms of effectiveness against sapstain and mould in rubberwood (Hong, 1980, 1989). Sodium pentachlorophenate (NaPCP), a cost-effective anti-sapstain formulation, usually used mixed with borax, was effective in the control of sapstain in green rubberwood logs and sawn timber (Hong 1980; 1989). This chlorinated phenol was however totally banned in Malaysia from 1 January 2000. Alternative novel formulations have since assumed prominence in the sapstain market, or at least tested by FRIM. These chemicals are readily grouped under the broad classes such as: carbamates, thiocyanates, benzothiazoles, organo-metallic compounds, triazoles, alkyl ammonium compounds, iodine-based compounds or phenolic compounds (Wong *et al.,* 1995).

Since 1928, most clonal studies were focused on inter clonal differences in latex production, vigour, major wind damage, diseases in the RRIM studies (LGM, 1998). Several clonal differences in harvesting and log characteristics (Ho, 1994; Gan *et al.,* 1987; Hong and Gan, 1995) have been reported, however, to date no study has been undertaken to examine interclonal differences in nitrogen, carbon, free sugars and starch composition. The present study will attempt to look into these possible clonal differences and to relate these differences to possible differences in

susceptibility of these rubberwood clones to a) *B. theobromae* and b) a selection of mould and non staining fungi.

1.4 BASIC PROPERTIES OF RUBBERWOOD

Freshly sawn rubberwood is white or pale cream in colour, sometimes with a pinkish tinge. It seasons to a light straw or light brown colour (Lim and Ani Sulaiman, 1994). Rubberwood discolours to a yellowish orange on exposure to light over a period of time (Minemura *et al.,* 1999). For the control of this color change, it was found that a coating of 5g m^{-2} polyethyleneglycol was effective in preventing photo-induced discoloration (Minemura *et al.,* 1999) of rubberwood. The grain ranges from straight to shallowly interlocking. Its planed surface is not particularly lustrous and may have zigzag pattern produced by the presence of wood parenchyma. It has no growth rings, but the presence of banded wood parenchyma bands may simulate growth rings. The texture is moderately coarse to coarse and even.

Wood vessels are moderately large to large size. Rays are moderately fine sized and are visible to the naked eye. Few vessels are solitary with the majority in radial pairs or multiples of up to 4, but occasionally with radial multiples of 5 to 8. The clustering of pores is common (Lim and Ani Sulaiman, 1994). The vessels are typically filled with tyloses and are sometimes abundant. Pores are round to oval in shape with tangential diameter ranging from 150 to 250 μm averaging 200 μm. Pores have simple perforation plates. Inter-vessel pits, with apertures and borders that are often elongated and almost scalariform to opposite, are 21-25 μm in diameter. Helical thickening is absent. Fibres are 1.20 mm to 1.65 mm in length, non-septate, with simple to minutely bordered pits mainly confined to the radial walls. In rubberwood the vessels and the parenchyma cells are interconnected by a series of pits in the secondary walls that permit passage of water and solutes among contiguous cells in living trees, these pits are also alternative invasion routes for sapstain fungi. Parenchyma consists of both apotracheal and paratracheal parenchyma. Apotracheal parenchyma is diffuse, diffuse-in-aggregates and in narrow, discontinuous lines of up to 3 cells wide. It often contains chambered crystals, and appears as 3 to 7 sometimes up to 9 celled strands. Paratracheal

parenchymas are relatively sparse, confined to the immediate vicinity of the vessels. Number of rays are 7-10 per mm, usually 2 to 3 cells wide; however, uniseriate or rays up to 4 cells wide may be present occasionally. Woody parenchyma appears as narrow and closely but irregularly spaced bands, joining ray to ray to form a net-like pattern. The rays are heterogenous, with 1 or 4 rows of square to upright marginal cells. Silica bodies are absent. Wood consists primarily of a series of thick-walled, elongated cells with the dual function of fluid conduction and structural support for the tree stem. Radial parenchymas are a prominent feature of many hardwoods, approaching 25% of wood volume in some species. The parenchyma cells are the major colonisation sites for the fungi that discolour the sapwood (Zabel and Morrell, 1992). It is probably the axial conducting elements which, because of their size and accessibility, provide the major routes for mycelial invasion (Rayner and Boddy, 1988).

Depending on the clone and age of the rubber tree, the basic density of rubberwood ranges from 430 to 620 kg/m^3 (FRIM, 1985). The air-dry density is between 560 – 650 kg/m^3 at 15% moisture content (FRIM, 1985). Hong and Gan (1995) in recommending the use of younger rubber trees to overcome current shortage of rubberwood for the wood industries found that rubberwood of 8 and 14 years old of PB260 and 8 year old RRIM600 do not differ significantly ($p>0.05$) from those of 25 to 30-year-old trees. However in the same study they also showed that the younger age group (less than 8 years) had significantly ($p<0.05$) lower basic density and is not recommended for the wood industry. For RRIM600 the 8 and 24 year-old-trees sampled had 480-603 kg/m^3 and 560-625 kg/m^3, respectively. Gnanaharan and Dhamodaran (1992) reported that the mechanical properties of air-dried rubberwood from a 35 year old plantation in the central region of Kerala posses medium strength properties.

Rubberwood logs are prone to have tension wood. In logs, tension wood may be in the form of concentric rings or crescent shaped arcs providing false rings to the logs. Tension wood differs from normal wood in physical, chemical and anatomical features. From the utilisation point of view, the presence of tension wood can have a major effect on the quality of the final products. The presence of unlignified wood fibres called gelatinous layer (or G - layer), which is cellulose in nature, makes

the wood surface lustrous, woolly and rough, causing various woodworking and finishing problems (Lim and Mohd Nadzri, 1995). Mean percent of tension wood in the various rubberwood clones is as shown in Table 1-2.

Table 1-2. Percentage of tension wood in rubberwood clones PB260 and RRIM600 at various age (Lim and Ani Sulaiman, 1994).

Age	**PB260**	**RRIM600**
3	44.0	Not available
8	30.0	33.0
14	45.0	Not available
24	Not available	30.0

The occurrence of tension wood is a common phenomenon in rubberwood and has been observed by many researchers (Huges, 1965; Panikkar, 1971; Rao *et al.,* 1983; Lim, 1985; Lim and Mohd. Nadzri, 1995: Renghu *et al.,* 1989). The proportion of tension wood in rubberwood may vary from tree to tree, and within the same tree, along the length of the trunk (Sharma and Kukreti, 1981). Proportion of tension wood ranged from 15 to 65%. However, the study by Lim and Ani Sulaiman (1994) on two clones of rubberwood (PB 260 and RRIM600) of different ages found that clone RRIM600 had a lower percentage of tension wood (about 30%) as compared to clone PB260 (more than 40%). For PB 260, the percentage of tension wood seemed to increase with height, whereas for clone RRIM600, the reverse was true, since the percentage and distribution of tension wood do not seem to follow a fixed pattern. Every effort was made in this present study to eliminate test samples known to contain tension wood. Tension wood can be recognised by dipping the cross section of the discs into a solution mixture of 1 g phloroglucinol in 50 ml ethanol (95%) and 50 ml concentrated hydrochloric acid for 1 minute (Lim and Mohd Nadzri, 1995). The tension wood zones, which lack lignin, do not stain whereas the normal wood zones turn crimson after reacting with the solution mixture.

Woody tissues comprise of cellulose, hemicellulose and lignins (Levy and

Dickinson, 1981). In softwoods, for example, the cellulose composition varies between 40 - 50%, while that of hemicellulose is around 20%, lignin 25 - 35% and the extractable fraction (including starch and tannins) is between 1 - 10% (Doimo, 1984). Normal hardwoods contain 42 ± 2% cellulose and the lignin content varies between 18 and 25%. In addition to cellulose and lignin both hardwood contains small amounts of pectin material, starch and hemicellulose (Kollmann and Coté, 1968). Rubberwood consists of 39.7% cellulose, 38.1% hemicellulose, and 17.8% lignin (Guha and Lee, 1972).

The types and distribution of the major chemical constituents in the wood cell walls vary among and within species (Zabel and Morrell, 1992). The minor components of wood include starch, lipids, tannins, oil, pectins, alkaloids and protein. The relative quantities of these components vary in different types of wood. Azizol and Rahim (1989) determined the content of free sugars in freshly cut as well as in seasoned rubberwood logs. Freshly cut logs contained 1.05 to 2.29% of free sugars and 7.53 to 10.17% of starch.

1.5 SAPSTAIN AND MOULDS

Sapstain is the bluish gray to black discolouration of wood that is caused by the pigmented hyphae of sapstaining fungi that grow into the wood. These pigmented hyphae grow primarily in the parenchyma tissues of the sapwood. Common sapwood discolorations are shades of yellow, pink, brown and gray. These stains are the results of pigments secreted from the fungal hyphae (Zabel and Morrel, 1992). The predominance of bluish discoloration (Table 1-3) and the restriction to the sapwood have resulted in the major stains being termed sapstain or blue stain. This stain damage to the wood does not reduce the strength of the wood but effects its appearance and causes large economic losses for the furniture and timber industries (Seifert, 1993).

Table 1-3 listed some of the important sapwood staining fungi reported in softwoods and hardwoods.

Table 1-3. Some important sapwood staining (blue stain) fungi.

Softwoods	Hardwoods
Ophiostoma (Ceratocystis) ips	*Ophiostoma (Ceratocystis) pluriannulatum*
Ophiostoma (Ceratocystis) piliferum	*Ceratocystis moniliformis*
Ophiostoma (Ceratocystis) picea	*Bortyodiplodia (Lasiodiplodia) theobromae*
Aureobasidium pullulans	*Ceratocystis coerulescens*
Leptographium lundbergii	*Graphium rigidum*
Alternaria (tenius) alternata	*A. sydowii*
Cephaloascus fragrans	*Penicillium citrinum*
Cladosporium spp.	
Bortyodiplodia(Lasiodiplodia) theobromae	
Phialophoara spp.	

Note: Scientific names which appear commonly in older literature are in parenthesis

Source: Balasundaran and Gnanaharan (1990); Florence *et al.* (1998) Scheffer and Lindgren (1940); Hong (1976); Hong and Wong (1994); Zabel (1954); Zabel and Morrel (1992).

The sapstain fungus penetrates through the cut ends of the logs within a week after felling and colonises the whole log. During the rainy season the infection is more severe than that in the dry season (Hong *et al.,* 1994). Sapstain defects is an important economic problem for the Malaysian rubberwood and furniture industry, due to the bluish black discolouration of the wood that is caused by pigmented hyphae of the sapstaining fungi. The cost of sapstain for the rubberwood furniture industry is not known but is due to a) the downgrading of products, to customers for non-specified product delivery and b) the opportunity costs lost due to failure to meet market needs. Rubberwood is reputed to be among the most susceptible sapwood species to fungal degradation, with less natural durability (in term of

weight loss) than even bamboo and oil palm trunk (Martawidjaja, 1971; Wong and Koh, 1991).

B. theobromae is a member of the Sphaeropsidales and produces dark-colour pycnidia, which contain brown ellipsoidal two celled conidia. The conidia are released from the neck of a pycnidium in thick, light-brown masses. Recently, however this common tropical blue-stain fungus has been reported to significantly degrade the wood cell wall in the sapwood of certain timber species (Encinas and Daniel, 1995; Wong and Singh, 1997), as well as the gelatinous fibres of 'tension wood cells' (Encinas and Daniel, 1997). These effects reduce certain wood strength properties (Arenas *et al.,* 1967). As has been reported in many other tropical countries *Botryodiplodia theobromae* (Syn: *Lasiodiplodia theobromae*) is the most important fungus causing sapstain in rubberwood in Malaysia (Hong, 1976; Hong and Wong, 1994; Wong *et al.,* 1999a). *B. theobromae* has also been recorded as the main fungus for causing sapstain in India (Florence *et al.*, 1998). Olofinboba (1974) reported *B. theobromae* is the blue stain causing fungus in *Antiaris africana*, an economically important tropical white colored wood. In Japan, Tsunoda *et al.* (1983) also isolated sapstain fungus *B. theobromae* from rubberwood. Pinheiro (1971) found that *B. theobromae* was the main pathogen causing blue stain in poplar wood. In Africa staining by *B. theobromae* on *Triplochiton scleroxylon* (abaci) caused excessive damage to the sapwood (Tabirih and Seehann, 1984).

There are other sapstain fungi found on rubberwood but they are of less importance. Florence *et al.* (1998) found that *Ceratocystis* spp. causes grayish-black stain in Kerala, India mostly during the rainy months of June to August. This genus is however more common in the temperate countries such as Britain (Bakshi, 1951), Southern United States (Verall, 1939), Spain (Troya and Navarrete, 1989) and Yugoslavia (Benko, 1983).

Moulds are generally a factor in very wet wood, such that wood that has been close piled for diffusion treatment or wood that has been stacked but has restricted airflow. Table 1-5 shows some of the common mould fungi and the colour of spore pigments. Most moulds are airborne, opportunistic fungi with hyphae that are normally colourless, but discolor the wood by forming masses of pigmented spores

on the wood surface (Zabel and Morrell, 1992). Mould discoloration can most often be removed by brushing or planing the wood surface, however the discoloration on hardwood is often deeper and may be persistent (Richardson, 1993; Florance *et al.*, 1998).

Table 1-4. Some important mould fungi and colour of pigmented spores.

Mould fungi	Color
Aspergillus spp.	Black
Fusarium spp.	Red or violet
Gliocladium spp.	Green
Monilia spp.	Orange
Penicillium spp.	Green
Rhizopus spp.	Black
Trichoderma spp.	Green
Curvularia	n.a.
Sphaeronaema	n.a.
Aspergillus terreus	n.a.

Sources: Ali Sujan *et al.* (1980); Hong (1981); Tsududa *et al.* (1983); Hong et al. (1987); Balasundaran and Gnanaharan (1990), Gnanaharan (1982) and Florence *et al.* (1998).

Surface moulds which infest rubberwood cause superficial staining on unseasoned or partially seasoned wood or logs. Examples are *Aspergillus niger, Fusarium* spp., *Penicillium* spp., *Sphaeronema* spp., *Trichoderma* spp., *Curvularia* spp. (Kaarik, 1974). *Aureobasidium pullulans* is a superficial stain fungus, and occurs commonly in temperate soils, wood and on plant surfaces. This fungus is one of the most frequently isolated sapstain fungi in temperate regions (Sharpe and Dickinson 1992). *Penicillium* spp. and *Aspergillus* spp. are the most commonly encountered moulds on the surface of rubberwood (Ananthanarayanan, 1971; Hong, 1976; Ali Sujan *et al.*, 1980; Forence *et al.*, 1998). Balasundaran and Gnanaharan (1990) also isolated *Aspergillus terreus*, *Aspergillus sydowii* and *Penicillium citrinum*, from the

surface of rubberwood treated with borax + boric acid + NaPCP.

Aspergillus niger v. Tieghem, is a primary mould, with non-pigmented hyphae. It represents a group of tropical moulds that compete for nutrients and space in the rubberwood, possibly reducing staining by other fungi. *A. niger* is a common spoilage mould which occurs all over the world (Onions, 1975). In culture, colonies spread rapidly with a white basal mycelium which frequently develops bright yellow areas, and as conidia are produced, the surface becomes black rusty and granular. The mop-like heads, which split in age to form columns, are borne at the apex of stalks (conidiophores) that arise from the substratum and may be 200 μ to several mm in length.

Trichoderma spp. is another common mould and is frequently found on timber and wood products (Onions, 1975). The taxonomy of *Trichoderma* is somewhat confusing and has led to incorrect identification of species within genus. The genus gives rise to typically green cushions of spore masses irregularly distributed over the agar (Eaton and Hale, 1993). *T. viride* is a pioneer fungus that rapidly invades exposed wood tissues and forms green masses of spores on the surface. The hyphae that penetrate the wood are colourless and do not adversely affect wood strength or colour. Lindgren and Harvey (1952) reported that applying *T. viride* provided considerable protection against stain and decay development in pine bolts. Spraying the wood with solutions of ammonium bifluoride appeared to favour the growth of *T. viride* on the log surfaces. Shields and Atwell (1963) have shown that *Trichoderma* spp. inoculations also protect several hardwood species. The potential of *Trichoderma spp.* for biological protection against both stain and decay fungi has become the subject of renewed interest (Seifert *et al.*, 1988; Benko and Highly, 1990a, 1990b).

1.6 DIFFERENCE BETWEEN SAPSTAIN AND DECAY FUNGI

Fungi are non-green plants; therefore they do not have chlorophyll and cannot synthesise food via photosynthesis. Fungi therefore require an already synthesised food source, such as wood. It is generally accepted that the blue stain fungi and mould utilise the readily available compound such a soluble carbohydrates (sugars

and starch), wood extractives (lipids) and proteins (Rayner & Boddy, 1988; Abraham & Breuil, 1993; Gao *et al.,* 1994; Gao and Breuil, 1995; Breuil, 1998). Mould fungi generally feed on material located on the wood surface, while sapstain fungi utilise food substances stored in the wood cells. It is also generally accepted that mould and sapstain fungi lack the necessary enzymes for degrading cellulose and lignified cell walls to cause significant loss in weight and strength.

Decay is the most destructive form of fungal attack on wood, which occurs in the form of brown, white and soft rots. Brown and white rots result from the growth of highly specialised higher fungi (of Basidiomycotina) with hyphae that ramify through the wood creating large boreholes in the cell walls. These fungi degrade the wood cell walls to derive nourishment and weaken infected areas. Soft rot is caused by another group of higher fungi (Ascomycotina and many Deuteromycotina) which produce fine boreholes with minimal enlargement (Walker, 1993).

Bacteria are also found to colonise wood in wet and moist environments. The role of bacteria is less important than fungi, however they are recognised as part of the spectrum of organism which are capable of invading wood (Eaton and Hale, 1993). Table 1-3 shows the characteristics of moulds, sapstain, bacteria, brown rot, white rot, and soft rot infected wood. The present study focuses on the prevention of sapstain fungi on rubberwood.

Table 1-5. Comparison of characteristics of moulds, sapstain/blue stain, bacteria, brown rot, white rot and soft rot infected wood.

Characteristics	Mould	Sapstain/Blue stain	Bacteria	Brown rot	White rot	Soft rot
Color	Black, green, orange or other shades.	Blue to black, gray or brown, some red, purple or yellow.	Brownish, inner zones have been noted to be greenish.	Initially lacks luster and appears dead then abnormal brown color develops.	Initially off-white, sometimes with black zone lines.	Darkened, dull brown to blue gray.
Preferred host	Surface of softwood: both sapwood and heartwood. Surface of hardwoods: sapwood preferred	Sapwood of both softwood and hardwoods.	Sapwood and earlywood more susceptible than heartwood and latewood.	Softwoods	Hardwoods	Softwoods and hardwoods.
Strength	No serious effect except toughness.	Only toughness reduced.	Significant reductions in many properties with prolonged exposure.	Significant, rapid reduction in many properties.	Gradual reduction except for toughness which is rapidly reduced.	Significant in thin members.
Surface	Can be brushed or planed from surface; shallow staining may result in hardwoods.	Discoloured blue to black even when planed.	Softened on surface; cross checking develops when dry.	Cross-grain checking, collapse or crumbling and abnormal shrinkage.	Nearly normal until advanced stages; white fibrous mass results.	Softened on surface; fine cracking and fissuring both with and across the grain when dry.
Other	Can occur on air-dried wood when relative humidity exceeds 90%.		Attacks water saturated or completely submerged wood.			Attacks surface of saturated wood.

Source: Cassen *et al.* (1995)

1.7 FACTORS AFFECTING SAPSTAIN SUSCEPTIBILITY

Several factors influence the microbial invasion and colonisation of wood. These include the nature of the wood as substrate, the environment to which the wood is exposed and the presence of any preservative treatment. The many chemical and physical factors that affect fungal growth are covered in detail in textbooks on fungal physiology (Cochrane, 1958; Griffin, 1981) and microbiology (Stainer *et al.*, 1986).

1.7.1 Routes of access: gross anatomy of rubberwood.

The activity of fungi and other organisms involved staining is partly dependent on the physical parameters of the wood. The first priority of a sapstain fungus is to gain access to the nutrient resources that are held within the wood. This may be achieved in two distinct stages a) entry into the wood itself (which may require passage across barriers) and b) movement within the wood. Access across barriers may either occur by direct mycelial penetration or following the natural system of passages provided by the various cellular elements through axial or radial passage systems e.g vessels and ray parenchyma.

In rubberwood the vessels provide the most effective axial passage so that their size, number and distribution are important. Fibres as structural cells are relatively thick, less pitted (than the parenchyma and vessel walls) and possess narrow lumina are thus ineffective as passage system. Living parenchyma cells in sapwood provide a direct route for radial passage. The distribution, size and type of medullary ray parenchyma cells in rubberwood are therefore important aspects in determining rate of mycelial penetration and colonisation.

1.7.2 Sapwood versus heartwood

The wood formed immediately inside the bark of a tree is called sapwood. Sapwood is light in colour and contains living cells that transport water from the roots to the branches and the leaves at the top of the tree. Heartwood is formed in the central part of the tree, as the water-conducting cells in the sapwood die.

Rubberwood is not known to form heartwood. Hence to understand the non-

durability of rubberwood we have to understand the formation and characteristics of heartwood found in many living tree species. Current theory on heartwood formation suggests successive formation of small air bubbles (embolisms) in the older vessels and tracheids, which isolate the adjacent parenchyma cells from food sources, ultimately resulting in their death. Significant chemical and structural changes occur during the transformation of sapwood into heartwood including the loss of starch, the deposition of extractives and the formation of tyloses. Unlike sapwood, heartwood is not susceptible to sapstain because of the reduced amount of starch and the deposition of extractives.

Rubberwood can be considered to be a sapwood specie since there is no clear distinction between sapwood and heartwood in rubberwood (Thomas and Landen, 1953). Like the sapwood of all timbers, rubberwood is rich in sugar and starch and is non-durable (Scheffer and Cowling, 1966; Hong et al., 1980; Mohd Dahlan and Tam 1987; Wong, 1988).

1.7.3 Nutrient contents.

As sapwood inhabiting organisms, sapstain fungi are generally thought to utilize the easily assimilable nutrients and not to damage the structural carbohydrates. The major source of nutrition for growth and development of sapstain fungi in wood is normally associated with the freely available non-structural wood compounds such as soluble carbohydrates (sugars and starch), wood extractives (lipids) and proteins (Rayner and Boddy, 1988; Abraham and Breuil, 1993; Gao *et al.,* 1994; Breuil, 1998).

The high susceptibility of rubberwood to sapstain and wood decay is due to the absence of heartwood and the high content of free sugars and starch (Azizol and Rahim, 1989). The sapwood of rubberwood provides a readily available nutrition for stain, mould and decay fungi (Ashari *et al.,* 1999; Breuil, 1998; Merrill and Cowling, 1966; Wong and Wilkes, 1988). Since these nutrients are stored in the parenchyma cells it could be possible that the differences in anatomical characteristics of these cells are also important in the relative susceptibility of the different rubberwood clones to sapstain fungi as mentioned in Section 1.7.1.

In the 70's, King *et al.* (1981, 1983) and Oxley *et al.* (1976) established that drying has an impact on the composition and distribution of water-soluble sugars as well as some biological effects of these phenomena. Later, Terziev (1995) showed that different drying schedules redistribute the low-molecular weight (LMW) sugars and nitrogenous compounds in different ways. This leads to different degrees of enrichment of the timber surface with nutrients and, consequently, different susceptibility to mould in laboratory and field tests can be expected due to redistribution of nutrients towards the surface of the wood (Terziev and Boutelje 1998).

The above information suggests that when discussing the susceptibility of timber to microbiological attack, the content of some substances, negligible at first sight, but important for the microbiological activities should be considered. It is therefore important to determine the amount of these relevant nutrients in the rubberwood clones and examined the variability of these nutrients in relation to the activity of *B. theobromae* and potential biological control fungi.

1.7.4 Moisture content and relative humidity

The activity of fungi and other organisms involved in staining is dependent on the physical parameters of the wood. The moisture content of the wood and the relative humidity of the surrounding environment are important factors that influence the colonisation of wood by fungi. Most fungi do not grow on wood with moisture content less than 30% (fibre saturation point) or on submerged wood (Schmidt and Gang, 1989). It was shown that a reduction in moisture content to that of 24% protects rubberwood from sapstain (Florence *et al.*, 1993). On the other extreme, saturated wood greater than 96% moisture content could similarly inhibit sapstaining (Colley and Rumbold, 1930). Damage to a log such as debarking causes rapid loss of moisture, which permits early infection. Similar damage to a standing tree may allow localised sapstain development in damaged zones (Butcher, 1974).

Relative humidity effects must be determined by growing the organism in the substrate, which is in equilibrium with the atmosphere. Agar and wood have been used. Unfortunately, quantitative growth on such material is difficult, and data are

rather imprecise. This imprecision makes it hard to decide whether relative humidity or the moisture content of the equilibrated substrate is the most important factor in fungus growth. Probably the amount of water in the substrate is the most important factor. Fungi tolerant of low humidity (85-90%) include *Stereum frustulosum*, *Schizophyllum commune*, and *Aspergillus* spp. (Cochrane, 1958). A few fungi attack substrate as low as 65% relative humidity (Cochrane, 1958), but most are limited to much higher humidity, 95% or more.

Florence (1991) studied the effect of relative humidity and moisture content of rubberwood on growth of *B. theobromae.* Florence (1991) found that at 20°C good growth of *B. theobromae* was noted at 100% relative humidity and no fungal growth was observed at 60%, 70% and 80% relative humidity. However at 30°C profuse growth of *B. theobromae* was noted at 90% and 100% relative humidity. At 40°C the growth of *B. theobromae* was not very much restricted but comparatively better growth was observed at 100% relative humidity. Another interesting finding was that when the relative humidity of the air is high (100%) the wood blocks having low moisture content absorb moisture from the surrounding air and facilitate the growth of *B. theobromae.*

1.7.5 Temperature

Staining fungi are particularly susceptible to high temperatures, and some species are killed by prolonged storage at 35°C (Zabel and Morrell, 1992). Eleven isolates of blue stain fungi including seven geographic strains of *Ceratocystis coerulea* were found to have maximum temperature tolerance ranging from 29 to 39°C (Lindgren, 1942). *B. theobromae* however can grow at a wide range of temperature in the laboratory. Hong (1980) tested the survival of *B. theobromae* on agar as well as on wood blocks and reported that the growth ceases after 2 days at 0°C and 50°C and after 3 days at 40°C on malt agar. Florence *et al.* (1998) observed that the optimum temperature for good growth of *B. theobromae* in agar medium is at 30°C.

Findlay (1959) found that *Botrydiplodia* spp. are more resistant to heat than other staining fungi and can survive for many hours after exposure to temperatures up to 65°C. Käärik (1980) also subscribed to this finding and reported that *B. theobromae*

can withstand temperatures above 65°C. Similarly Florence *et al.* (1998) also reported fungal growth in PDA at 50°C. Hong (1980) reported an optimal temperature of about 30°C for *B. theobromae* growth, and recommended kiln-drying temperatures at 65°C for at least 3 hours to kill the fungus. This shows the duration of exposure to high temperature is also important in ensuring survival of fungi.

1.7.6 pH

All wood-damaging fungi show, in their spore germination and growth, a distinct preference for an acidic environment. Most plant pathogens grow best in media with an initial pH of 5.0 to 6.5 (Cochrane, 1958). Wood decaying Basidiomycotina have pH optimum range from 3 to 6 (Zabel and Morrell, 1992). Brown-rot fungi have the lowest optimum (around pH 3). Wood-stain fungi are highly pH sensitive, and their growth often diminishes or ceases as pH exceeds 5 (Zabel and Morrell, 1992). The optimum pH for growth of sapstain fungi *Ophiostoma* species were reported to be between 3.5 - 6.5 (Kaarik, 1980).

1.8 CONTROL OF SAPSTAIN – AND RELATED PROBLEMS

Rubberwood in Peninsular Malaysia is usually sawn by band saw at a permanent sawmill. Most of the trees are extracted from rubber smallholders in remote locations. Yahya (1998) estimated the production of rubberwood in 1998 at approximately 1.951 million m^3 from rubber estates (21.6%), and 6.685 million m^3 from smallholdings (78.4%) giving a total of 8.636 million m^3. This shows that more than 75% of the rubberwood comes from the rubber smallholders which are scattered throughout Peninsular Malaysia covering a total area of 1.2 million ha (76.9% of total area). For rubber small holdings the simplest and lowest cost effective harvesting method is to cut rubberwood on site using mobile band saws (Gan *et al.*, 1987).

Wood preservation and protection have been well researched for much of the 20th century. Several reviews on the effective method of treatment with preservatives have been well documented and published including the earlier but still relevant work of Cartwright and Findlay (1958), Findlay (1959) and Levi (1973). More recent publications are by Richardson (1993), Cassens *et al.* (1995) and Palfreyman *et al.*

(1996). Standard specification of chemicals recommended for preservative treatments and methods of treatments have also been well documented since early 1950's and well accepted as standard practice by the wood preservation industry including the use of NaPCP in preventing sapstain which was introduced in the 1950's.

Rubberwood preservation treatments are necessary at three steps in the service life of wood products. Firstly, immediately after felling to prevent attack by moulds and sapstain fungi; secondly, before product use to inhibit attack by rotting fungi or insects during service life and thirdly, during use when remedial treatment is required due to an observed attack by decay agent (Palfreyman *et al.*, 1996). In the past the use of NaPCP as temporary protection has been very effective in controlling sapstain in rubber wood if the sawn timber are dipped in a 3% mixture of NaPCP and borax.

For more than 20 years the industry has relied on NaPCP. These chlorinated phenols are very effective fungicides for treating rubberwood (Hong *et al.*, 1994). Unfortunately the compounds are quite toxic to fish, potentially carcinogenic and are sometimes contaminated with extremely toxic dioxin as by-product of the manufacturing process (Bray, 1981; Jones, 1981). For these reasons the use of NaPCP has been banned in Malaysia since 1 January 2000. Research on alternative chemicals to NaPCP to control of sapstain has received great attention since mid 1980's. Several trials of environmentally acceptable preservatives to replace NaPCP have been conducted to determine acceptable levels of efficacy of these formulations for sapstain control (Hong, 1983; Wong *et al.,* 1995; Wong and Woods, 1997; and Wong *et al.,* 1997). Wong *et al.* (1995) reported that alkyl ammonium compounds (AAC) tested in Malaysia appear to be only of moderate efficacy although these compounds are among the potential chemicals for anti-sapstain and above ground applications in the developed countries (Barnes, 1993). The anti-sapstain compound 2-(thiocyanomethylthio) benzothiazole (TCMBT) tested alone and in combination with methylene-bis-thiocyanate (MBT) in Malaysia demonstrated comparable efficacy with NaPCP. Copper-8-quinolinolate (oxine copper) efficacy appears to match that of NaPCP/borax mixture. Due to its low effective dosage and its extremely low toxicity, United States Food and Drug Administration (USFDA) has

approved the use of oxine copper for wood in contact with food (Wong *et al.*, 1995).

Although several sapstain control measures are adopted by the Malaysian rubberwood industry in overcoming the above mentioned NaPCP problem, and have significantly prevented and reduced blue-stain development in rubberwood, the new challenge is to identify "environmentally benign" alternatives to NaPCP to prevent sapstain. It is timely, therefore to evaluate alternative systems to prevent staining by the *B. theobromae.*

1.9 Alternative sapstain control systems including biological control

Several alternative chemicals have been tested and proposed as alternative to NaPCP as mentioned in Section 1.12. Unfortunately, none of these preservatives satisfies the industry that has become used to the effective chlorinated phenols. These new preservatives still suffer from a combination of the following problems (Seifert, 1993): reduced efficacy, higher effective concentration, unwanted discolouration, increased cost, corrosion of equipment, high fish toxicity, skin sensitivity and worker resistance. At the same time pressure to develop benign systems and sustainable technologies are already demanding alternative approaches to wood preservation. Design, use of physical barrier systems, development of biologically based strategies which utilise the natural ecology of the wood decay process, the development of highly targeted preservatives and the use of 'natural' products for increasing durability of wood are some of the possible alternatives (Palfreyman *et al.*, 1996).

Biological control treatment is one of the non-chemical approaches of preventing sapstain. As expected the upsurge in the research of the topic is due to increase in public awareness and concern over environmental toxicity of chemical biocides including wood preservatives (Bruce, 1992). Smith in 1919 was the first to propose the term 'biological control' (Campbell, 1989). Baker and Cook (1974) described biological control or biocontrol as 'the reduction of inoculum density or disease producing activities of a pathogen or parasite in its active state or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists'. It has only been in the past 70 years that there has been a significant accumulation of

information on the biological control of plant pathogens and only over the last 15 – 20 years that extensive worldwide research has occurred.

Most studies on sapstain have been concerned with softwoods, although the phenomenon also occurs in hardwoods. Earlier studies of sapstain examine biological control potential of bacteria and culture filtrates from species including *Bacillus subtilis* (Bernier *et al.,* 1986; Siefert *et al.,* 1987; Florence, 1991). Florence (1991) reported that *Bacillus subtilis*, a bacterium isolated from rubberwood was found to be antagonistic against several stain and mould fungi. The efficacy of this bacterium was tested in the field and proved to be effective in controlling sapstain up to a level of 80% in green lumber. However Silva and Morrell (1998) showed that *B. subtilis* is not capable of completely inhibiting growth of the target fungus *Ophiostoma picea*. It is unclear whether this incomplete protection reflects on the inability of *B. subtilis* to completely colonize the substrate but did suggest that both the control and target organisms germinate and colonise portions of the wood surface and this probably led to protected and unprotected zones. There are also reports on the use of mould and fungi as biological control agent of sapstain on other wood species. Croan (1996) showed that non-pigmented isolates of *Ceratocystis* spp. and *Ophiiostoma* spp. could also be used for the biological logical control of sapstain on yellow pine logs. More examples on biological control of sapstain on softwoods are given in Section 7.4.2.

The green moisture content of rubberwood is approximately 60%, therefore the freshly cut lumber is always damp. Under tropical humid climate as in Malaysia rubberwood requires proper stacking and good air ventilation and must be air dried or kiln dried to below 24% moisture content within 24 hours. If all the logs were to be processed within 24 hours the sawn timbers produced would be free from sapstain. Therefore sapstain control without the use of chemical is possible if an efficient and integrated management system involving careful harvesting, efficient delivery, kiln drying and storage could be implemented. However in Malaysia, with 76% of the rubberwood logs coming from the smallholders, this ideal system is sometimes not practicable especially when felling is carried out in scattered and/or remote locations. In addition, due to logistics and poor co-ordination especially between felling contractors and smallholders, delay often occurs in the delivery of logs/sawn timber to the treatment plant, sawmill, particleboard or MDF plant

where the desired stacking/drying/treatment could be carried out. Similarly unscheduled supply/arrival of sawn timber at the treatment plant or mill could also delay drying or sapstain treatment. Any delay in delivery of these logs (without or with improper pre–treatment) is currently the major cause of sapstain in rubberwood. This is currently the major problem of preventing sapstain of rubberwood in Malaysia. This could be a possible solution if a better management and coordinating system in the felling and replanting of rubber tree owned by smallholders could be effectively implemented.

In temperate countries during winter when fungi inoculum and insect activity are lowest, logs are often floated down the river to lumber mills during transportation (Seifert, 1993) or stored in ponds up to a year before processing. A similarly widely used practice in temperate countries involves storage of log piles and prevention of sapstain by sprinkling (Webber, 1990). These methods are however not possible for rubberwood in the hot and humid tropical climates of Malaysia. The average air temperature and relative humidity are approximately 30^0C and 80% respectively are optimum for fungal growth (Florence *et al.,* 1998).

The next possible non-chemical alternative is to use rubberwood naturally resistant to sapstain by emulating the success of RRIM in selecting rubberwood clones that could produce high latex or/and timber yield. Similar breeding selection programme could be worked out to breed and select rubberwood clones of high resistance against sapstain. There lies great potential and possible non-chemical solution in overcoming sapstain problem of rubberwood in Malaysia.

1.10 Possibility of clonal variation in rubberwood

From Section 1.4 it was shown that there are differences in percentage of tension wood in clone PB260 and RRIM600. The proportion of tension wood ranged from 15 to 65%. However, the study by Lim and Ani Sulaiman (1994) on two clones of rubberwood (PB 260 and RRIM600) of different ages found that clone RRIM600 had a lower percentage of tension wood (about 30%) as compared to clone PB 260 (more than 40%). It was also noted that there were differences in basic density with age and clone (PB260 and RRIM600). Depending on the clone and age of the rubber

tree, the basic density of rubberwood ranges from 430 to 620 kg/m^3 (FRIM, 1985). What is more significant is that since 1928, the RRIM through clonal breeding and selection, is successful in increasing latex yield from 550 kg/ha/yr to more than 2500 kg/ha/year (LGM, 1998). With such quantum increase in latex yield, there is the possibility of a corresponding increase in variability of the rubberwood clones in term of its physical, chemical and anatomical characteristics. For example the high latex yield could be associated with a) active and relatively higher quantity of living parenchyma cells or b) large diameter vessels or c) storage of nutrients like starch in the stem. There has been no study so far been conducted to examine these factors which could be related to the susceptibility of rubberwood to sapstain fungi.

Unlike other timber species, the rubber tree is unique as it has both latex and wood that could be exploited. Consequently, alternatives involving both latex and timber extraction should be considered. The RRIM has demonstrated the success of tree breeding through selection of better clones that provide good timber and latex yield. While growth characteristics such as bole volume, vigour, trunk appearance, resistance to wind damage, and major leaf diseases are important, other clonal differences such as a) clonal variation in physical and anatomical properties, b) differences in carbon, nitrogen, free sugars and starch, and c) susceptibility of rubberwood to sapstain fungi could also be examined in the future. Selection of clones more resistant to sapstain fungi could help overcome the major problem of sapstain in the wood industry.

Thus there lies enormous potential of research into future clones to include also aspects related to physical properties, anatomical properties, and basic chemical composition of rubberwood clones. The information gathered would provide basic knowledge that will help us understand sapstain fungi activity and sapstain susceptibility of rubberwood clones. With possible differences there is also the potential of examining differences in sapstain and biological control agent activities in the selected rubberwood clones rather than in agar media.

1.11 Aims and Objectives

Sapstain fungal growth on rubberwood is an important economic problem for the Malaysian wood industry. In the current situation it is important to evaluate all possible ways of reducing/eliminating sapstain. In this current study clonal differences in susceptibility to sapstain fungi are evaluated in conjunction with potential biological control systems. The specific objectives of the study are:

- To examine the physical and anatomical features of the selected rubberwood clones GT1, PB217 and RRIM600.
- To determine the amount of nitrogen, carbon and sugar contents in the selected rubberwood clones.
- To evaluate several methods of quantitative assessment of sapstain fungi (*B. theobromae*) as an alternative to the normal subjective qualitative assessment of sapstain.
- To examine sapstain susceptibility using a quantitative method of assessment on the selected rubberwood clones.
- To screen several moulds and non-staining fungi using a range of agar techniques for potential biological control activities.
- To determine, quantitatively, biological control activity (of the screened fungi) against the sapstain fungus *B. theobromae* on wood samples of the three selected clones.

CHAPTER 2

2. GENERAL MATERIALS AND METHODS

2.1 SOURCE OF RAW MATERIALS

Based on the available clones for felling and replanting, three rubberwood (*Hevea brasiliensis* (A Juess) Muell. Arg.) clones were selected for this study. Two clones (PB217 and RRIM600) were obtained from the Bukit Pilah Estate, Negeri Sembilan, Malaysia and the third clone (GT1) came from the Tangkah Estate, Johor, Malaysia (Figure 2-1). The trees were sampled from locations of similar planting density (400 stem/hectare), soil type (loamy), terrain (undulating to hilly) and month of the year. The wood materials were felled in November 1997 (end of dry season).

2.1.1 Field procedure

Five trees were felled at random from each clone. The standing height was measured from the base to the top. Each stem was then measured and marked to indicate the breast-height (1.3 m from soil level at highest point) before felling for diameter measurement. After felling, the diameter at breast height over bark, total height and merchantable height (height to first branch) were measured (Figure 2-2). Discs samples of about 2 cm thickness at breast height, and at the different percent heights (20%, 50% and 80%) to first branch were also obtained. The remaining parts of the stem (below the first branch) were cut into 1 m to 2 m billets. The cut surfaces of the stem were coated with paint to minimize drying and to prevent insect and fungal attack. The samples were immediately transported to the laboratory for further assessment.

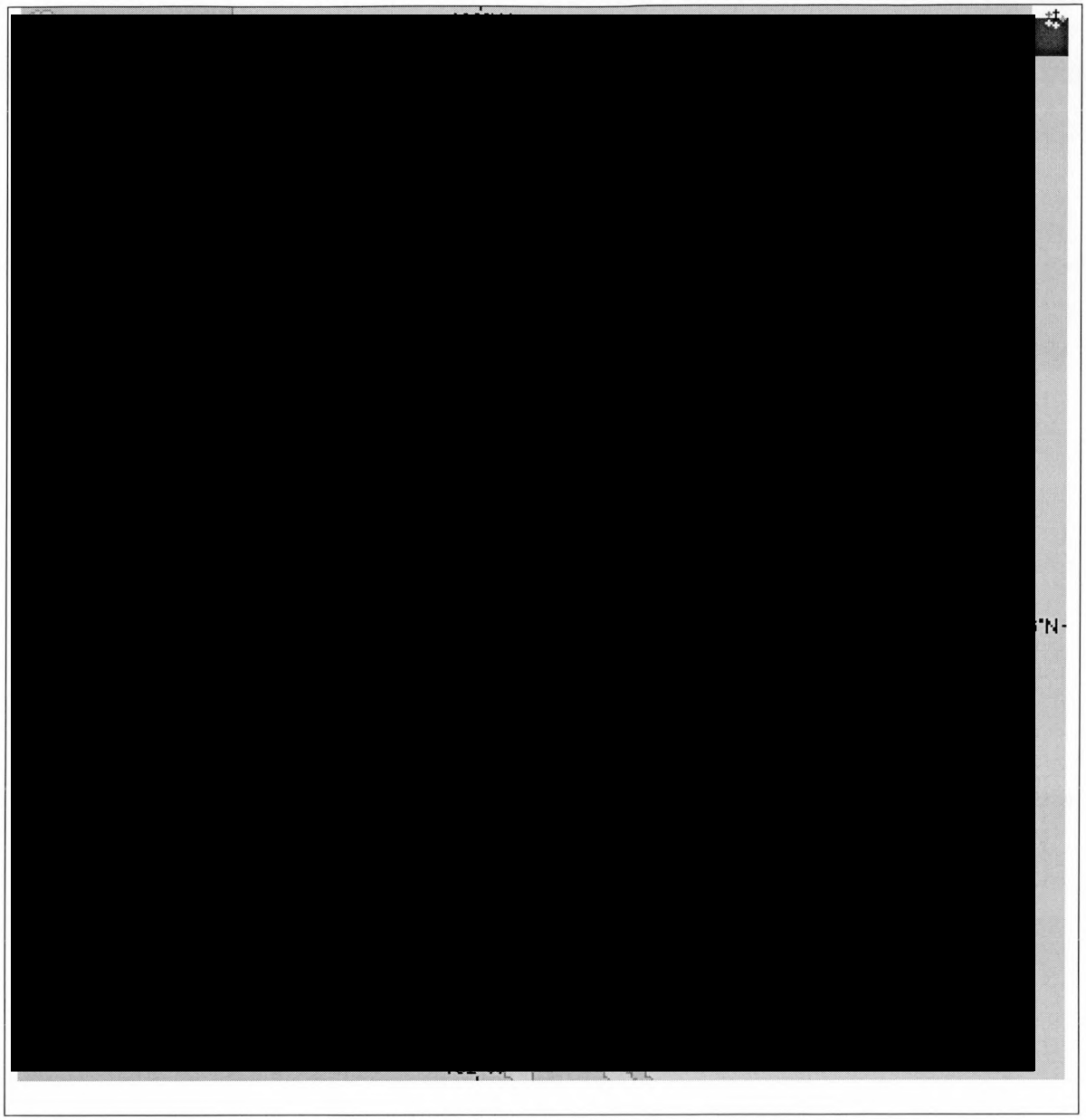


Figure 2-1. Map of Peninsular Malaysia indicating locations where clone samples were obtained.

2.1.2 Materials preparation

2.1.2.1 Billets

At the sawmill division in FRIM the remaining billets were sawn to 2.5 x 2.0 x 100 cm size and dried to below 10% moisture content at 45°C for 48 hours. About 25 pieces were selected at random for the drying experiment and the remaining pieces of wood blocks (5 x 20 x 40 mm) were then prepared, double sealed in plastic bags and sterilised by gamma radiation (25 kGrey dosage).

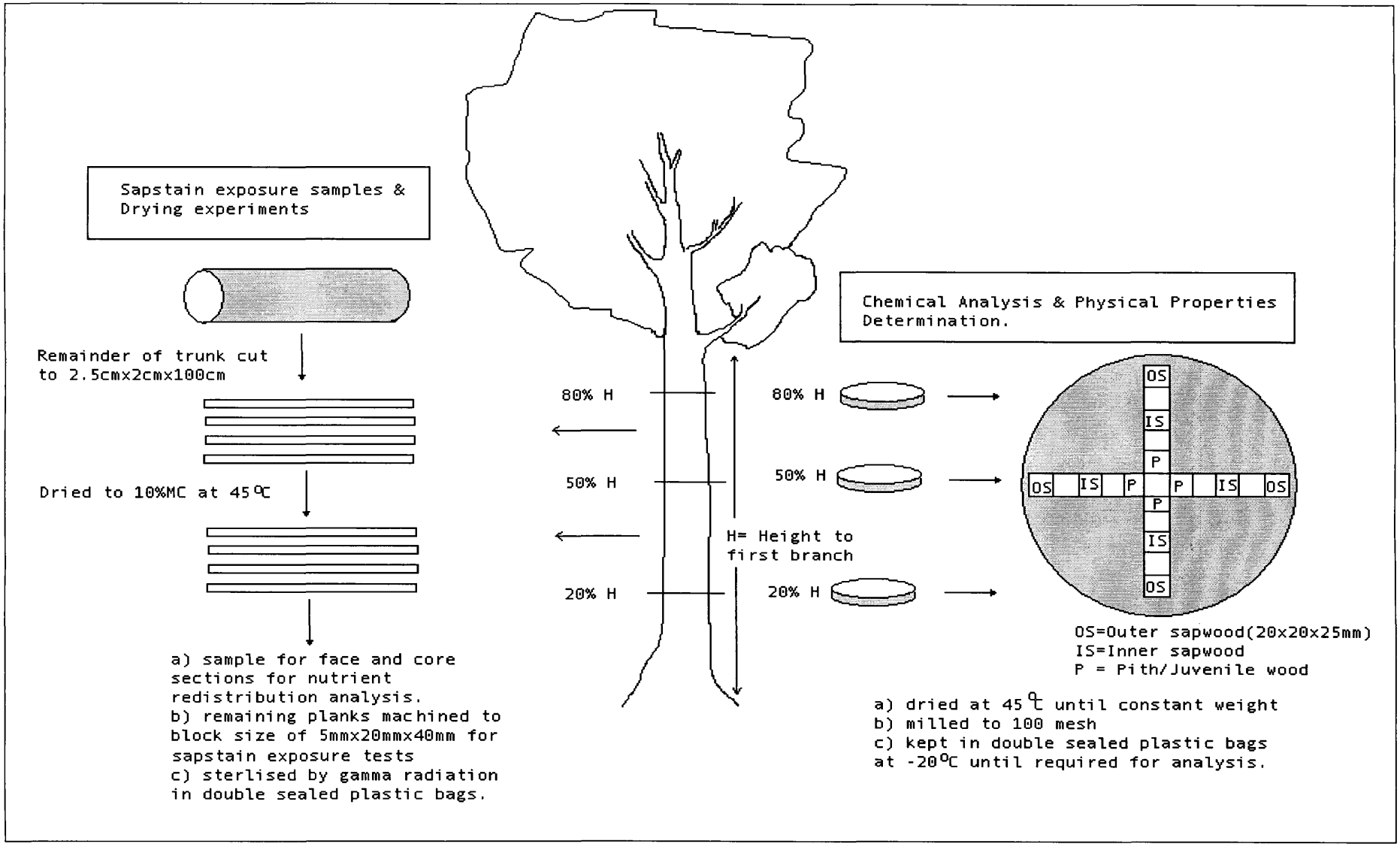


Figure 2-2. Preparations of test materials.

2.1.2.2 Disc samples at different height portions

Immediately after arrival in the laboratory, samples (10 x 10 x 20 mm) were cut from each disc to represent the outer, middle and inner sapwood sections. The wood specimens were then dried in the oven overnight at 45°C. The dried samples were then milled (Willey mill), screened (40 mesh), then placed in separate airtight containers and stored in the freezer until required for chemical analysis (King *et al.*, 1981).

2.1.3 Sampling for basic physical properties

For the determination of physical basic density and moisture content, the disc samples from the 3 height portions were used.

2.1.3.1 Determination of basic density

Technical Association of Pulp and Paper Industry (TAPPI) standard Method T-18 (TAPPI, 1978) for the determination of basic density of wood was used. From each disc, 3 samples of approximately 2cm x 2cm x 2cm size were obtained. The volume of the green sample V_g was accurately measured by the water displacement method as follows: a) the wood sample was initially weighed (w_1), b) the same sample was then reweighed while completely submerged under water (w_2) c) the difference in weight ($w_1 - w_2$) g is the green volume of the sample in (V_g) cm^3.

After the determination of the green volume, the samples were placed in an oven at 105 °C ± 2 until constant weight (W_o). The basic density was calculated as follows:

Basic density (D) = W_o/V_g kg/m^3 where,

W_o = oven dried weight (kg) and

V_g = Volume of the green sample as determined by water displacement (m^3).

2.1.3.2 Determination of moisture content

The determination of moisture content was based on TAPPI Standard Method (TAPPI, 1978). Fresh duplicate samples, from each breast height (BH) disc from the three clones were immediately weighed and dried in an oven at a temperature of 103 ± 2°C until constant weight. The samples were then cooled for 30 minutes in a

desiccator prior to weighing. The moisture content of the test samples were expressed as a percentage loss in weight of the final oven-dry weight using the following calculation:

Moisture content (%) = $[(W_g - W_o)/W_o] \times 100$ where,

W_g is the green weight and W_o is the oven-dry weight in g.

2.2 CHEMICAL ANALYSES

2.2.1 Nitrogen

The wood samples from three height and sapwood portions of GT1, PB217 and RRIM 600 were sampled for analysis of nitrogen. The nitrogen contents were determined by the micro-Kjedahl method (Kjeldahl, 1883; Pomeranz and Moore, 1975) using a) the Kjeldatherm™ digestion system KT-40 (C. Gerhardt, Bonn, Germany) b) the Vapodest-5 (C.Gerhardt, Bonn, Germany) distillation and titration unit.

2.2.2 Sample standards preparation

2.2.2.1 Standard nitrogen solution 1000 ppm (1 mg/g)

Ammonium sulphate was dried at 105°C for two hours and then weighed accurately into a 600 ml beaker. 200 ml of distilled water was added and stirred until the particles were completely dissolved. The solution was then transferred to a 1 litre volumetric flask and made up to the mark with distilled water.

2.2.2.2 Nitrogen working standard

50 ml of standard nitrogen solution was pipetted into a 100 ml volumetric flask and made up to the mark and then thoroughly mixed. This gave a standard solution of 500 ppm.

2.2.3 Determination of nitrogen content

2.2.3.1 Digestion of sample

The wood meals were dried to constant weight in a dessicator. Approximately 0.5 g of wood meal was accurately weighed and placed into a 100ml digestion tube and 10 ml of 98% concentrated sulphuric acid was added, followed by addition of 2.5 g catalyst (K_2SO_4:SeO_2 ;1000:1). The sample in the digestion tube was then soaked overnight. After soaking, the sample was then placed in the Kjeldatherm™ digestion system. The water jet pump and the Turbog™ scrubber unit were switched on. The temperature of the heating block was gradually increased (50°C each time) until the temperature reached 400°C. The sample was then allowed to digest under reflux for 3 to 4 hours. When digestion reached the end point (as indicated by the development of a clear liquid), the reaction mixture was then cooled to room temperature.

2.2.4 Distillation and titration of samples

The digest solutions were then analysed for nitrogen using Vapodest-5 distillation and automatic titration unit.

Distillation and titration of sample solutions were programmed using the Vapodest-5 distillation unit. The programmed methods were as follows: (i) diluting water: 3 sec. (20ml per 5ml sulphuric acid used in digestion) (ii) adding sodium hydroxide: 5 sec. (35ml per 5ml sulphuric acid used) (ii) distillation time: 4 min. (recommended for the catalyst used) (iii) suction time (receiver): 10 sec. (iv) suction time (digestion tube): 20 sec. (v) Adding boric acid: 9 sec. (vi) the end point was determined automatically (the calculated pH value in the receiver at the beginning of the distillation will be taken as the end point of the titration).

During the distillation in the Kjeldatherm™, nitrogen which was converted to ammonium sulphate for the duration of the digestion process, was released as ammonia when excess sodium hydroxide was added . The ammonia was collected in 2% boric acid solution. The ammonia collected was titrated with standard 0.01M HCl. No indicator was needed as titration was done automatically based on the

continuously measured pH. The calculated pH value in the receiver at the beginning of the distillation was taken as the end point of the titration.

Ammonium sulphate standards at 500 and 1000 ppm were run with each set of wood samples.

2.2.5 Nitrogen content calculation

The unit was programmed to calculate the results based on the formula:

%N (w/w) = 1.4007 x titre / weight of wood (g) analysed

= 1.4007 x (xx.xx ml used – xx.xx ml blank value) / weight of wood

Therefore the percent nitrogen were expressed on the basis of the original dry weight of the wood sample.

2.2.6 Total carbon

2.2.6.1 Test for presence of inorganic carbon

A small amount of finely ground rubberwood sample (rubberwood flour) was placed on a spot plate, and moistened with a few drops of water. 4N HCL was added drop wise to the wetted sample, and observed for any effervescence (about 5 minutes).

2.2.6.2 Total organic carbon

The total organic carbon contents were determined by the Walkley-Black procedure (Walkley and Black, 1934; Walkley, 1947; Peech *et al.,* 1947, Greweling and Peech 1960). An accurate amount of 0.1 to 0.2 g of rubberwood flour was placed in a 500-ml wide mouth Erlenmeyer flask. 10 ml of 1N potassium dichromate ($K_2Cr_2O_7$) was added and the flask was swirled gently to disperse the wood fibre in the solution and 50 ml of concentrated sulphuric (H_2SO_2) was rapidly added, directing the stream into the suspension. The flask was then swirled gently until the sample and the reagents were mixed and then swirled vigorously for a total of 1 min. The flask was then allowed to stand on a sheet of asbestos for about 30 min. Then 200 ml of water was added to the flask and filtered. 3 to 4 drops of o-phenanthroline indicator were added as indicator and the solution was titrated with 0.5N ferrous sulphate ($FeSO_4$). As the end point approached, the solution took on a greenish cast and then changed to a

dark green. At this point, ferrous sulphate heptahydrate was added drop by drop until the colour changed sharply from blue to red (maroon colour in reflected light against a white background). A blank determination was made in the same manner, but without the wood sample to standardize the $Cr_2O_7^{2-}$.

The percentage organic carbon (C%) were calculated to the following formula, using a correction factor of f = 1.30.

C % = [(meq$K_2Cr_2O_7$ - meq $FeSO_4$) (0.003)(100) x f] / weight of oven dried wood (g)

2.2.7 Total free sugars

2.2.7.1 Extraction procedures

Wood samples milled to 100 mesh were dried to constant weight at 45°C and then placed in the desicator over concentrated sulphuric acid. 3 g of wood samples were placed in 50ml deionised water in Erlenmeyer flasks (250 ml). The flasks and contents were allowed to equilibrate for five minutes and were then placed on an orbital shaker at 200 r.p.m at 30°C. After sixty minutes, the contents were filtered through filter paper (Whatman's No.1) and then filtered through a membrane filter (pore size, 0.45 μm) to remove any residual wood. The pH of the filtered solutions was determined and recorded using Mettler™ Teledo pH meter and Mettler™ Teledo Intlab 413 electrode.

2.2.7.2 Injection of samples

The clear solution (60 μl) of each sample was drawn into a syringe and injected into a Shimadzu LC-10AT high performance liquid chromatograph equipped with a 5 μm, 4.6 mm (I.D.) x 250 mm spherical Apex™ carbohydrate column. After injection and passing through the column, the sample was mixed with a steady stream of nebulising nitrogen gas (flowing at a rate of 2 standard litre/min), forming an aerosol. This was immediately followed by solvent vaporisation in the drift tube (recommended temperature, 79°C) to produce a cloud of solute droplets, which entered a light scattering detector. The sample particles scattered the laser light, but

the evaporated mobile phase (acetonitrile:water 80/20) did not. The scattered light was detected by a silicone photodiode located at a 90° angle from the laser source. The photodiode produced a signal, which was sent to the analog output for collection via a Schimadzu™ chromatopac C-R6A data processor.

Free sugar contents were calculated by comparison to standards. The standards injected were 0.4 mg/ml each of xylose, arabinose, fructose, glucose and sucrose. After injection the syringe was rinsed 5 times in deionised water. Two replicates were undertaken for each sample extract.

2.2.8 Determination of starch content

Rubberwood samples for analysis were prepared by grinding the transverse cross-sectional faces of samples with a disc-sander passing through a 200 mesh sieve and followed by drying for 3 days in a desiccator over concentrated sulphuric acid. 4.7 ml (7.2M) of perchloric acid ($HClO_4$) was added to 400 mg of the dried finely ground rubberwood sample. Samples were agitated for 15 min at 150 rpm. Distilled water was added to makeup 50 ml in and samples were centrifuged for 15 minutes at 2000 rpm. 10ml of the supernatant was poured into a 50 ml volumetric flask. 2 drops of phenolphthalein indicator were added followed by 2-3 drops of 2M sodium hydroxide until the solution turned pink. Excess 2M ethanoic acid was then added until the solution turned colourless (2.5 ml). 0.5 ml 10% potassium iodide (KI) was added followed by 5ml potassium iodate (KIO_3). The solutions were then allowed to stand for 15 minutes. Finally distilled water was added to make 50 ml. The solution was labelled and ready for starch determination by the colorimetric method.

The colorimetric method depends ultimately on the reaction of the amylose in wood starch with iodine. Absorbance was read at 650 nm in 10 mm light path glass curvettes in an Ultrospec II ™ spectrophotometer using a method, which was adapted from Humphreys and Kelly (1961). The standard reference was obtained using A.R. potato starch in which the amylose/ amylopectin ratio is similar to that the starch present in wood. A range of standard starch solutions (0.1 – 1.0) gl^{-1} was prepared and 10 ml solutions of known concentration were treated as above to produce a calibration curve for absorbance. The percentage of starch is calculated by

using the following formula:

% starch = [0.36778 (E. reading + 0.008) x 50] / [10 (Oven dried weight of sample)]

2.3 SOURCE AND MAINTENANCE OF FUNGI

Cultures of the blue stain fungus *Botryodiplodia theobromae* Pat. (isolated from blue stained rubberwood, A.A.H. Wong, FRIM) and *Paecilomyces variotii* Banier (F02) were provided by A. A. H. Wong from the Forest Research Institute of Malaysia (FRIM), the stain fungus *Aureobasidium pullulans* (de Bary) Arnauld (UiTM-630) (F5) and the mould fungi F013 (*Trychosporone* sp.), *A. niger* v. Tiegham (F14), F15 (unknown), F16 (unknown), *P. chrysogenum* (F17), *A. oryzae* (F18), *Fusarium moniliforme* Thom. (F30), *Aspergillus flavus* Link ex Fr. Pers. ex S.F. Gray (F31) *Aspergillus fumigatus* Fres. (F32), *Mucor* spp. (F33) and *Penicillium* spp. (F43) were provided by H. K. Lee of Universiti Teknologi MARA, Shah Alam, Malaysia (UiTM). Additional pure fungal and bacterial cultures were obtained from University Kebangsaan Malaysia (e.g. *Trichoderma reesei* (F03), *Trichoderma viridae* Pres. Ex Gray 1821 (F04), *Trichoderma* spp. (F06), and *Penicillium* spp. (F01). All fungi were grown on 2% malt agar plates at 25° C in the dark for approximately 14 days. The organisms were sub-cultured at regular intervals.

2.3.1 Screening of test fungi

Test A. Sensitivity test and interaction assay.

Mycelia plugs removed from the growing margins of culture *B. theobromea* was placed at one side of petri dish containing malt extract agar. Plates were incubated at 25° C for 4 days. After 4 days culture of each of the test fungi was placed at the opposite end of each petri dish to interact with the *B. theobromae* under sterile condition. The plates in triplicates were incubated under the same condition for 4 weeks. Plates were examined daily to determine the outcome of interaction between the fungi.

Test B. *B. theobromae* plug on MEA seeded with test fungi spores (kept in MEB)

Mycelia plugs removed from the growing margins of test fungi were placed in Malt Extract Broth (MEB) in a\ 250 ml flasks under sterile condition and placed on a shaker for 72 hours at 27°C to stimulate spore germination and were then seeded on petri dishes containing malt extract agar.

Test mycelia plugs were removed from the growing margins of *B. theobromae* and placed at the centre of each petri dish already seeded with respective test fungi spores.

Test C. Test fungi plug on MEA seeded with *B. theobromae* spores (in MEB)

Mycelia plugs removed from the growing margins of *B. theobromae* were placed in MEB in a 250ml flask under sterile condition and placed on a shaker for 72 hours at 27°C to stimulate spore germination and were then seeded on a petri dish containing malt extract agar.

Test mycelia plug were removed from the growing margins of *B. theobromae* and placed at the centre of each petri dish already seeded with test fungi spores Diameter growth of the test fungi was measured after 3 days.

Test D. Rapid screening test after inhibition of test fungi spores (kept in distilled water).

Mycelia plugs removed from the growing margins of *B. theobromae* were placed in distilled water in a 250 ml flask under sterile condition and placed on a shaker for 72 hours at 27°C to inhibit spore germination and was then seeded on a petri dish containing MEA.

Test mycelia plug were removed from the growing margins of *B. theobromae* and placed at the centre of each petri dish already seeded with respective inhibited test fungi spores. Diameter growths of the respective test fungi were measured after 3 days.

Test E. Rapid screening test after inhibition of BT spores (kept in distilled water).

Mycelia plugs removed from the growing margins of *B. theobromae* were placed in distilled water in a 250 ml flask under sterile condition and placed on a shaker for 72 hours at 27°C to inhibit spore germination and was then seeded on a petri dish containing MEA.

Test mycelia plugs were removed from the growing margins of test fungi and placed at the centre of each petri dish already seeded with *B. theobromae* spores.

Test F. Screening on malt extract agar (MEA).

Agar discs were cut out from the test fungal culture and placed on MEA plates seeded with the target fungus *B. theobromae* (from actively growing culture). Diameter growths of the respective test fungi were recorded daily for 1 week.

Test G. Screening on potato dextrose agar (PDA)

Agar discs were cut out from the test fungal culture and placed on PDA plates seeded with the target fungus *B. theobromae* (from actively growing culture). Diameter growths of the respective test fungi were recorded daily for 1 week.

All assay plates were incubated at 27°C and 80% relative humidity. Diameter growths of the test fungi were measured after 3 days.

2.4 SAPSTAIN EXPOSURE TEST PROTOCOL

Mycelia fragments/spore suspensions of sapstain fungi and moulds were prepared separately by blending the fungal colony agar mixture with 400 ml of sterilized distilled water in a Waring™ blender followed by filtering through sterilized cotton gauze. Five replicate samples of each rubberwood clone were dipped for 5 seconds in a given treatment regime, then placed in pairs into glass petri dishes. The paired samples (with similar fungal treatment) were placed under sterile condition on nylon mesh over moistened filter paper in the dish. Samples were incubated at 25°C for 4 weeks.

2.4.1 Spore count

Before inoculation the spores in suspension were counted using a haemocyometer grid. The minimum number of spores required was 10^6 spores per ml as with this amount of spore the estimated number of spores binding to the rubberwood sample surface per 5 second dip is approximately 1600 spores per m^2.

2.4.2 Inoculum

Two sapstain fungi used as inoculum were *A. pullulans* and *B. theobromae*. The mould fungi were *A. niger, T. viridae, T. reesei* and *P. variotii.*

2.4.3 Testing against sapstaining fungi and moulds

Rubberwood samples were then treated with spore inoculum of sapstain or mould fungi or mixture. The spore mixtures were prepared by mixing equal proportions of suspensions of individual fungal spores. The rubberwood samples were inoculated with various treatment regimes by dipping into the respective spore suspension for 5 seconds. Treated samples of each clone were positioned on an inert plastic mesh placed in a petri dish over moistened filter paper to maintain humid conditions and reduce drying out of the wood. The mesh supported the samples above the moist surface to prevent leaching of nutrient and water-logging (which might depress fungal growth) of the wood samples. The samples were incubated at 30°C for 4 weeks, with observations and assessments being made everyday for the first week. The surface coverage of fungi was visually assessed daily based on a 5-point FRIM rating scale (Wong *et al.,* 1999b) with 0 =sound, 1 = 0- 5% coverage, 2 = 6 to 20% coverage, 3 = 21 to 35% coverage, 4 = 36 to 50% coverage, and 5 = more than 50% coverage, until mycelia coverage exceeds 50% of the test surface.

2.5 QUALITATIVE AND QUANTITATIVE SAPSTAIN EVALUATION

2.5.1 Visual stain assessment

Visual assessment of sapstain was carried out after drying of sapstain exposed samples and minimal sanding (Section 2.5.2.1) based on the 5-point scale mentioned in 2.4.3.

2.5.2 Quantitative stain assessment

After 4 weeks, the wood samples from the sapstain exposure tests were dried to below 10% moisture content in an oven at 60°C for 48 hours to prevent any further fungal growth.

2.5.2.1 Sanding of sapstain exposed samples

Sanding of samples was done manually using 120-grit sandpaper. The samples were then manually sanded to remove approximately 0.5 mm of the surface layer to remove the mycelium and fungal spores on the rubberwood surface. The percentage darkness determined after sanding is the discoloration as a result of pigmented fungal mycelium at the subsurface layer.

2.5.2.2 Spectrophotometer (Spectroflash™500)

Spectroflash™ 500 is a fast, accurate dual beam spectrophotometer designed to measure the percent reflectance or transmittance of samples in the visible wavelength region. The transmission of the light beam and the detection of the amount of light reflected from the rubberwood surface were fully computerised.

The spectrophotometer was calibrated by measuring the zero reflecting light trap (or blocking the lens for transmission), and using a black calibration tile (minimum reflectance) and a white calibration tile (maximum reflectance) to establish the lower and upper reflectance limits. Measurements were made using a 6 mm viewing area.

Samples were placed against the reflectance measurement aperture and the sample test description was keyed into the computer. Light reflected from the sample was collected, measured and recorded on a CIE standard daylight illuminant on a scale of 0–100. The darkness mean was represented by percent of light absorbed (inverse expression of percent of light reflected and measured). Two readings were obtained for each sample

2.5.2.3 Colorimeter (Minolta™ Croma Meter CR200)

The Minolta™ Croma Meter CR200 is a compact tristimulus colour analyser for measuring reflective colour of surfaces. The measuring head had an 8mm-diameter measuring area and uses diffuse illumination and a 0^{o} viewing angle.

Unlike the spectrophotometer the tristimulus colorimeter takes measurements under only standard illuminant C or Standard Illuminant D65, both of which represent daylight and which have very similar spectral power distribution (Minolta, 1994). Calibration of the colorimeter involved setting the readings to a standard (Y; x; y) readings using the calibration plate (white) provided. For standard illuminant C the (Y; x; y) were set to (94.2; 0.3131; 0.3201) and for standard illuminant D65 the (Y; x; y) were set to (94.2; 0.3156; 0.3329).

Readings were obtained by placing the "observer" of the portable colorimeter on the sample surface, once the reflectance measurements were taken, the reflectance values were automatically calculated based on CIE standard daylight illuminant on a scale of 0–100. Similarly darkness means were represented by percent of light absorbed (inverse expression of percent of light reflected and measured).

2.5.2.4 Use of the Densitometer

Calibration of the densitometer involves establishing a zero value in the reflectance value of unstained wood and a maximum value, i.e. the reflectance of the darkest sample. All other samples were evaluated against this scale. Two readings were obtained for each sample as follows: the amount of light reflected from the sample surface was evaluated by scanning crosswise. The average value obtained represents the average percent reflectance. Darkness was represented by percent of light absorbed = (100 - percent reflectance).

2.6 SCANNING ELECTRON MICROSCOPY (SEM)

For more detailed observation of the fungal mycelium the rubberwood samples were observed by scanning electron microscopy. Before viewing by the SEM, small sections from wood samples (10 mm x 5 mm x 5 mm) were removed for observation and processed and viewed as described in 2.6.1 – 2.6.2.

2.6.1 Coating of specimens

Two random samples were selected to represent the untreated rubberwood sample, samples treated with *B. theobromae, A. niger, T. viridae, B. theobromae+ T. viridae* and *B. theobromae + A. niger* and prepared for SEM examination. The small wood specimens were gold coated using the SPI-module™ sputter coater using the manufactures instructions.

2.6.2 Viewing of SEM images.

Samples were viewed using the LEICA S440™ which is a software-controlled scanning electron microscope using the manufacturers instructions. The specimen chamber holds the specimen to be viewed in such a way that it may be freely maneuvered during examination. The Cartesian stage permits movement of 100 mm in both X and Y directions, 20 mm in the Z direction, 0^{o} to 90^{o} tilt and 360^{o} rotation.

The specimens observed were approximately 5 mm x 10 mm x 10 mm. Because of the small size of the specimen, there were limited number of vessels and rays that could be examined per sample. Vessel diameter and ray characteristics (ray height and width) were determined from 5 replicates per sample.

2.7 STATISTICAL ANALYSIS

The statistical analysis was carried out using the Minitab Statistical Software (Minitab Relase 13.1) and the Statistical Package Software (SPSS). Analysis of variance (ANOVA).

Standard procedures were carried out to examine each datafile for possible outliers or data entry errors prior to statistical analysis. Normality and skewness tests were also performed before every analysis of variance (ANOVA) and post hypothesis testing.

Tukeys' test were used to compare treatments with the control (e.g. *B. theobromae* exposed samples) while Duncan Multiple Range Test (DMRT) were used to examine differences between treatments due to the main effect an interactions (Waller and Duncan, 1969).

CHAPTER 3

3. VARIABILITY IN PHYSICAL AND ANATOMICAL PROPERTIES OF RUBBERWOOD CLONES

3.1 INTRODUCTION

This chapter will examine the clonal variation in the physical and anatomical properties of the three selected rubberwood clones GT1, PB217 and RRIM600. Physical properties examined were basic density (oven dry weight/green volume), green moisture content and pH. Where possible the effect of height and section of the stems are taken into account in the analysis of these variables. The objective of this chapter is to gather new information on differences in physical and anatomical characteristics of rubberwood clones that could influence the susceptibility of the selected rubberwood clones GT1, PB217 and RRIM600 to sapstain fungi.

3.2 MATERIALS AND METHODS

The basic density and moisture content of rubberwood clones were determined as described in Section 2.1.3 and method for the determination of vessel and ray parenchyma dimensions were as described in Section 2.6.2

3.3 RESULTS

Table 3-1 shows the mean height, diameter at breast height and merchantable length of GT1, PB217 and RRIM600 rubber trees used in this study. GT1 was significantly taller ($p<0.05$) and with larger diameter that PB217 and RRIM600. There is no significant difference ($p>0.05$) in merchantable length.

Table 3-1. Mean* height, diameter at breast height and merchantable length of clones GT1, PB217 and RRIM600.

Clone	Height (m)	DBH (cm)	Merchantable length (m)
GT1	20.30(0.66)b	26.98(3.65)b	4.68(0.47)a
PB217	18.33(0.39)a	23.44(2.28)a	3.60(0.41)a
RRIM600	19.42(0.42)b	24.43(1.62)a	4.49(0.62)a

Note: Number in parenthesis refers to standard deviations.

* = Same letter down the column (between clones) denotes non-significant difference ($p>0.05$)

3.3.1 Basic density

Table 3-2 summarises the mean basic density of the rubberwood clones GT1, PB217 and RRIM600 showing the mean values of clone, height and clone versus height interaction. The table shows that clone PB217 has significantly ($p<0.05$) the lowest basic density (582.2 kg/m^3) though the value for the bottom section of PB217 is similar to those of all the height sections of GT1 and RRIM600. There is however no significant ($p>0.05$) difference between clone RRIM600 (628.9 kg/m^3) and GT1 (615.6 kg/m^3).

Table 3-2. Summary of results showing basic density (kg/m^3) means of main effects and interaction for rubberwood clones GT1, PB217 and RRIM600.

MAIN EFFECTS:		BASIC DENSITY*
CLONE (n = 9):	GT1	615.6b
	PB217	582.2a
	RRIM600	628.9b
HEIGHT (n = 9):	Top section	608.9a
	Middle section	601.1a
	Bottom section	616.7a
INTERACTION (n = 3): CLONE	HEIGHT	BASIC DENSITY*
GT1	Top section	616.7a
	Middle section	600.0a
	Bottom section	630.0a
PB217	Top section	566.7a
	Middle section	576.7a
	Bottom section	603.3b
RRIM600	Top section	643.3a
	Middle section	626.7a
	Bottom section	616.7a

Note: * same letter down the column denotes not significantly different ($p>0.05$)

3.3.1.1 Statistical significance

Table 3-3 shows the analysis of variance (ANOVA) of the basic density at the three height sections (top, middle and bottom) for the three selected clones GT1, PB217 and RRIM600. It shows that effect of clone, and effect of two-way interaction of height-clone on the basic density is significant ($p<0.05$), however the effect of height is not significantly different ($p>0.05$).

Table 3-3. ANOVA of the basic densities at 3 height sections for clones GT1, PB217 and RRIM600.

Source of variation	df	Mean square	F
Clone	2	5200.0	32.65*
Height	2	544.4	3.42NS
Clone x Height	4	877.8	5.51*
Error	18	159.3	

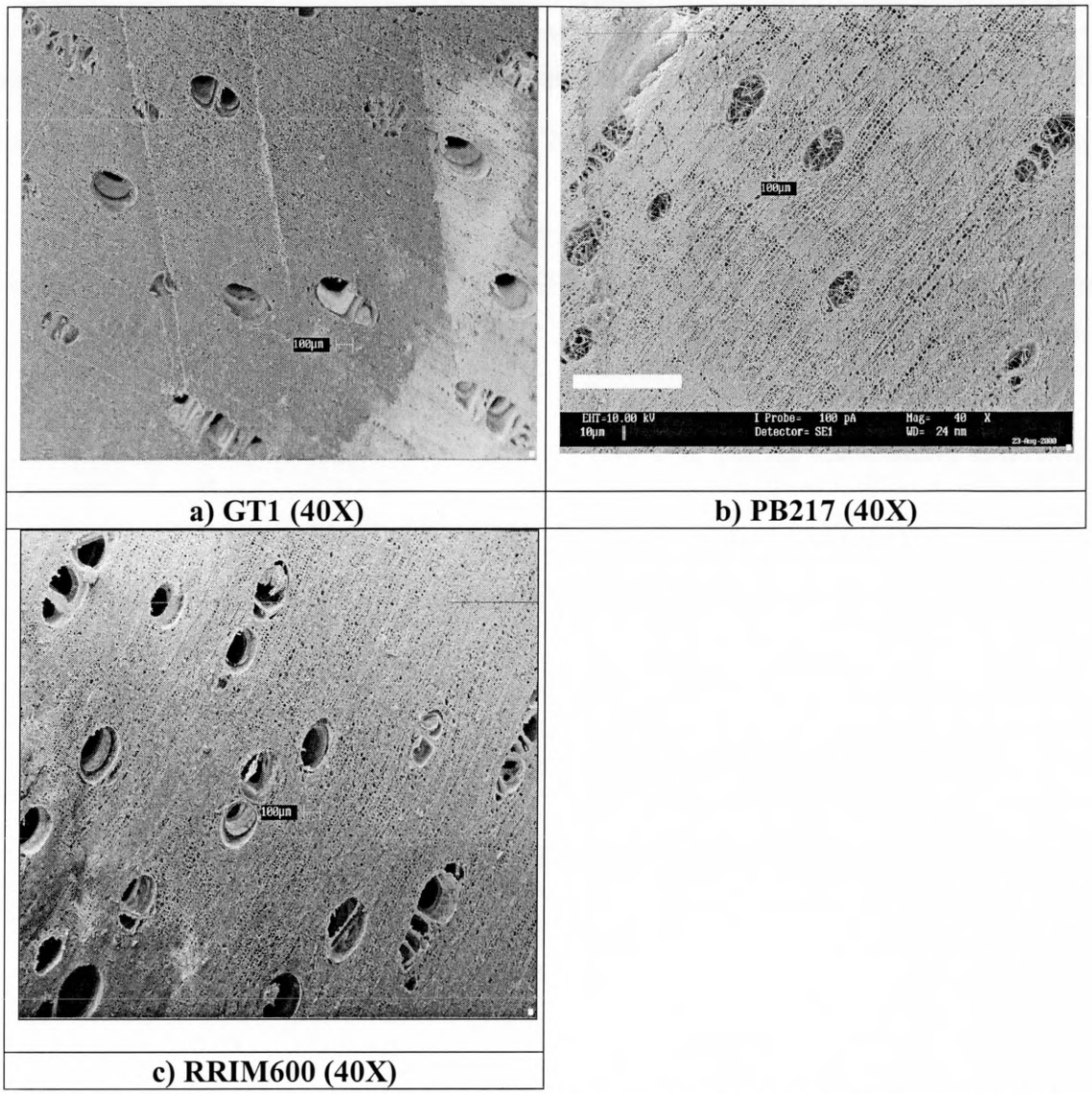
* denotes significantly different difference ($p<0.05$), and NS denotes non-significant difference ($p>0.05$).

3.3.2 Anatomical features

3.3.2.1 Examination of pores

Ten (10) small blocks of wood samples were examined for pore size and dimensions. Plate 3-1 shows the relative size of the vessel pores as seen on the cross-section of the three clones at 40x magnification. The three photos show the presence of solitary vessels and vessels occurring in pairs or multiples. Menon (1993) reported radial pairs of up to 4 or more. Vessels that occur in pairs and multiples are generally smaller and are more variable in diameter than the solitary vessels. These features were observed in all the three rubberwood clones. The vessels vary considerably thus making precise estimation of the vessel diameter difficult with the limited number of samples.

The mean pore diameter from twenty measurements for clone GT1 ranges from 170-270 μm with a mean diameter of 220.3 μm., clone PB217 ranges from 170-300 μm with a mean of 227.5 μm, while for clone RRIM600 the diameter ranges from 180-310μm with a mean of 250.5 μm. This shows that clone RRIM600 has relatively larger diameter pores than the other two clones.



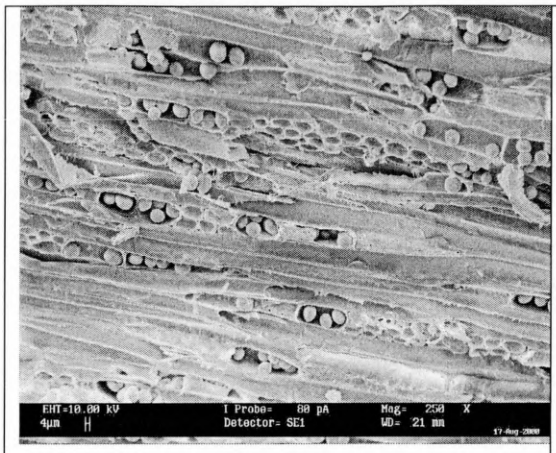
a) GT1 (40X) | **b) PB217 (40X)**

c) RRIM600 (40X)

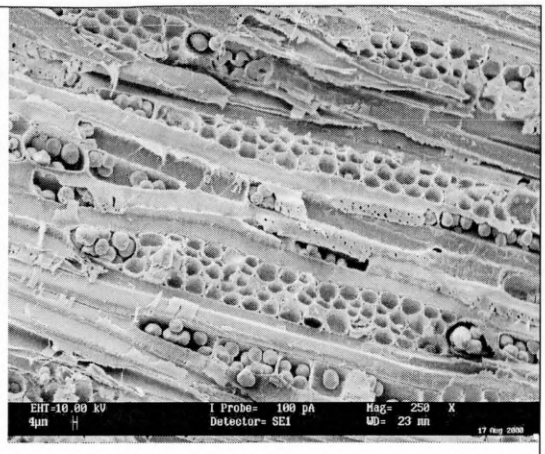
Plate 3-1. The relative size and distribution of vessel pores in the clones a) GT1, b) PB217 and c) RRIM600 (40x magnifications: 100μm scale as shown).

3.3.2.2 Examination of Rays

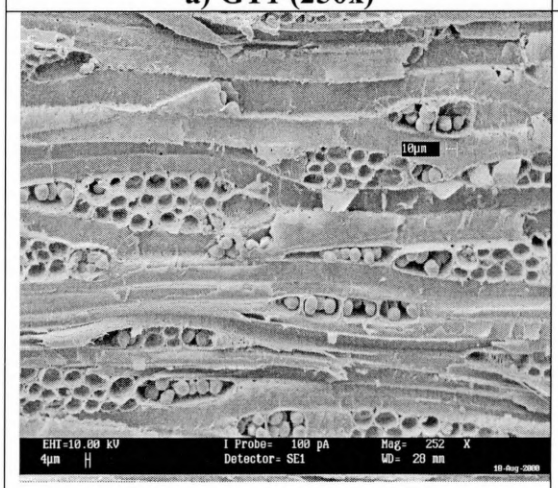
Plate 3-2 shows the tangential cross section (250x) SEM images of the three selected rubberwood clones samples a) GT1, b) PB217 and c) RRIM600. Rays in GT1 are 2-4 cells wide and 15- 20 cells long. The rays of PB217 are from 3-5 cells wide and 28 – 34 cells long. Those of RRIM600 are from 3-4 cells wide 10-15 cells long. The wider ray structure could also be observed in the cross section (Plate 3-1) where it can be seen that clone PB217 has the widest ray, followed by RRIM600 and GT1. Rays of PB217 are also longer (1450 µm) than the other two clones. These images are typical of the sections of the rubberwood clones examined under the SEM.



a) GT1 (250x)



b) PB217 (250x)



c) RRIM600 (250x)

Plate 3-2. The three photos above show the relative size of the ray parenchyma of clones **a)** GT1, **b)** PB217 and **c)** RRIM600 (magnifications 250x: 10μm scale as shown).

3.3.3 Green moisture content

Table 3-4 summarises the mean moisture contents for the rubberwood clones GT1, PB217 and RRIM600 showing the mean values of clone and height and means of interactions of clone and height. The table shows that clone GT1 has significantly ($p<0.05$) the lowest green moisture content (56.16%) and that this low level is uniform throughout the trunk. The clone with the highest moisture content is PB217 (64.09%); however it is not significantly different ($p>0.05$) from that of RRIM600 (61.09%).

Table 3-4. Summary of results showing mean moisture contents (%) of main effects and interactions for clones GT1, PB217 and RRIM600.

MAIN EFFECTS:		MOISTURE CONTENT*
CLONE:	GT1	56.16a
	PB217	64.09b
	RRIM600	61.09b
HEIGHT:	Top section	59.45a
	Middle section	62.73a
	Bottom section	59.45a
INTERACTION: CLONE	HEIGHT	MOISTURE CONTENT
GT1	Top section	54.44a
	Middle section	57.97a
	Bottom section	56.08a
PB217	Top section	65.70a
	Middle section	67.25a
	Bottom section	59.31a
RRIM600	Top section	68.23a
	Middle section	62.96a
	Bottom section	62.07a

Note: * Same letter down the column denotes non-significant difference ($p>0.05$).

3.3.3.1 Statistical significance

Table 3-5 shows the ANOVA table of the moisture contents at 3 height sections (top, middle and bottom) for the three selected clones GT1, PB217 and RRIM600. It shows that clonal effect is highly significant ($p<0.01$) on the moisture content, however the effect of height and height vs. clone interaction are not significantly different ($p>0.05$).

Table 3-5. ANOVA of moisture contents at 3 different height sections for clones GT1, PB217 and RRIM600.

Source of variation	df	Mean square	F
Clone	2	131.66	5.93*
Height	2	34.68	1.56NS
Clone x Height	4	22.88	1.03NS
Error	18	22.19	

* denotes significant difference ($p<0.05$), and NS denotes non-significant difference ($p>0.05$)

3.3.4 The pH of water extract from rubberwood clones.

Table 3-6 summarises the results of pH measurements of water extracts from the three selected rubberwood clones GT1, PB217 and RRIM600 showing the mean values of the main effects of clone, height, radial section (RSEC) and effect of two-way interactions of clone, height and radial section. The table shows that clone RRIM600 has significantly ($p<0.05$) the highest pH (6.17). The lowest pH is associated with clone GT1 (5.78); however it is not significantly different ($p<0.05$) from that of PB217 (5.89).

Table 3-6. Summary of results showing mean pH of main effects and interaction for clones GT1, PB217 and RRIM600.

MAIN EFFECTS (n = 18):					pH			
CLONE:			GT1 (GT)		5.78a			
			PB217 (PB)		5.89a			
			RRIM600 (RR)		6.17b			
HEIGHT:			Top section (T)		5.99a			
			Middle section (M)		5.93a			
			Bottom section (B)		5.93a			
RSEC:			Pith/Juvenile wood (P)		5.93a			
			Inner sapwood (IS)		5.94a			
			Outer sapwood (OS)		5.98a			
INTERACTION (n = 6): CLONE x HEIGHT			CLONE X RSEC			HEIGHT X RSEC		
Clone	Height	pH	Clone	Rsec	pH	Height	Rsec	pH
GT	T	5.82a	GT	P	5.80a	T	P	6.02a
GT	M	5.77a	GT	IS	5.71a	T	IS	6.03a
GT	B	5.77a	GT	OS	5.85a	T	OS	5.92a
PB	T	6.02a	PB	P	5.83a	M	P	5.89a
PB	M	5.86a	PB	IS	5.95a	M	IS	5.89a
PB	B	5.80a	PB	OS	5.90a	M	OS	6.01a
RR	T	6.13b	RR	P	6.18b	B	P	5.87a
RR	M	6.15b	RR	IS	6.17b	B	IS	5.91a
RR	B	6.21b	RR	OS	6.15b	B	OS	6.01a

Note: * Same letter down the column denotes non-significant difference ($p>0.05$).

3.3.4.1 Statistical significance

Table 3-7 shows the ANOVA table of the pH at three height sections and three radial sections for the three selected clones GT1, PB217 and RRIM600. It shows that clonal effect is highly significant ($p<0.01$) on the pH, however the effect of height, radial sections and all interactions are not significantly different ($p>0.05$).

Table 3-7. ANOVA of the pH at three different height sections and radial sections for clones GT1, PB217 and RRIM600.

Source of variation	df	Mean square	F
Clone	2	0.69870	20.69*
Height	2	0.02460	0.73NS
Radial section	2	0.01196	0.36NS
Height x Rsec	4	0.03544	1.05NS
Clone x Rsec	4	0.02100	0.62NS
Clone x Height	4	0.03600	1.07NS
Clone x Height x Rsec	8	0.01087	0.32NS
Error	27	0.03377	

Notes: ** denotes significant difference ($p<0.05$), and NS denotes non-significant difference ($p>0.05$)

3.4 DISCUSSION

3.4.1 Basic density of rubber wood clones GT1, PB217 and RRIM600

Several researchers have studied the basic density of the stem wood of locally grown rubber trees and the data published is summarised in Table 3-8. Lim (1996) found that the variation in density of branch and stem woods is negligible in rubberwood, therefore wood properties related to density such as strength and hardness are not likely to be much affected. Lim and Fujiwara (1995) found that density decreased by 4 % with each (0.6 m x 0.6 m) increase in planting spacing. The mean density of clone PB235 decreased from 622 kg m^{-3} (2.2 x 2.4 m spacing) to 575 kg m^{-3} at (3.7 x

3.7 m spacing) and in clone PB260, it decreases from 599 kg m^{-3} (2.2 x 2.4 m spacing) to 546 kg m^{-3} (3.7 x 3.7 m spacing). The effect of spacing in the current study is negligible as the three clones were planted at the same spacing of 400 stem/ha.

Table 3-8. Basic density of rubberwood clones as reported in literature.

Age (years)	Clone	Density (kg/m^3)	Source
3	PB 260	508-547	Ani Sulaiman & Lim (1992)
8	PB 260	526-565	Ani Sulaiman & Lim (1992)
14	PB 260	503-553	Ani Sulaiman & Lim (1992)
8	RRIM600	480-603	Ani Sulaiman & Lim (1992)
24	RRIM600	560-625	Ani Sulaiman & Lim (1992)
23	RRIM600	560-650	Ani Sulaiman & Lim (1992)
29	RRIM600	565-650	Lim (1996)
29	RRIM600 (branches)	555-635	Lim (1996)
24	RRIM600	616-643	Ashari *et al.* (2000)
24	PB217	567-603	Ashari *et al.* (2000)
24	GT1	600-630	Ashari *et al.* (2000)

3.4.1.1 Effect of rubberwood clones

Figure 3-1 shows the mean basic density at different height portions for each clone. The figure shows that the basic density for the three rubberwood clones ranges from 567 – 643 kg/m^3. The range and the mean basic density of RRIM600 is consistent with those reported (Table 3-4) by Lim (1996) and Ani Sulaiman and Lim (1992). The density of cone GT1 and PB217 are also within the ranges reported.

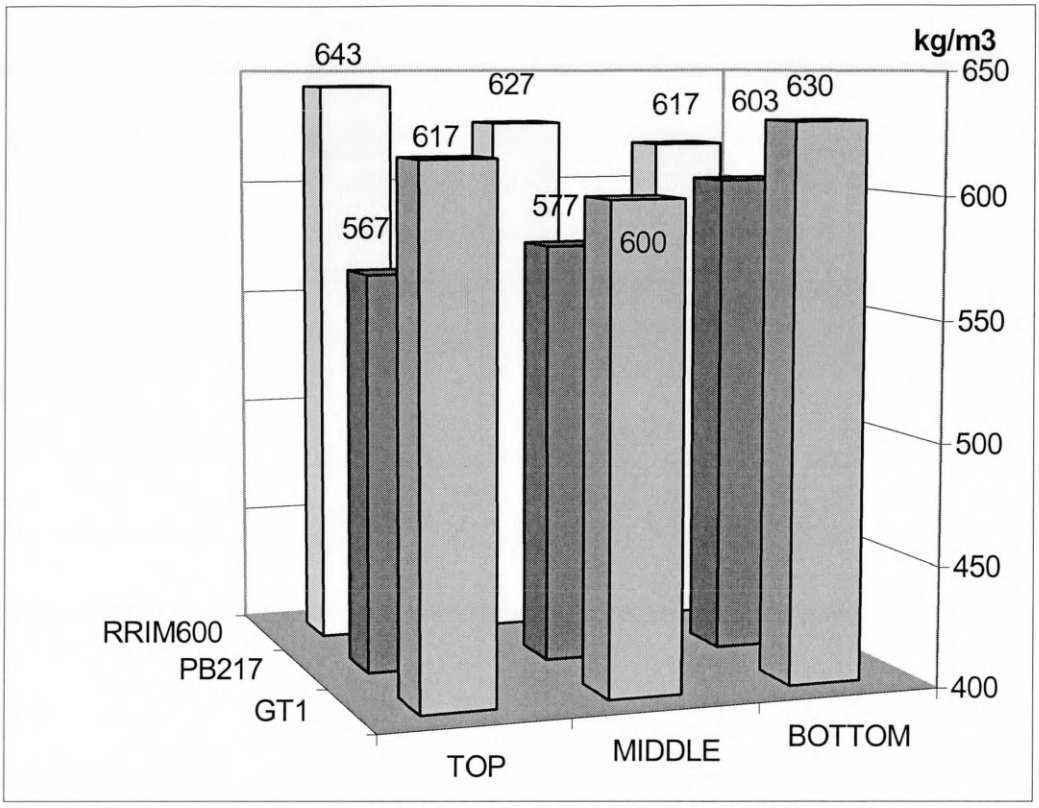


Figure 3-1. Basic density at different height portions for clones GT1, PB217 and RRIM600.

Variation in the density of wood is due to differences in the structure and the presence of extraneous constituents. The structure in softwoods and hardwoods is characterised by the proportional amount of different cell types such as fibres, tracheids, vessels, resin ducts, wood rays and by their dimensions, especially the thickness of the cell walls (Kollmann and Coté, 1968). The present study shows that the rubberwood clones differ in the proportion of vessels, fibres, length and with of ray parenchyma. Clones of lower basic density has less cell wall material and therefore either has more pores or is composed of higher percentage of parenchyma cells. It was observed that the ray bundles of PB217 are relatively wider and longer compared to the other two clones. The low basic density of PB217 in this case could be due to the larger number of epithelial parenchyma cell (which have thinner wall) in the rays.

3.4.1.2 Effect of clone and height interactions

Figure 3-1 illustrates the interaction of the three selected clones GT1, PB217 and RRIM600 versus height. Table 3-2 shows that there is highly significant ($p<0.01$) clone and height interaction. As a result of the highly significant ($p<0.01$) interaction of clone and height, there is a different trend in basic density for the three clones with respect to height. Clone RRIM600 shows increasing basic density from the bottom of the stem to the top, thus the top portion of clone RRIM600 is higher in density than the bottom portion. Conversely for clone PB217 the basic density decreases from the bottom to the top while GT1 shows no definite trend since the mid section has lower basic density than the top and bottom section of the stem. The opposing trends in wood density between clone GT1 and clone RRIM600 indicates that variation in density between heights lack any consistent trend as shown in the analysis of variance table (Table 3-3).

3.4.2 Anatomical features

3.4.2.1 Vessel diameter and frequency

The pore diameter (averages of 20 measurements) for clone GT1 ranges from 170 to 220 μm with a mean diameter of 220 μm. The pore diameter of clone PB217 ranges from 170 to 300 μm with a mean diameter of 230 μm and for clone RRIM600 the diameter ranges from 180 to 300 μm with a mean diameter of 250 μm. Due to the large range of vessel diameter however the differences are not significant ($p>0.05$). For a better comparison of porosity (amount of vessel lumen) between clones it is better to use the mean basic density, which basically is an estimate of the amount of cell wall material. From the relatively larger vessels in RRIM600 observed (Plate 3-2) and with its high basic density, it could be postulated that the cell wall of RRIM600 is relatively thicker than GT1 and PB217.

3.4.2.2 Rays

The ray tissue in rubberwood has been described in Chapter 1. Wood rays are either uniseriate (one cell wide) or multi seriate (two or more cells wide). There are two types of parenchyma, the procumbent (horizontally elongated) parenchyma cell and

the square or upright (vertically elongated) parenchyma cell. The uniseriate parenchyma is homogenous and composed of upright parenchyma cells. The multi seriate parenchyma is heterogeneous and is composed of the upright parenchyma cells and procumbent parenchyma cells.

Plate 3-2 clearly shows that the rays in the three rubberwood clones GT1, PB217 and RRIM600 are made up of uniseriate or multi seriate rays. Menon (1993) and Lim and Ani Sulaiman (1994) also described a similar observation. The procumbent cells that made up the rubberwood ray parenchyma are both wider and longer for clone PB217 (3 to 6 cells width) as compared to RRIM600 (3 to 4 cells wide) and GT1 (2 to 4 cells wide). The ray parenchyma of PB217 is also longer (about 1450 μm). It was also noted that the starch granules are found in the ray parenchyma cells. It was observed that the starch granules are mostly located in the upright cells rather than the procumbent cells. To date most of the reports (Menon, 1993; Lim & Ani Sulaiman, 1994) generally mentioned that starch granules are stored in the parenchyma cells, however to the best of the author's knowledge this is the first time that it is being reported that starch granules are only found in the upright cells of the parenchyma in all the three rubberwood clones examined (starch granules are known to be stored in all parenchyma cell types in rubberwood).

3.4.3 Green moisture content

Table 3-5 clearly shows that there is a significant ($p<0.05$) difference in the green moisture content of the three rubberwood clones. There is however no significant difference ($p>0.05$) in the green moisture content with respect to height. Table 3-4 shows that the overall mean green moisture content of clone PB217 (64.09%) is significantly ($p<0.05$) the highest followed by RRIM600 (61.09%) and GT1 (56.16%). The relative higher moisture content of clone PB217 corresponds well with its observed lower basic density and relatively wider and longer ray parenchyma (Plate 3-2). Figure 3-1 shows the variation of green moisture content by clone and height. Although there is a significant ($p<0.05$) difference in green moisture content between clones, it can be seen from Figure 3-1 that the top portion of GT1 is considerably and significantly ($p<0.05$) lower in moisture content than those of the

other two clones. This is probably due to fewer parenchyma cells in GT1 and smaller pore diameter.

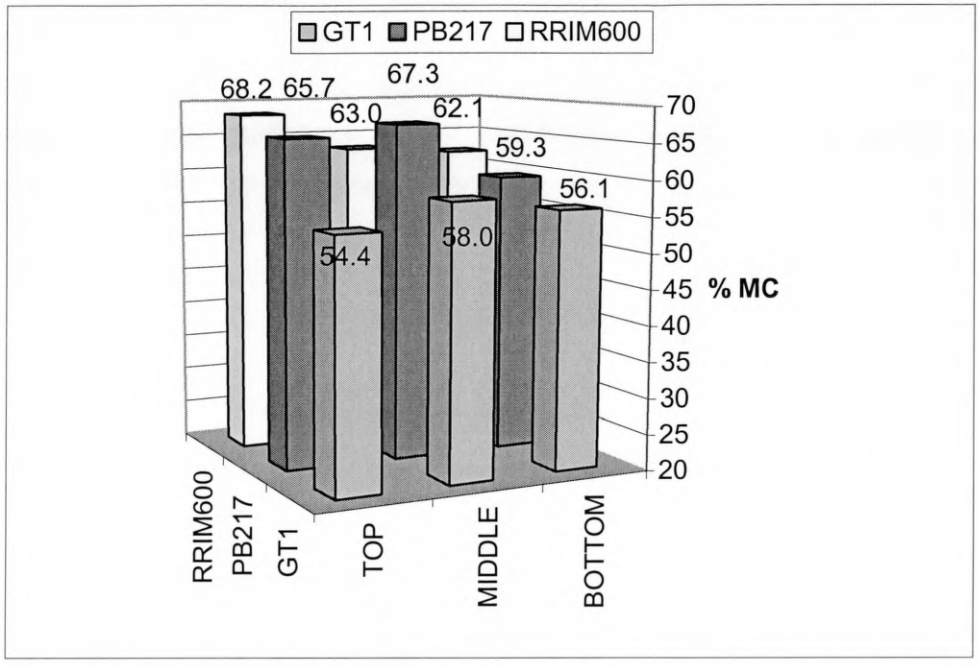


Figure 3-1. Mean percent green moisture content (MC) of clones GT1, PB217 and RRIM600 by height.

3.4.4 The pH of cold water extract of rubberwood clones.

The pH (cold water extract) reported in this study used a similar method of determination as reported by Wong (1980). Table 3-7 shows that clonal effect is the only factor that significantly ($p<0.05$) influences the pH. Table 3-6 shows that pH of clone RRIM600 (6.17) is significantly ($p<0.05$) higher than extracts of clone GT1 (5.78) and clone PB217 (5.89). However there is no significant difference ($p>0.05$) between pH of GT1 and PB217.

In general fungi grow best within a pH range of 3 to 6, whereas many have optimal growth within a pH range of 5 to 6.5. Wood decaying Basidiomycotina have pH optimum range from 3 to 6. Brown-rot fungi have the lowest optimum (around pH 3). Wood-stain fungi are highly pH sensitive, and their growth often diminishes or ceases as pH exceeds 5 (Zabel and Morrell, 1992). Therefore the pH of the rubberwood clones is high as far as sapstain fungi is concerned especially for clone RRIM600. Based on this information the pH of all the rubberwood clones are within

the optimal pH range. However significantly higher ($p<0.05$) pH of RRIM600 (6.17) is high enough to affect rate of fungal growth knowing that sapstain fungi are highly pH sensitive.

This is the first report on the pH of extracts from rubberwood clones (this is however not the first report of pH of rubberwood (Wong, 1993). Since pH is an important factor in fungal growth, these results will provide useful information of the microenvironment in which the sapstain fungi and biological control microorganisms will face in the various rubberwood clones GT1, PB217 and RRIM600.

3.5 CONCLUSION

This chapter clearly shows that there is significant ($p<0.05$) variability in the three rubberwood clones in term of basic physical and anatomical characteristics. The results and findings of this chapter as in Table 3-9 on the physical and anatomical characteristics of the three selected rubberwood clones are related and relevant to Chapters 5, 6, and 7 which will examine the activity of sapstain and mould fungi on the selected rubberwood clones GT1, PB217 and RRIM600.

Table 3-9. Summary of physical and anatomical properties of rubberwood clones GT1, PB217 and RRIM600.

	GT1	PB217	RRIM600
Basic density* (kg/m^3)	615.6b	582.2a	628.9b
Green MC* (%)	56.16a	64.09b	61.09b
pH*	5.78a	5.89a	6.17b
Ray width	2-4 cells	3-6 cells	3-4 cells
Ray height	Shortest	Longest	Medium
Vessels diameter (μm) range	200-250	200-240	250-300
Vessels diameter (μm)	220	228	250
Vessels frequency (per field of view 40X)	12-15	10-12	16-18

Note: * Same letter across the row denotes non-significant difference ($p > 0.05$).

The major conclusions from these physical and anatomical studies are:

- Clone PB217 is significantly ($p<0.05$) the lowest in basic density (582 kg/m^3) as compared to the other two clones GT1 (616 kg/m^3) and RRIM600 (629 kg/m^3)

- In term of green moisture content GT1 is significantly ($p<0.05$) the lowest (56.2%) as compared to the other two clones PB217 is the highest (64.1%) and RRIM600 (61.09%).

- The amount of parenchyma cells and diameter of pores influence the green moisture content. The higher green moisture content is due to greater number of parenchyma cells due to wider and longer radial parenchyma in clone PB217. For clone RRIM600 the higher green moisture content is probably due to larger diameter pores.

- The pH of water extracts of the clones is significantly highest ($p<0.01$) for RRIM600 (6.17) as compared to GT1 (5.78) and PB217 (5.89).

CHAPTER 4

4. NUTRIENT CONTENT OF RUBBERWOOD

4.1 INTRODUCTION

Fungi and most bacteria posses no chlorophyll and therefore require a food source or substrate that can provide three major needs: a) energy, b) a pool of metabolites for the synthesis of the wide range of compounds needed for growth (for example, chitin, glucans, nucleotides, enzymes, proteins and lipids); and c) required vitamins, minor elements, CO_2, and nitrogen (Zabel and Morrell, 1992).

The results presented in Chapter 3 showed that the three rubberwood clones not only differ in basic density, moisture content, pH, but also in width and length of ray parenchyma cells and diameter of vessel pores. Results in chapter 3 show that most of the starch is present as granules in the upright cells of the parenchyma. It is postulated that with differences in the number of parenchyma cells there will be difference in, not only the amount of starch but also the amount of cytoplasm and cell nutrients present in the parenchyma cells. This will determine the amount and composition of nutrients in the rubberwood clones and thus influences susceptibility of the rubberwood clones to sapstain fungi.

Chapter 4 will examine the nutrient components of the three rubberwood clones GT1, PB217 and RRIM600, the focus being on the carbon:nitrogen ratio and the readily available nutrients for growth of sapstain fungi. The chapter is subdivided into 3 sections namely a) carbon and nitrogen b) sugar content and c) starch content. The procedures for sampling and analysis of these chemical constituents in rubberwood were already described in Chapter 2.

4.2 RESULTS

4.2.1 Carbon content of rubberwood clones

Table 4-1 summarises the mean percent carbon in the three selected rubberwood clones GT1, PB217 and RRIM600 showing the means of the main effects of clone, height, radial section (RSEC) and effects of two way interactions of clone, height and radial section on percent carbon. Essentially there is no difference between the clones in the overall levels of carbon. However the data shown in Table 4-1 indicates that in PB217 the inner sapwood is significantly higher ($p<0.05$) in percent carbon (47.12%) compared with the outer sapwood (45.81%). For the other two clones, GT1 and RRIM600 however there is no significant difference in percent carbon between the outer sapwood and the inner sapwood. Table 4-1 also shows that there is no significant difference between height portions and clones, however the mean percent carbon for inner sapwood (46.63%) is significantly ($p<0.05$) higher than that of the outer sapwood portion (45.27%) of the rubberwood stem.

Table 4-1. **P**ercent carbon means of main effects (clone, height and radial position) and respective interactions for clones GT1, PB217 and RRIM600.

MAIN EFFECTS :						CARBON (%)		
CLONE (n = 8):			GT1 (GT)			46.2a		
			PB217 (PB)			46.46a		
			RRIM600 (RR)			45.18a		
HEIGHT (n = 12):			Top section (T)			45.46a		
			Bottom section (B)			46.44a		
RSEC (n = 12):			Inner sapwood (IS)			46.63b		
			Outer sapwood (OS)			45.27a		
INTERACTION (n=4): CLONE x HEIGHT			(n = 4) CLONE X RSEC			(n=6) HEIGHT X RSEC		
Clone	Height	%C	Clone	Rsec	%C	Height	Rsec	%C
GT	T	46.86a	GT	IS	46.11a	T	IS	46.57b
GT	B	45.54a	GT	OS	46.29a	T	OS	46.30b
PB	T	47.54a	PB	IS	47.12b	B	IS	46.68b
PB	B	45.38a	PB	OS	45.81a	B	OS	44.23a
RB	T	44.91a	RR	IS	46.65a			
RB	B	45.45a	RR	OS	43.71a			

Note: * Same letter down the column denotes non-significant difference (p>0.05).

4.2.1.1 Statistical significance

Table 4-2 shows that all interactions, height x radial section; clone x radial section; clone x height and clone x height x radial section are not significantly different.

Table 4-2. ANOVA of percent carbon with respect to height and radial sections for clones GT1, PB217 and RRIM600.

Source of variation	Df	Mean square	F
Clone	2	3.652	2.01NS
Height	1	5.767	3.17NS
Radial section	1	11.055	6.08*
Height x Rsec	1	7.141	3.92NS
Clone x Rsec	2	4.850	2.67NS
Clone x Height	2	3.885	2.12NS
Clone x Height x Rsec	2	0.204	0.11NS
Error	12	1.819	

Notes: * denotes significant difference with $0.01 < p < 0.05$, and NS denotes non-significant difference with $p > 0.05$.

4.2.2 Nitrogen content of rubberwood clones

Table 4-3 summarises the mean percent nitrogen in three selected rubberwood clones GT1, PB217 and RRIM600 showing the means of the main effects of clone, height, radial section (RSEC) and effect of two way interactions of clone, height and radial section on percent nitrogen. Clone GT1 has significantly ($p<0.05$) the highest (0.106%) level of nitrogen. The lowest mean percent nitrogen is in clone PB217 (0.064 %) however it is not significantly different from RRIM600 (0.077%). Generally the bottom portion (0.100%) of the rubberwood stem contains significantly higher ($p<0.05$) mean percent nitrogen than the top portion (0.066%) of the stem except for clone GT1 where there is no significant ($p>0.05$) difference between the top and bottom portion.

Table 4-3. Means percent nitrogen of main effects and interactions for clones GT1, PB217 and RRIM600.

MAIN EFFECTS:					NITROGEN %			
CLONE (n = 12):			GT1 (GT)		0.106b			
			PB217 (PB)		0.064a			
			RRIM600 (RR)		0.078a			
HEIGHT (n = 18):			Top section (T)		0.066a			
			Bottom section (B)		0.100b			
RSEC (n = 18):			Inner sapwood (IS)		0.089a			
			Outer sapwood (OS)		0.076a			
INTERACTION (n = 4): CLONE x HEIGHT			(n = 4) CLONE X RSEC			(n = 9) HEIGHT X RSEC		
Clone	Height	%N	Clone	Rsec	%N	Height	Rsec	%N
GT	T	0.090b	GT	IS	0.119b	T	IS	0.075a
GT	B	0.122b	GT	OS	0.093b	T	OS	0.056a
PB	T	0.037a	PB	IS	0.058a	B	IS	0.103b
PB	B	0.092b	PB	OS	0.071a	B	OS	0.096b
RR	T	0.070a	RR	IS	0.090b			
RR	B	0.085b	RR	OS	0.065a			

Note: * Same letter down the column denotes non-significant difference (p>0.05).

4.2.2.1 Statistical significance

Table 4-4 shows the ANOVA of mean percentage nitrogen at two height sections and two radial sections for the three selected clones GT1, PB217 and RRIM600. It shows that all two-factor interactions have no significant effect on mean percent total nitrogen except for clone vs. radial section interaction.

Table 4-4. ANOVA of total nitrogen content (%) at two height sections and two radial sections for clones GT1, PB217 and RRIM600.

Source of variation	df	Mean square	F
Clone	2	0.005433	15.74**
Height	1	0.010253	29.70**
Radial section	1	0.001394	4.04NS
Height x Rsec	1	0.000349	1.01NS
Clone x Rsec	2	0.001339	4.17*
Clone x Height	2	0.001151	3.33NS
Clone x Height x Rsec	2	0.0009229	2.67NS
Error	24	0.000345	

Notes: * denotes significant difference ($0.01 < p < 0.05$), and NS denotes non-significant difference ($p > 0.05$).

4.2.3 Carbon:nitrogen ratio

Table 4-5 shows the calculated carbon:nitrogen ratio (C:N ratio) for the three rubberwood clones. All clones have a C:N ratio of greater than 400. The highest C:N ratio is for PB217 (721.4) while the C:N ratios for both GT1 (434.4) and RRIM600 (581.5) are significantly ($p < 0.05$) lower.

Table 4-5. Carbon:nitrogen ratio of clones GT1, PB217 and RRIM600.

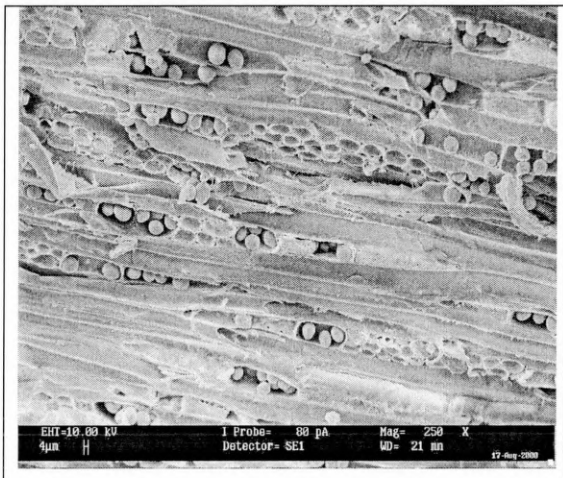
Clone	Percent nitrogen	C:N ratio
GT1	0.1061a	434.4a
PB217	0.0644b	721.4b
RRIM600	0.0777a	581.5a

Note: * Same letter down the column denotes non-significant difference ($p > 0.05$).

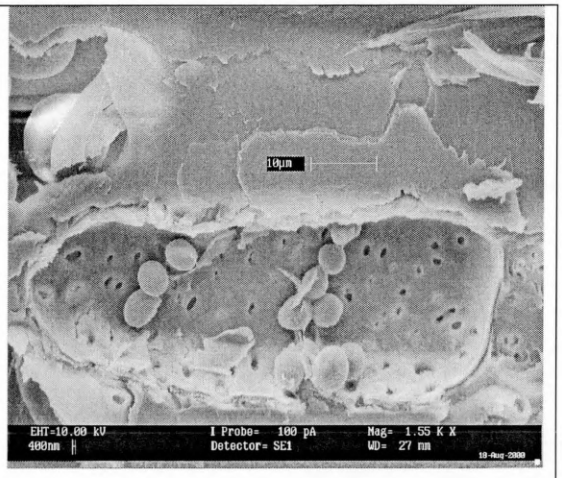
4.2.4 Starch content of rubberwood clones

4.2.4.1 SEM Examination of starch granule in the rubberwood samples.

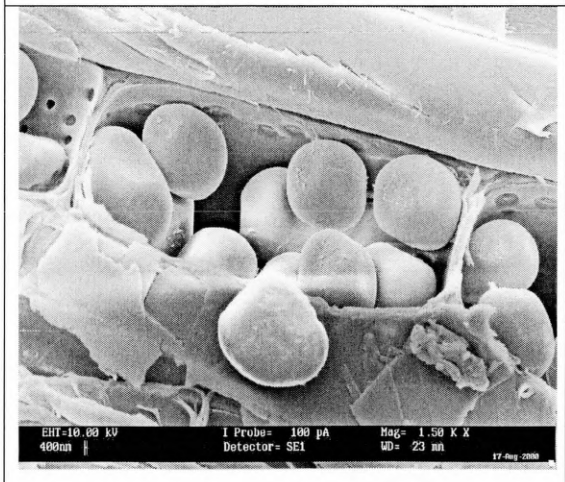
Plate 4-1 shows the SEM images of starch granules in the three rubberwood clones. Starch granules were essentially stored in the upright cells of the ray parenchyma, apotracheal parenchyma and the longitudinal parenchyma. The diameter of the starch granule is approximately 10 to 13 μm.



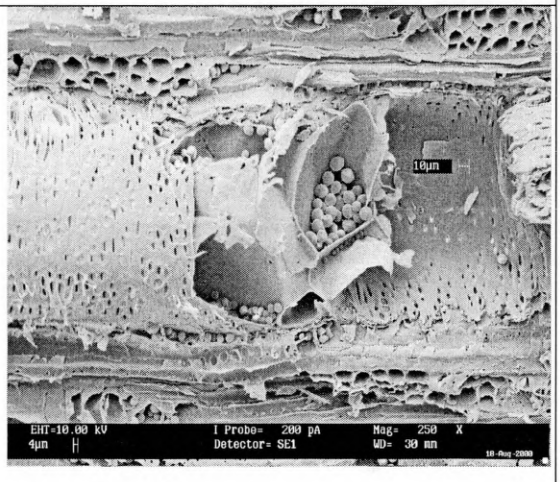
a) Starch granules in ray parenchyma of clone GT1 (250x)



b) Starch granules in longitudinal parenchyma cells of clone RRIM600 (1500x)



c) Starch granules in longitudinal parenchyma cells of cone PB217 (1500x)



d) Starch granules in apotracheal parenchyma cells of clone RRIM600 (250x)

Plate. 4-1. Starch granules in clones GT1, PB217 and RRIM600.

Table 4-6 summarises the results of mean percent starch in the three rubberwood clones showing the mean values of the main effects of clone, height, radial section (RSEC) and effect of two way interactions of clone, height and radial section on percent starch. The starch content is significantly highest($p<0.05$) in clone RRIM600 (5.81%) followed by GT1 (4.30%) and the lowest is in PB217 (3.64%). Starch is also found in greater quantity in the top section of the stem than the bottom portion. There is however no significant difference ($p>0.05$) in the amount of starch between the inner sapwood and the outer sapwood portions. The high starch content in RRIM600 is attributed to the high percentage of starch in the younger upper (7.1%) part of the stem.

The starch content in RRIM600 is also higher in the outer sapwood (6.2%) than the inner sapwood (5.4%). The pattern of radial distribution of starch was examined in 5 trees of *Fagus sylvatica* L. in relation to discoloured wood by Magel and Holl (1993). In two of the 5 trees Magel and Holl (1993) observed that all sapwood shows higher amount of starch. The youngest wood zone (outermost sapwood) of all trees contained highest concentration of starch. This observation is generally in agreement with the differences observed in RRIM600.

Table 4-6. Summary of results showing percent starch means of main effects and interactions for clones GT1, PB217 and RRIM600.

MAIN EFFECTS (n =16):						STARCH (%)		
CLONE (n = 16):			GT1 (GT)			4.30b		
			PB217 (PB)			3.64a		
			RRIM600 (RR)			5.81c		
HEIGHT (n = 24):			Top section (T)			4.97b		
			Bottom section (B)			4.20a		
RSEC (n = 24):			Inner sapwood (IS)			4.47a		
			Outer sapwood (OS)			4.70a		
INTERACTION (n = 8) : CLONE x HEIGHT			(n = 8) CLONE X RSEC			(n = 12) HEIGHT X RSEC		
Clone	Height	%	Clone	Rsec	%	Height	Rsec	%
GT	T	4.75b	GT	IS	4.32a	T	IS	4.91b
GT	B	3.85a	GT	OS	4.29a	T	OS	5.02b
PB	T	3.06a	PB	IS	3.66a	B	IS	4.03a
PB	B	4.22b	PB	OS	3.62a	B	OS	4.37a
RR	T	7.08d	RR	IS	5.43b			
RR	B	4.54c	RR	OS	6.19c			

Note: * Same letter down the column denotes non-significant difference ($p>0.05$).

4.2.4.2 Statistical significance

Table 4-7 shows the ANOVA for percent starch in the selected rubberwood clones GT1, PB217 and RRIM600. Effect of clone and height are highly significant ($p<0.01$), however there is no significant difference ($p>0.05$) in percent starch between outer and inner sapwood. Clone and height interaction is highly significant ($p<0.01$); clone and section interaction is significant ($p<0.05$). However section and height interaction is not significant ($p>0.05$). The highly significant ($p<0.01$)

height vs. clone interaction can be seen in table 4-6 that shows higher percentage of starch in the upper stem portion of clone GT1 and RRIM600 but lower percentage starch in the bottom stem portion of PB217.

Table 4-7. ANOVA of percent starch at two height portions and two radial sections of clones GT1, PB217 and RRIM600.

Source of variation	df	Mean square	F
Clone	2	19.8019	81.26**
Height	1	0.9693	28.60**
Radial section	1	0.6188	2.54NS
Height x Rsec	2	0.1553	0.64NS
Clone x Rsec	2	0.8404	3.45*
Clone x Height	1	13.7831	56.56**
Clone x Height x Rsec	2	0.7347	3.02NS
Error	36	0.2437	

Notes: NS, *, ** - Mean square is non-significant, significant ($0.01 > p > 0.05$) and highly significant ($p < 0.01$), respectively.

4.2.5 Free and total sugar contents of rubberwood clones

Three sugars, which were detected in all the rubberwood extracts, eluted through the HPLC column in the following order: fructose, glucose and sucrose. Table 4-8 summarises the results of the fructose, glucose, sucrose and total sugars of the three selected rubberwood clones showing the mean values of the main effects of clone, height, radial section (RSEC). Clone PB217 contains the highest amount of fructose (1.3428mg/g) followed by RRIM600 (0.909mg/g) and GT1 (0.443mg/g). PB217 also contains the relatively higher amount of glucose (1.227 mg/g) as compared to clone GT1 (0.275) and RRIM600 (0.558). There are however no significant differences ($p < 0.05$) in the sucrose and total sugar contents among the rubberwood clones as shown in the ANOVA table (Table 4-10).

Table 4-8. Summary of results showing means (mg/g) of fructose, sucrose, glucose and total sugar means (main effects) for clones GT1, PB217 and RRIM600.

MAIN EFFECTS:	(n=27)	Fructose	Glucose	Sucrose	Total
CLONE:	GT1	0.443a	0.275a	8.477a	9.195a
	PB217	1.343c	1.227b	7.593a	10.163a
	RRIM600	0.908b	0.558a	7.471a	8.938a
HEIGHT:	Top section	1.146b	0.896b	7.571ab	9.614b
	Middle section	1.092b	0.792b	8.935b	10.819b
	Bottom section	0.455a	0.372a	7.036a	7.863a
RSEC:	Pith/Juvenile wood	0.832a	0.789a	9.163b	10.783b
	Inner sapwood	0.780a	0.550a	7.290a	8.620a
	Outer sapwood	1.082a	0.721a	7.089a	8.892a

Note: * Same letter down the column denotes non-significant difference ($p>0.05$).

Table 4-9 summarises the results of free sugars in water extracts from the three selected rubberwood clones GT1, PB217 and RRIM600 showing the mean values of the effect of two way interactions of clone, height and radial section. The table shows that the bottom section of clone GT1 is significantly lower ($p<0.05$) in fructose and glucose while the middle and top sections of clone PB217 is significantly higher ($p<0.05$) in fructose and glucose. The high fructose content in PB217 is only in the outer sapwood. On the other hand the high glucose content PB217 is found mainly in the top and middle section. Generally sucrose is abundant in the middle juvenile portion of the trunk while fructose in the top outer sapwood portion. Similar observations were reported by Magel and Holl (1993). Magel and Holl (1993) observed that sucrose is the dominant sugar in the youngest wood zone (outer sapwood) of *Fagus sylvatica.* They also reported higher amounts of sucrose but lower amount of fructose and glucose.

Table 4-9. Summary of results showing means (mg/g) of fructose, sucrose, glucose and total sugars and all two-way interactions for clones GT1, PB217 and RRIM600.

INTERACTIONS (n=9): CLONE X HEIGHT		Fructose	Glucose	Sucrose	Total
GT1	Top section	0.527a	0.260a	8.753a	9.540a
	Middle section	0.546a	0.318a	9.357a	10.221a
	Bottom section	0.257a	0.247a	7.322a	7.824a
PB217	Top section	1.762c	1.850b	6.741a	10.353a
	Middle section	1.750c	1.470b	8.948a	12.169a
	Bottom section	0.516a	0.361a	7.091a	7.968a
RRIM600	Top section	1.151b	0.579a	7.219a	8.948a
	Middle section	0.981b	0.587a	8.499a	10.067a
	Bottom section	0.595b	0.508a	6.694a	7.797a
CLONE X RSECTION		Fructose	Glucose	Sucrose	Total
GT1	Pith/Juvenile wood	0.374a	0.289ab	11.373b	12.035a
	Inner sapwood	0.471a	0.253a	7.583a	8.307a
	Outer sapwood	0.485a	0.283ab	6.475a	7.244a
PB217	Pith/Juvenile wood	1.116a	1.390b	7.410a	9.916a
	Inner sapwood	1.237ab	0.959ab	6.954a	9.150a
	Outer sapwood	1.675b	1.333b	8.416ab	11.423a
RRIM600	Pith/Juvenile wood	1.007a	0.688ab	8.704a	10.399a
	Inner sapwood	0.632a	0.439ab	7.334a	8.404a
	Outer sapwood	1.088a	0.547ab	6.375a	8.010a
HEIGHT X RSECTION		Fructose	Glucose	Sucrose	Total
TOP	Pith/Juvenile wood	0.936ab	1.015a	7.990a	9.841a
	Inner sapwood	1.059ab	0.760a	7.124a	8.943a
	Outer sapwood	1.545b	0.913a	7.599a	10.056a
MIDDLE	Pith/Juvenile wood	1.137ab	0.909a	10.551b	12.597a
	Inner sapwood	0.933ab	0.585a	8.309a	9.826a
	Outer sapwood	1.208ab	0.881a	7.945a	10.034a
BOTTOM	Pith/Juvenile wood	0.523ab	0.442a	8.947a	9.912a
	Inner sapwood	0.348a	0.305a	6.439a	7.092a
	Outer sapwood	0.495a	0.369a	5.722a	6.586a

Note: * Same letter down the column denotes non-significant difference ($p>0.05$).

4.2.5.1 Statistical significance

Table 4-10 shows the ANOVA table of fructose, glucose, sucrose and total sugars at 3 height sections and 3 radial sections for the three selected clones GT1, PB217 and RRIM600. It shows that clonal effect is highly significant ($p<0.01$) on the content of fructose and glucose but not significant ($p>0.05$) for sucrose and total sugars. The effect of height is highly significant ($p<0.01$) on the content of fructose, glucose and total sugars but significant ($p<0.05$) for sucrose. The effect of radial section is not significant ($p>0.05$) on the amount of fructose and glucose, highly significant ($p<0.01$) on the amount of sucrose and significant ($p<0.05$) on the total sugars. There is highly significant ($p<0.01$) clone vs. height interaction.

Table 4-10. ANOVA showing mean square values of fructose, glucose, sucrose and total sugar contents at 3 different height sections for clones GT1, PB217 and RRIM600.

Source of variation	df	Fructose	Glucose	Sucrose	Total
Clone	2	5.4721**	6.4539**	8.142NS	11.278NS
Height	2	3.9894**	2.0771**	25.892*	59.644**
Radial section	2	0.7065NS	0.4090NS	35.312**	37.479*
Height x Rsec	4	0.3690NS	0.0363NS	5.056NS	7.997NS
Clone x Rsec	4	0.3184NS	0.1144NS	20.715*	23.145*
Clone x Height	4	0.7960NS	1.6696**	2.183NS	2.811NS
Clone x Height x Rsec	8	0.1903NS	0.2544NS	5.101NS	8.074NS
Error	54	0.3553	0.3849	5.651	8.729

Notes: ** denotes highly significant difference ($p<0.01$), and NS denotes non-significant difference ($p>0.05$).

4.2.6 Nitrogen and sugar contents in sawn wood before and after drying

4.2.6.1 Distribution of nitrogen after drying

Table 4-11 summarises the mean nitrogen contents in the three selected rubberwood clones GT1, PB217 and RRIM600 showing the mean values of the main effects of clone and surface position after drying and interactions. After drying the lowest mean nitrogen is in clone GT1. The surface (down to 5mm) nitrogen is significantly ($p<0.05$) higher than the inner core (5mm zone).

Table 4-11. Summary of results showing percent nitrogen means of main effects and interactions for clones GT1, PB217 and RRIM600 after drying.

MAIN EFFECTS :		% NITROGEN
CLONE:	GT1	0.0536a
	PB217	0.0832b
	RRIM600	0.0727b
SURFACE POSITION:	SURFACE	0.0840b
	CORE	0.0556a
INTERACTIONS: CLONE	HEIGHT	% NITROGEN
GT1	SURFACE	0.1082b
	CORE	0.0432a
PB217	SURFACE	0.0639a
	CORE	0.0582a
RRIM600	SURFACE	0.0800a
	CORE	0.0654a

Note: * Same letter down the column denotes non-significant difference ($p>0.05$).

Table 4-12 shows the ANOVA table of percent nitrogen with respect to the surface and core of rubberwood samples of the three selected rubberwood clones GT1,

PB217 and RRIM600 after drying. It shows that the main effect clone is significantly different ($p<0.05$), the effect of surface position is highly significant ($p<0.01$) and all the effect of interaction is not significantly different ($p>0.05$).

Table 4-12. ANOVA of percent nitrogen with respect to the surface and core of rubberwood samples of clones GT1, PB217 and RRIM600 after drying.

Source of variation	df	Mean square	F
Clone	2	0.00207	4.69*
Surface position	1	0.00567	12.83**
Clone x surface position	2	0.00085	1.92NS
Error	23	0.00044	

Notes: ** denotes significant differences ($p<0.01$), * denotes significant difference ($0.01< p<0.05$), and NS denotes no significant difference ($p>0.05$).

4.2.6.2 Distribution of sugar after drying

Three sugars, which were detected in the surface and core samples of all the rubberwood extracts, eluted in the following order: fructose, glucose and sucrose. Table 4-13 shows the mean sugar contents for fructose, glucose, sucrose and total sugars after air-drying for the three selected rubberwood clones GT1, RRIM600 and PB217. It shows that surface sugar content is higher on the surface of the wood than the core. The highest total sugar is on the surface of clone RRIM600 (13.54mg/g) and the lowest is on the surface of GT1 (4.10mg/g). It is also the same for glucose and sucrose. However the highest fructose is on the surface of clone PB217 (2.88mg/g) and the lowest on the surface of GT1 (0.23mg/g)

Table 4-13. Surface and core means (mg/g oven dry weight) for fructose, glucose, sucrose and total sugars for clones GT1, PB217 and RRIM600 after drying.

		n	Fructose	Glucose	Sucrose	Total
GT1	Core	12	0.000	0.000	1.003	1.003
	Surface	12	0.465	0.269	3.363	4.098
	Mean	**24**	**0.233**	**0.135**	**2.183**	**2.551**
PB217	Core	12	1.717	0.684	2.771	5.173
	Surface	12	**2.878**	1.159	7.439	11.477
	Mean	**24**	**2.298**	**0.922**	**5.105**	**8.335**
RRIM600	Core	12	0.521	1.079	2.967	4.566
	Surface	12	1.237	1.104	11.205	13.546
	Mean	**24**	**0.879**	**1.091**	**7.086**	**9.056**
Surface position*						
Core		12	0.746 a	0.588 a	2.247 a	3.581 a
Surface		12	1.527 b	0.844 b	7.336 b	9.707 b

Note: * Means having the same letter down the column show non-significant difference ($p>0.05$) according to the Waller-Duncan multiple range tests. Results are expressed as a percentage of the initial dry weight of wood.

4.2.6.3 Statistical Significance (after drying)

Statistical analysis of the data (Table 4-14) shows that clone effect is highly significant ($p<0.01$) for fructose, glucose and sucrose and significant ($p<0.05$) for total free sugars. Position showed highly significant ($p<0.01$) effect for fructose, and sucrose, and total free sugars but not significant ($p>0.05$) for glucose. Interaction of clone and position is highly significant ($p<0.01$) for sucrose, significant ($p<0.05$) for fructose and total sugars but not significant ($p>0.05$) for glucose.

Table 4-14. Mean squares from the ANOVA of percent fructose, glucose, sucrose and total sugar for air-dried samples.

Source	df	**Fructose**	**Glucose**	**Sucrose**	**Total**
Clone	2	72.625 **	9.457**	48.482 **	226.000 **
Position	1	11.116 **	1.460NS	155.632 **	312.951 **
Clone x Position	2	3.592 *	0.750 NS	17.317 **	25.767 *
Error	18	0.958	0.724	2.145	5.44

Notes: ** denotes significant differences ($p<0.01$), * denotes significant difference ($0.01< p<0.05$), and NS denotes no significant difference ($p>0.05$).

4.3 DISCUSSION

The results of nutrient contents from sample taken at 3 stem heights and three radial sections were evaluated to give an overall comparison of the amount of nutrients in the standing tree of the three selected rubberwood clones. For two main nutrient components, nitrogen and, free and total sugars the chemical composition of the sawn wood after drying was evaluated to examine possible redistribution during drying as reported by King *et al.* (1981, 1983) and Terziev (1995). Any redistribution of these nutrients after drying will affect their composition in the wood blocks used in the sapstain exposure tests and therefore has to be examined in detail.

4.3.1 Percentage of carbon in rubberwood clones.

Wong (1993) reported that the total carbon content of rubberwood as 37.5%. The means for the three rubberwood clones GT1 (46.2%), PB217 (46.5%) and RRIM600 (45.2%) are therefore higher than that reported by Wong (1993). Encinas and Daniel (1999) found slightly higher values for birch, Scots pine and Carribean pine of 48.8%, 49.7% and 49.6% respectively. Encinas and Daniel also observed that the amount of carbon in wood samples was almost constant for each wood species and ANOVA values for fungal inoculated and non-inoculated samples showed no significant difference ($p>0.05$).

4.3.2 Total nitrogen

The amount of nitrogen in clone PB217 (0.064%) and RRIM600 (0.077%) are comparable to birch and Scots pine. However the amount of nitrogen in GT1 (0.106%) is relatively high. The nitrogen content determined by the Kjeldahl method agreed with previous data (Merill and Cowling, 1966; Abraham and Breuil, 1993), falling in the expected range of less than 0.1% of the dry weight of wood. Encinas and Daniel (1999) reported that the amount of nitrogen in birch, Scots pine and Carribean pine is 0.068%, 0.064% and 0.034% respectively. There is no comparable data for rubberwood.

4.3.2.1 Affect of clone and height.

The top portion of stem wood has significantly lower ($p<0.05$) mean nitrogen content (0.07 %) than the bottom portion (0.10 %); a trend similarly found in lodgepole pine as reported elsewhere (Abraham and Breuil 1993). However Abraham and Breuil (1993) provide no reason for the observed difference. The higher nitrogen content in the lower portion of rubberwood could be associated with the tapping panel. Regular tapping causes injury to the bark; as a result this part of the stem is full of enzymatic activity related to healing of the injured tissues. Since enzymes are proteins therefore they contribute to the higher nitrogen content of the tapped portion of the stem. From the observation of the stem during felling clone PB217 is more heavily tapped than the other two clones. This could be the reason for PB217 showing greatest difference between the top and bottom portions.

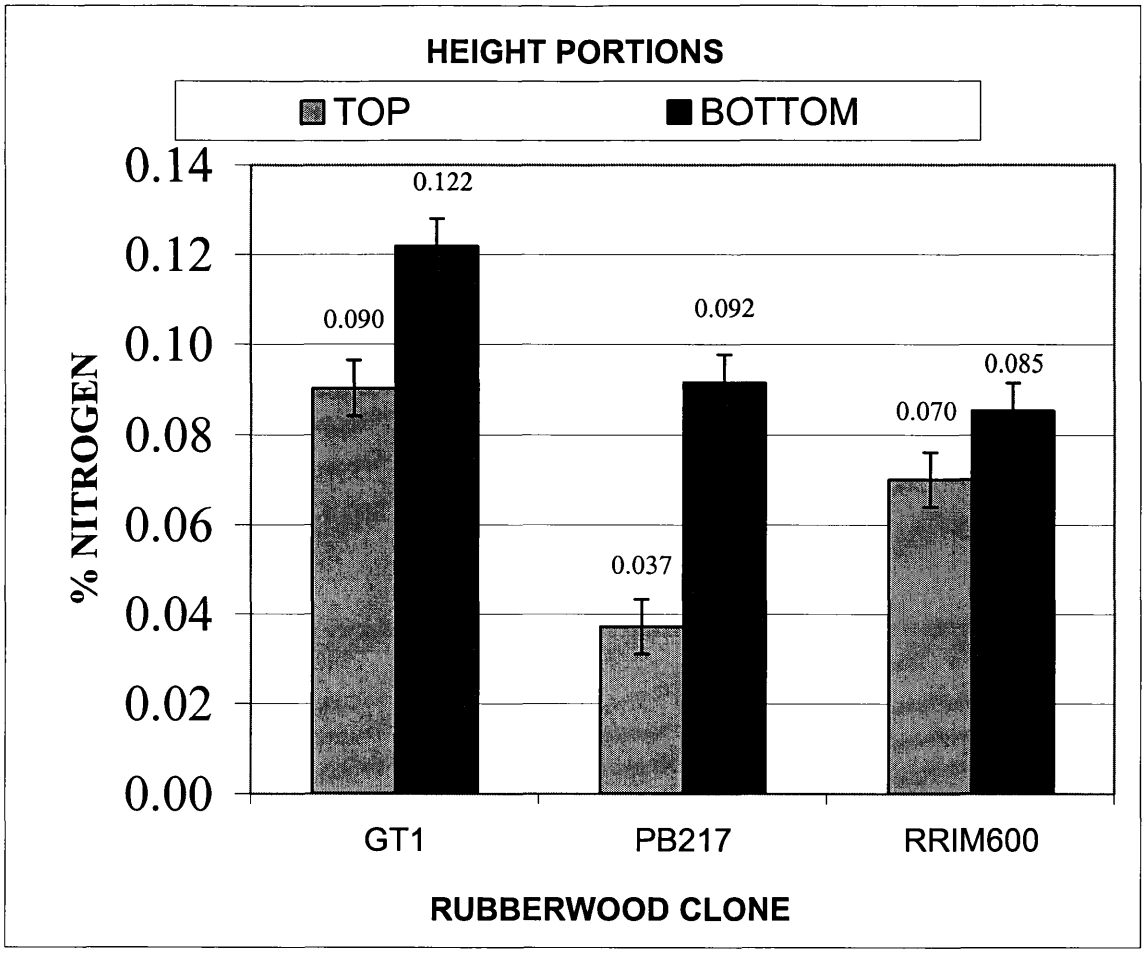


Figure 4-1. Nitrogen content (%) in various stem portion of rubberwood clones GT1, PB217 and RRIM600.

4.3.3 Nitrogen content in inner and outer sapwood.

Table 4-3 shows that there in no difference in total nitrogen between the inner sapwood (0.089%) and the outer sapwood (0.076%) region, this is also shown in Table 4-4. Breuil (1998) found that nitrogen concentrations range from 0.045 % to 0.049% in mature wood samples taken at different positions within a 45-year-old lodgepole pine. Nitrogen content is highest in the cambial zone and in the pith and lowest just outside the pith region (Cowling, 1970). Abraham and Breuil (1993) observed that the nitrogen content decreases slightly from the cambium to the heartwood and were higher in the branches, with a concentration of 0.066%. The values obtained for the rubberwood clones are higher than those obtained by both earlier findings.

The C:N ratio of wood varies from about 350:1 to 1,250:1, depending on the tree

species, the part of the tree, the location of the tree, and the time of the year (Merill and Cowling, 1966). Levi and Cowling (1969) attributed the low wood nitrogen levels as growth-limiting factors for fungi. Levi and Cowling (1969) showed that for C:N ratios of 4:1 to 400:1 the amount of mycelial growth was not limited by nitrogen but limited by the total amount of C available, whereas at higher C:N ratios growth was limited by the amount of nitrogen available. All the clones has C:N ratios above 400 in which case the mycelial growth as suggested by Levi and Cowling (1969) will be limited by the amount of nitrogen

4.3.4 Starch content of rubberwood clones

There is no record of seasonal variation in starch content in rubberwood. However Breuil (1998) reported that starch is stored as granules in the ray and axial parenchyma cells, it was reported that amount of starch increases steadily during the summer and decreases in the fall concentrations are higher in sapwood than in heartwood; 1.0% and 0.4%, respectively (Breuil, 1998). The concentrations of these substances were also reported to vary greatly with season and among wood species (Fischer and Höll, 1992; Hillis, 1987; Kramer and Kozlowski 1979). Although all these report are on temperate species for timbers exposed to variation in summer and winter climates, variation in starch could possible be observed in rubberwood with respect to the variation in dry and wet season. Information provided by the Meteorology Department described the monthly rainfall pattern of Bahau and Tangkak with two periods of maximum rainfall. The primary maximum generally occurs in October-November while the secondary maximum generally occurs in April-May while the primary minimum occur Jun-July with the secondary minimum in February. The seasonal variation of the selected clones were minimised by harvesting the rubberwood clones in December, to ensure all the three clones experienced minimal differences in rainfall pattern.

Starch is a molecular polymer of glucose. Its structure changes during certain procedure such as autoclaving. The result of this change due to high pressure and temperature treatment, the availability of starch as a nutrient to potential organisms may change. Raw starch for example will not support the growth of the fungus *Phymatotrichum omnivorum* (Blank and Talley 1941). For this reason the samples

used in this study were not sterilised by autoclaving and test specimens were sterilised by gamma radiation. By avoiding the use of steam and high pressure the starch granule in the rubberwood samples are intact as shown in Plate 4-1. The photos in Plate 4-1 also show that the starch granules in the rubberwood samples are still intact after drying and have diameters of approximately 10 to 13 μm. In section 4.2.4.1. it was shown that the inter parenchyma cells pit opening in the rubberwood clones is approximately 0.5μm. Therefore starch granules being enclosed in the upright cells of the parenchyma (pit size smaller than the starch granules) are unlikely to be redistributed during drying at temperature of 45°C.

4.3.4.1 Overall differences between clones.

The percentage of starch obtained in this study ranges from 3.64% to 5.81%, much lower than that reported by Azizol and Rahim (1989). Azizol and Rahim (1989) found that rubberwood (clone not mentioned) contains about 7.53 – 10.17% of starch. The higher percentage could be due to a different clone of rubberwood being examined by Azizol and Rahim (1989), as the exact clone was not mentioned, or could be due seasonal variation. Percentages obtained by the present study were very much higher if compared to kiln dried sapwood of Birch (0.005%), Scots pine (0.087%) and Caribbean pine (0.192%) as found by Encinas and Daniel (1999). The much lower values by Encinas and Daniel (1999) cannot be compared directly with the values obtained in this study as the current samples were dried at 45°C. Furthermore the samples used by Encinas and Daniel (1999) were imported from several countries and lacked information concerning both the history of the tree from which the samples were derived as well as the harvesting season. Each of these aspects is known to affect contents of non-structural compounds in wood (Fengel and Wegener, 1984). The high percentage of starch in rubberwood would be likely to render the material susceptible to primary inhibiting organisms such as sapstain fungi.

4.3.4.2 Percent starch in relation to height portion.

Figure 4-2 shows the distribution of starch in relation to the stem length for the three rubberwood clones GT1, PB217 and RRIM600. Azizol and Rahim (1989) found that

the top portion of the stem contains significantly higher ($p<0.05$) amount of starch (4.97%) than the bottom (4.20%). The distribution of starch in the two clones RRIM600 and GT1 therefore is in agreement with the finding of Azizol and Rahim (1989) but it is not the case for clone PB217.

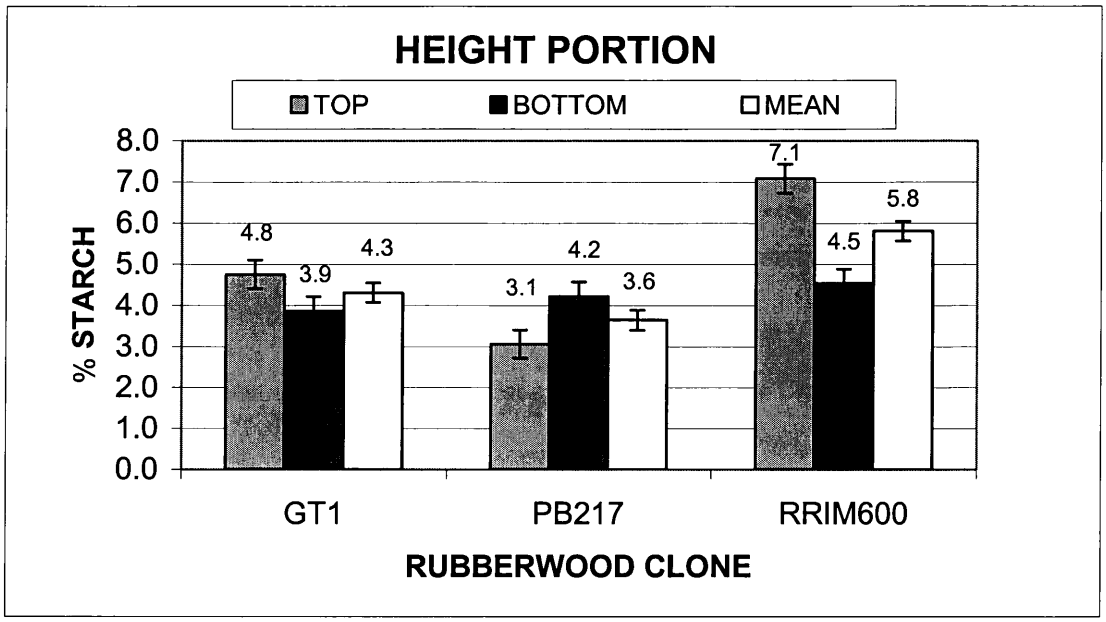


Figure 4-2. Distribution of starch in relation to height (top and bottom) for rubberwood clones GT1, PB217 and RRIM600.

Starch is a polysaccharide. Polysaccharides can be divided into two broad classes (Cochran 1958): a) reserve or nutrient polysaccharides (e.g., starch, glycogen and inulin) and b) structural polysaccharides (e.g. glucose, pectic materials and chitin). Starch is the principal reserve polysaccharide in wood and functions as the storage compound for glucose. Thus it is also an important carbon source for many wood attacking insects and fungi (Findlay, 1985). The differences in the amount of starch in the selected rubberwood clones indicated that there were different compositions of nutrients available to the sapstain fungi and other microorganisms in the different rubberwood clones. Therefore it is postulated that there will be differences in susceptibility to sapstain fungi between the rubberwood clones. This will be examined and discussed in Chapters 6 and 7 in relation to sapstain susceptibility and sapstain exposure tests to potential biocontrol fungi.

4.3.5 Free and total sugar contents of rubberwood clones

The monosaccharide D-glucose is utilised essentially by all fungi and is a common carbon source in many cultural media. Galactose, mannose, and fructose also are used by many fungi but appear to be initially converted to glucose 6-phosphate before following the same metabolic pathways as glucose in the respiration or fermentation processes (Zabel and Morrell, 1992). In tissue culture malt is a preferred medium for many wood-decay fungi, providing both glucose and vitamins.

This section will investigate variability in the major nutrient components mainly the readily available sugars, which are important for the growth of sapstain fungi. The differences in free and total sugar composition if any could impact on the susceptibility of the three selected rubberwood clones to sapstain fungi.

4.3.5.1 Effect of clones.

Table 4-8 shows that clone PB217 contained significantly higher ($p<0.05$) amounts of fructose (1.34 mg/g) and glucose (1.23 mg/g) as compared to the other two clones. Clone GT1 had the lowest fructose (0.44 mg/g) and glucose (0.28 mg/g) contents. There are no similar studies on free sugar and total sugar contents in either rubberwood or specific rubberwood clones for comparison. However similar studies in green spruce showed fructose and glucose were the predominant sugars and that the content of glucose and fructose together ranged from 2 mg/g to 15 mg/g (Nayagam, 1987). The amount of fructose and glucose are therefore much lower in rubberwood than in spruce though the levels found are comparable to those reported in birch, Scots pine and Caribbean pine (Table 4-15). The table also shows that the levels of fructose and glucose in clone PB217 are comparable to birch, while those of RRIM600 and GT1 are comparable to Scots pine and Caribbean pine. Sucrose contents in all the three clones are about 10 times higher than those of Scots pine and are higher than the other specimen.

Table 4-15. Fructose, glucose and sucrose contents of rubberwood as compared to birch, Scots pine and Carribean pine.

Wood species	Fructose mg/g	Glucose mg/g	Sucrose mg/g	Reference
Birch*	1.37	2.37	0.219	Encinas & Daniel (1999)
Scots pine*	0.243	0.241	0.743	Encinas & Daniel (1999)
Carribean pine*	0.300	0.255	0.072	Encinas & Daniel (1999)
Spruce	2 to 15			Nayagam (1987)
GT1	0.443	0.275	8.477	Ashari *et al.* (2000)
PB217	1.343	1.227	7.593	Ashari (2000)
RRIM600	0.909	0.558	7.471	Ashari *et al.* (2000)

* Care should be taken in interpreting this information as some examples are from kiln dried timber.

4.3.5.2 Variation of sugar with respect to height.

The higher amount of fructose, glucose and sucrose in the middle and upper portion could be due to more active growth (Figure 4-3). The relatively lower amount of nutrient in the lower potion of the stem could be because the tapping panel is located at the reachable part of the stem, which is in its lower portion. The effect of daily tapping of latex lowers the sugar levels as energy and resources of the meristem is focused on repairing the damaged bark.

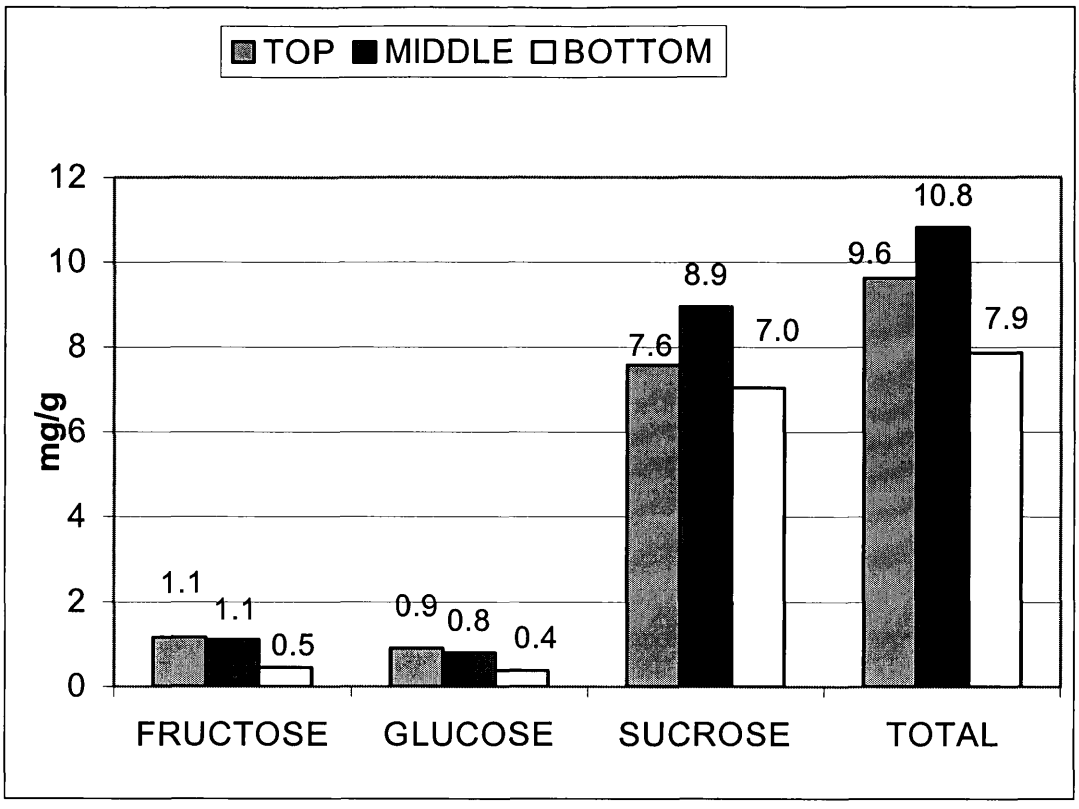


Figure 4-3. The amount of fructose, glucose, sucrose and total sugars (mg/g) with respect to height in rubberwood clones GT1, PB217 and RRIM600.

4.3.5.3 Variation of sugar with respect to sapwood portion

There is a significantly higher ($p<0.05$) amount of fructose and sucrose in the pith/juvenile portion of the stem of the rubberwood clones (Figure 4-4). There is however no significant difference ($p>0.05$) between the outer and the inner sapwood portions. Nayagam (1987) found that concentration of soluble nutrients in spruce decreases with increasing distance from the cambium. The difference is that the spruce as examined by Nayagam (1987) contained heartwood and therefore the innermost portion is the heartwood while in the rubberwood in the present study was mainly composed of sapwood.

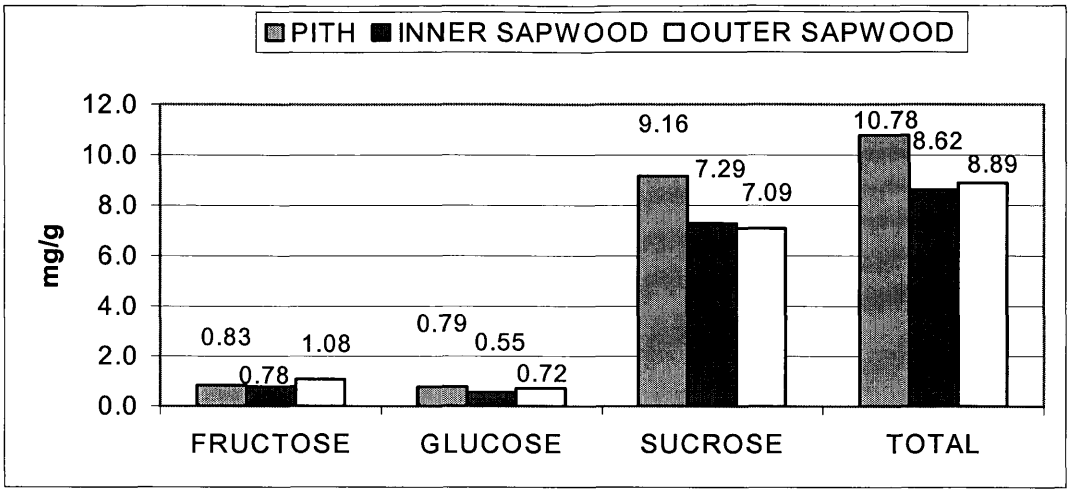


Figure 4-4. Graph showing the amount of fructose, glucose, sucrose and total sugars (mg/g) with respect to sapwood portions in rubberwood clones GT1, PB217 and RRIM600.

4.3.6 Distribution of nitrogen and sugars after drying

4.3.6.1 Mean nitrogen content after drying (face vs. core)

Figure 4-5 shows that the surface percent nitrogen in all clones is significantly higher ($p<0.05$) than the core. Similar observations has been recorded by King *et al.,* (1981, 1983) and Nayagam (1987) they showed that the outer surface layers of a radial cut, air dried, plank had a higher nitrogen level than the inner parts of the same plank. However Figure 4-5 also reveals that the mean percent nitrogen on the surface is not significantly different ($p>0.05$) from the mean nitrogen of the respective clones before drying.

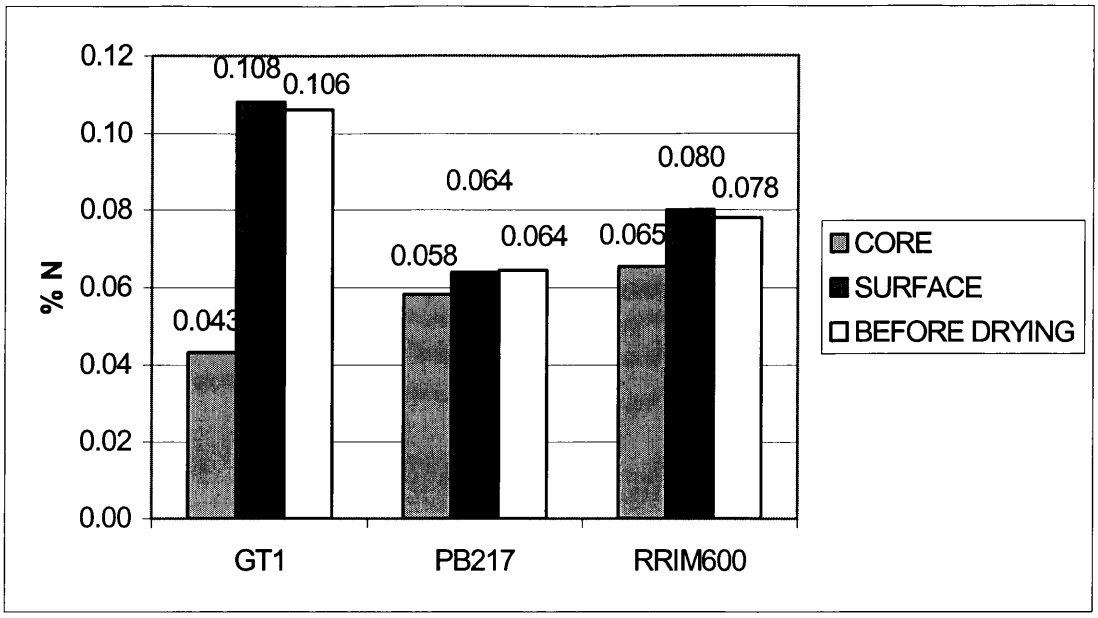


Figure 4-5. Figure showing mean (%) nitrogen content of surface and core; and overall mean for rubberwood clones GT1, PB217 and RRIM600.

King *et al.* (1981) showed that nitrogen level of wood dried at 20°C and high relative humidity corresponded to nitrogen levels of matching samples, which had been extracted in cold water for 48 hours to remove soluble nitrogen and soluble material from wood, suggesting that most of the nitrogen in wood samples is insoluble nitrogen.

King and co-workers (1983) also found that the outer surface layer of a radial cut air-dried plank had a higher nitrogen level (0.220%) than the inner layer (0.041%). It was found in this current study that the mean surface layer nitrogen is significantly higher ($p<0.05$) than the core layer for all the three clones with an overall mean of 0.0840% for the surface layer and 0.0556% for the core layer.

Longitudinal permeability is, as a result of wood anatomy, considerably greater than radial or tangential permeability (Smith, 1960; Siau, 1971). Ray tissue, despite the generally higher volume fraction in hardwood is not particularly efficient in promoting radial flow. According to Siau (1984) the openings in the operative pit membrane (with a mean or 30nm) are much smaller than the vessel diameter. Smith (1960) showed that in red beech hardwood the longitudinal permeability is 65,000 times that tangential permeability. By contrast in softwoods longitudinal flow is

through tracheids lumens and passing through pit openings that are normally bordered pits. In softwood radial flow through the ray cells with simple pits is also less restrictive.

This however is not necessarily true in rubberwood or any other hardwoods. In the rubberwood specimen the pit opening was observed to range from 1-8 μm (Plate 7-6). In rubberwood, as in most hardwoods, the vessels are large (200 – 220 μm) and therefore play a much bigger role in fluid flow during drying (Langrish and Walker, 1993). This higher longitudinal permeability could result in a higher amount of the nitrogen in the wood to be redistributed longitudinally rather than transversely.

The proposed differences in redistribution between hardwoods and softwoods are illustrated graphically in Figure 4-6. In the case of nitrogen the core is left with lower nitrogen and the wood surface with an insignificant increase of nitrogen levels.

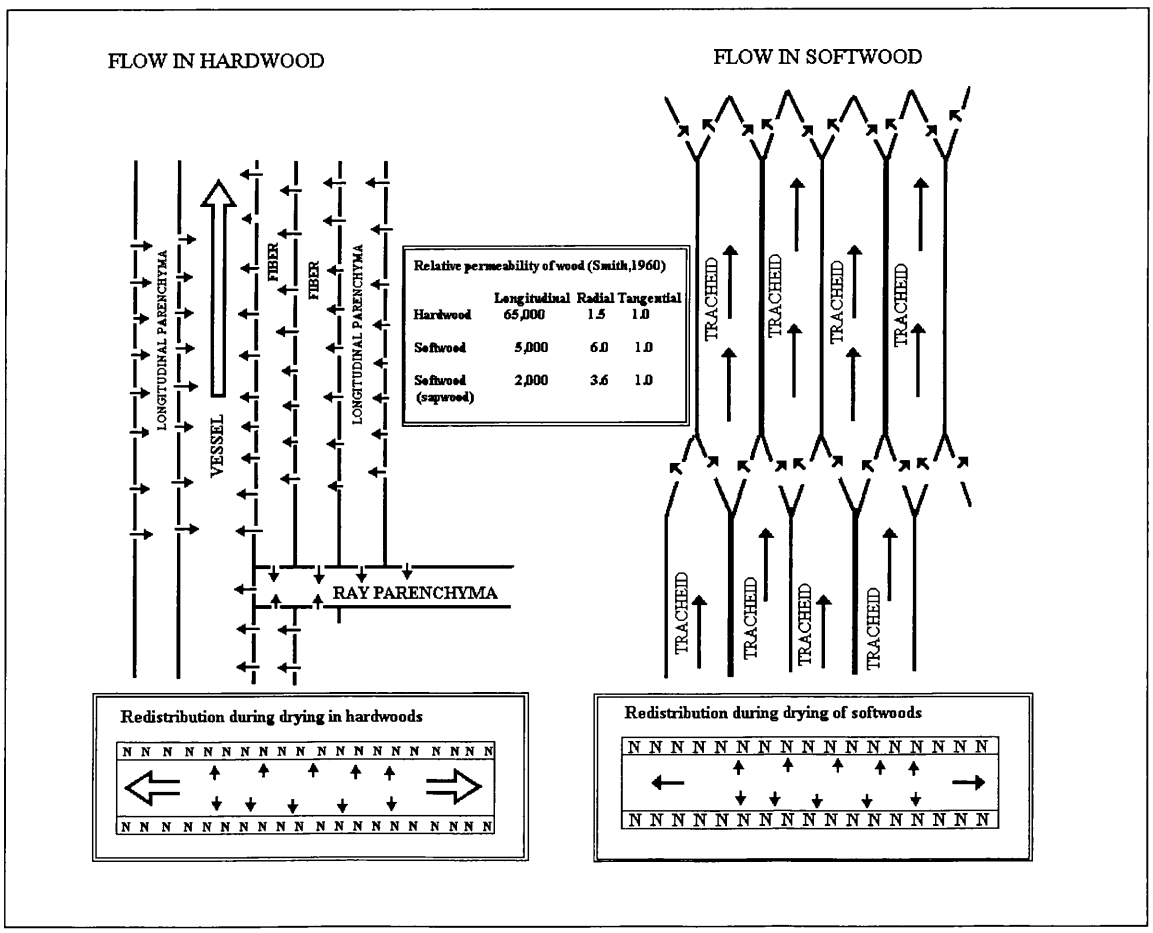


Figure 4-6. Diagram illustrating the various flow pathways for water in hardwoods and softwoods to explain redistribution of nitrogen on the wood surface during drying. The relative magnitude of flow is indicated by the size of the arrows. (Adapted from Siau, 1971).

4.3.7 Distribution of sugars after drying (face vs. core)

4.3.7.1 Clonal variation of sugar contents after drying

Figure 4-7 shows that after drying, the surface of clone PB217 contained significantly the highest ($p<0.05$) means of fructose (2.88mg/g), sucrose (11.21mg/g) and total sugar (13.55mg/g) as compared to those of clones GT1 and RRIM600.

Although there is no significant difference ($p>0.05$) in overall mean sucrose content before drying, after drying clone RRIM600 contains the highest amount of sucrose on the surface (11.21mg/g) followed by PB217 (7.44mg/g) and GT1 (3.36mg/g). A similar trend was also observed for total sugars, highest in RRIM600 (13.55mg/g) followed by PB217 (11.45mg/g) and GT1 (4.10mg/g).

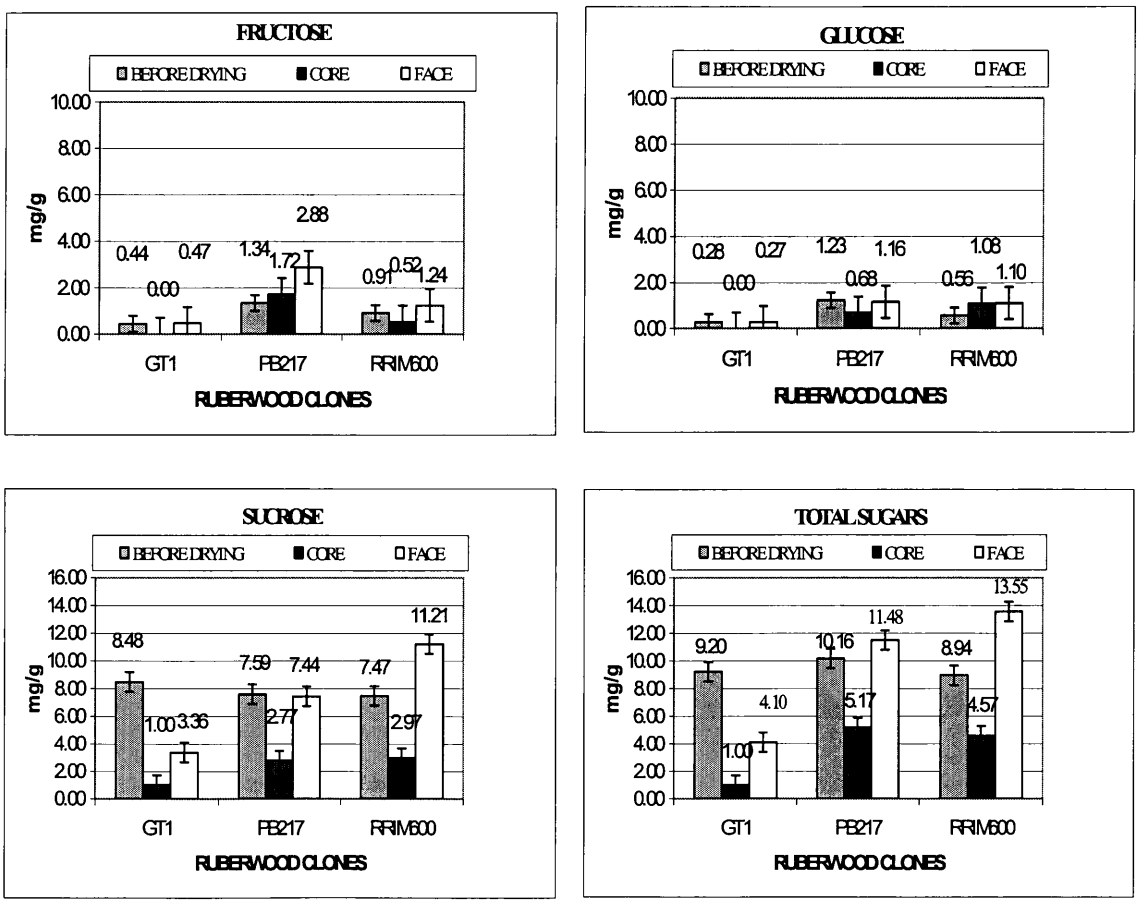


Figure 4-7. Mean (mg/g) fructose, glucose, sucrose and total sugar contents in the three rubberwood clones GT1, PB217 and RRIM600 before drying and individual value for face and core after drying. **Note:** The 'BEFORE DRYING' values refer to the overall mean from disc samples.

Nayagam observed an increase in amount of sugars on the wood surface of spruce after drying. A similar increase of total sugar on the wood surface after drying is only observed in clone RRIM600. There was no significant difference ($p>0.05$) for clone PB217 but GT1 shows a decrease in total sugar levels after drying on the wood surface.

Fructose, glucose, and sucrose are present together with starch in the ray cells (Kollmann and Cote, 1968). Unlike starch fructose, glucose and sucrose are simple sugars and are soluble in water. Since sugars are mainly stored in the living parenchyma cells of the sapwood, radial flow is dependent on the amount of ray cells. The higher rate of longitudinal fluid flow than the transverse movement in rubberwood like most hardwood resulted in different level of sugar on the rubberwood surface after drying. An opposite trend to that reported by Nayagam is observed in clone PB217 and RRIM600.

The differences in terms of longitudinal and transverse flow of sugars during drying could also be as described in section 4.3.6.1. The differences in redistribution of nitrogen and sugars in the rubberwood clones remained to be evaluated. Potential differences in redistribution on the tangential or radial surface will depend on molecular size, solubility of the mobilised molecule, liquid vs. vapour diffusion drying, the location where these nutrients are mostly found (in the upright ray parenchyma cells or in the procumbent ray parenchyma cells), the size and number of ray parenchyma.

Table 4-16 shows the summary of the results. Among the nutrients in the rubberwood clones analysed in this present study, starch was the least affected by drying. Fructose, glucose, sugars and total sugars are sensitive to temperature and also tend to be redistributed after drying. The amounts of these sugars (0.4 – 8 mg/g) are also relatively lower as compared to starch (36 mg/g –50 mg/g). Similarly nitrogen is also redistributed after drying. With these factors taken into account, it is postulated that the rubberwood susceptibility to sapstain may be dependent on the starch content. Among the three rubberwood clones examined in this current study RRIM600 would probably be the most susceptible to sapstain fungi because it is the highest in starch content coupled with lower C:N ratio before and after drying. GT1

will be the least susceptible with higher C:N ratio after drying and significantly lower ($p<0.05$) starch than that of RRIM600 and lower fructose and glucose contents than those of PB217. This is however without taking the anatomical features into consideration.

Table 4-16. Summary of results of the current study.

CHARACTERISTICS OBSERVED	GT1	PB217	RRIM600
Carbon (%)	46.2a	46.46a	45.18a
Nitrogen (%) (stem)	0.1060b	0.0644a	0.0777a
Nitrogen ratio (stem)	433a	713b	591b
After drying (sawn timber)	919b	574a	656a
Starch (%)	4.30a	3.64a	5.81b
Fructose (mg/g)	0.443a	1.434c	0.909b
Glucose (mg/g)	0.275a	1.227b	0.558a
Sucrose (mg/g)	8.477a	7.393a	7.471a
Total sugars (mg/g)	9.195a	10.163a	8.938a

Note: * Same letter across row denotes non-significant difference ($p>0.05$)

4.4 CONCLUSION

The rubberwood clones in this study, as most other sapwood material provide a readily available nutritional resource for stain and mould fungi (Breuill, 1998; Merrill and Cowling, 1966). The composition of these compounds especially the free sugars and starch in these rubberwood clones, are important factors that could influence the microbial activity of the sapstain fungi and potential biological control agents. The findings of this chapter are as follows:

- There are differences in term of nutrient contents in the selected rubberwood clones GT1, PB217 and RRIM600.
- The rubberwood clones differ significantly ($p<0.05$) in term of C:N ratio, the amount of starch and total free sugars.
- The percentage of carbon is not significantly different ($p>0.05$) in the 3 selected rubberwood clones. The mean percent of carbon for the three clones GT1, PB217 and RRIM600 are 46.2%, 46.5% and 45.1% respectively. The mean value for the three clones is 45.93%.
- The nitrogen content differs in the three selected rubberwood clones and therefore its C:N ratio also differs significantly ($p<0.05$). The C:N ratios in increasing order are GT1 (433), RRIM600 (591) and PB217 (713).
- C:N ratios of all the three selected clones are all greater than 400 indicating that nitrogen is limiting to fungal growth.
- Starch content is found to be highest in clone RRIM600 (5.81%), followed by GT1 (4.30%) and PB217 (3.63%)
- Starch granules are mostly found in the upright parenchyma and are not redistributed during drying.
- There are no significant differences ($p>0.05$) for sucrose and total sugar contents among clones.
- The clone PB217 contain significantly higher ($p<0.05$) amounts of fructose (1.34 mg/g) and glucose (1.23 mg/g) as compared to those of clones GT1 and RRIM600. Clone GT1 has the lowest fructose (0.44 mg/g) and glucose (0.27

mg/g) contents.

- After drying the total sugar content on the surface of the wood differ significantly ($p<0.05$) between clones. The highest mean total sugar content after drying is also observed in clone PB217 (13.15mg/g) followed by RRIM600 (8.65mg/g) and GT1 (2.56mg/g).

CHAPTER 5

5. SAPSTAIN CAUSED BY *BOTRYODIPLODIA THEOBROMAE, AUREOBASIDIUM PULLULANS* AND *ASPERGILLUS NIGER*

5.1 INTRODUCTION

Primary moulds and sapstain fungi are passive colonizers of wood (Levy, 1982) and are similar to sugar fungi that feed mainly on sugar and simple carbohydrates present in the parenchyma cells of the sapwood (Garret, 1951). They are considered primary or initial wood colonisers because they invade and assimilate the easily available carbon and nitrogen nutrients in the ray parenchyma cells (Ballard *et al.,* 1982). *Aspergillus niger* is an example of a primary mould with non-pigmented hyphae and it is used in this study as it is postulated that *A. niger* is a rapid coloniser and therefore a potential competitor for nutrients and space in the rubberwood. The competition for nutrients and space could reduce the staining effect of the sapstain fungus *Botryodiplodia theobromae.* Sapstain fungi are sometimes referred to as blue stain fungi because of the dark blue stain produced on infected wood, and have also been previously reported to grow on simple sugars (Sharpe and Dickinson, 1992). Sharpe and Dickinson (1992) reported that the sapstain fungus *Aureobasidium pullulans* could utilize the simple sugars well, but not the oligosaccharides. *A. pullulans* was also able to utilize well several lignin precursor compounds. Sapstain and mould fungi grow mainly on the nutritive substances in the parenchyma cells of the sapwood, and stain the wood by forming grains of melanin within and around the hyphae (Brisson *et al.*, 1996; Zheng *et al.*, 1994). Many stain fungi are specific to a geographical region or wood species (Zabel and Morell, 1992). This indicated that nutrients or physical factors could have significant impact on differences between growth of sapstain fungi on sapwood and heartwood (Breuil, 1998).

Previous reports evaluated mycelial coverage of sapstain fungi on the surface in assessing the sapstain susceptibility of wood species and in efficacy studies of biocides. The following study will assess the stain caused by fungal mycelium below the wood (after minimal sanding approximately 0.5 mm), that is to evaluate the

staining on wood as a result of hyphal penetration or diffusion of clonal metabolites. The stain will be assessed using spectrophotometry and a visual method of assessment.

Chapters 3 and 4 of this study show that there are differences in basic physical characteristics and chemical characteristics (C:N ratio and composition of sugars and starch) in the three rubberwood clones. This chapter reports a preliminary study to examine sapstaining activity of *B. theobromae, A. niger and A. pullulans* in the three selected rubberwood clones (Clones GT1, PB217 and RRIM600). The objective of the study was to determine whether the variability in physical and anatomical characteristics, the C:N ratio, starch and sugar contents shown in Chapter 3 have any effect on the levels of staining in the three rubberwood clones.

5.2 MATERIALS AND METHODS

In this and all subsequent experiments reported in this chapter, rubberwood blocks (5 x 20 x 40 mm) were used and the fungi (*A. niger, A. pullulans* and *B. theobromae*) were cultivated for 14 days at 25^0C in the dark as described in Chapter 2. Randomly selected samples of each clone were exposed to sapstain fungi *A. niger, A. pullulans* and *B. theobromae* as described in the sapstain exposure test protocols (Section 2.4). Seven combinations (Table 5-3) of fungal inocula were used as the treatment culture regimes comprising of single inoculum, dual and tri-culture mixed inocula. Control samples were either a) treated with 2% sodium pentachlorophenate (NaPCP), then inoculated with *B. theobromae* or b) left not inoculated. The sapstain test was carried out as described in Section 2.4. Evaluation of results (after 4 weeks) was as described in Section 2.5. The surface coverage of fungi was visually assessed daily based on a 5-point FRIM rating scale (Section 2.4.3). Visual assessments (Section 2.5.1) and quantitative assessments (Section 2.5.1) were made after 4 weeks.

5.3 RESULTS

5.3.1 Mean number of days to develop stain.

5.3.1.1 Statistical analysis

Table 5-1 shows the ANOVA table for the mean number of days to achieve mycelial coverage score of 2, 3 and 5 for rubberwood clone samples exposed to sapstain fungi *B. theobromae, A. niger* and *A. pullulans* treatments regimes. The table shows that there is a significant ($p<0.05$) effect of clone, fungal treatment and its interaction on number of days to achieve score of 2, 3 and 5.

Table 5-1. Mean number of days to achieve mycelial coverage scores of 2, 3 and 5 on rubberwood samples exposed to various fungal treatments.

Source	df	Score 2	Score 3	*Score 5*
Clone	2	8.590*	12.658*	26.175*
Fungi treatments	3	16.074*	16.414*	24.475*
Clone x treatment	6	0.795NS	2.664*	10.442*
Error	108	0.5469	0.7264	2.2453

* denotes significant difference ($p<0.05$), and NS denotes no significant difference ($p>0.05$)

5.3.1.2 Effect of clones.

Table 5-2 shows the overall mean number of days taken to develop mycelial coverage scores of 2 (6-23% coverage) or 3 (24-35% coverage) or 5 (more than 50% coverage) on the wood blocks of the three selected rubberwood clones. Clone RRIM600 (2.2 days) is the fastest to achieve a score of 2, followed by GT1 (2.7 days) and PB217 (3.2 days). Clone RRIM600 took 2.7 days to achieve score 3, significantly earlier ($p<0.05$) than clone GT1 (3.6 days) and PB217 (3.8 days). Similarly clone RRIM600 took 4.3 days to achieve a score of 5 significantly ($p<0.05$) earlier than clone GT1 (5.8 days) and PB217 (5.6 days). There is however no significant difference ($p>0.05$) between clones GT1 and PB217 in the number of days to achieve scores of 3 and 5.

After 4 weeks incubation it was observed that all samples exposed to *B. theobromae*

achieved visual ratings of 5 (> 50% mycelial coverage) after 7 days. Growth of *A. pullulans* was slowest on PB217, but with no significant difference (p>0.05) between the other two clones. The rate of mycelial coverage for each fungus differs between the rubberwood clones tested.

Table 5-2 shows that growth of *A. niger* was slowest in clone GT1 (score of 5 in 6.9 days) but was faster in either clones PB217 (4.2 days) or RRIM600 (3.3 days). However for *B. theobromae* fastest growth was observed on clone RRIM600 (5.1 days) as compared to GT1 and PB217, however there was no significant difference (p>0.5) between clone GT1 (6.5 days) and PB217 (7.6 days). *A. pullulans* is the only fungus that showed no significant difference (p>0.05) in mycelial growth rates between these three clones.

Table 5-2. Mean number of days* to develop score 2, 3 and 5 by *A. niger, A. pullulans and B. theobromae* on the wood blocks of clones GT1, PB217 and RRIM600.

CLONE	Score 2	Score 3	Score 5
GT1	2.7b	3.6b	5.8b
PB217	3.1c	3.8b	5.6b
RRIM600	2.2a	2.7a	4.3a
B. CLONE vs. FUNGI 1. GT1 vs. fungi			
A. niger	2.6a	3.9a	6.9b
A. pullulans	2.5a	3.3a	5.0a
B. theobromae	3.7b	4.3a	6.5ab
2. PB217 vs. fungi			
A. niger	2.2a	2.7a	4.2a
A. pullulans	3.3b	3.9b	6.0ab
B. theobromae	4.2c	5.1b	7.6b
3. RRIM600 vs. fungi			
A. niger	1.6a	1.9a	3.3a
A. pullulans	2.4ab	3.0b	5.2b
B. theobromae	3.2b	3.6b	5.1ab

* = Same letter down the column (between clones and fungal treatment by clone comparisons) denotes non-significant different for mean days (p>0.05)

5.3.2 Mycelial coverage on the rubberwood surface

Since the clonal and fungal interaction is highly significant ($p<0.01$, the clonal effect of fungi were examined individually. The effects of fungi on the rubberwood clones were examined by plotting the daily mean percent coverage of mycelium for the first 7 days.

5.3.2.1 Percent coverage of the fungi on the three selected rubberwood clones

Figure 5-1 shows the mean daily mycelia coverage (FRIM commercial rating as denoted in Section 5.2 and Chapter 2) of *A. niger*, *A. pullulans, B. theobromae* and a mixed inoculum of these stain fungi on the wood surface among three rubberwood clones. For both *A. pullulans* and the mix inocula there were no significant differences ($p>0.05$) in rate of coverage on the rubberwood clones. *A niger* showed significantly faster ($p<0.05$) initial rate of coverage on GT1 but this was slower on PB217 and RRIM600, while *B. theobromae* has faster initial coverage rate on GT1 but slowest on PB217.

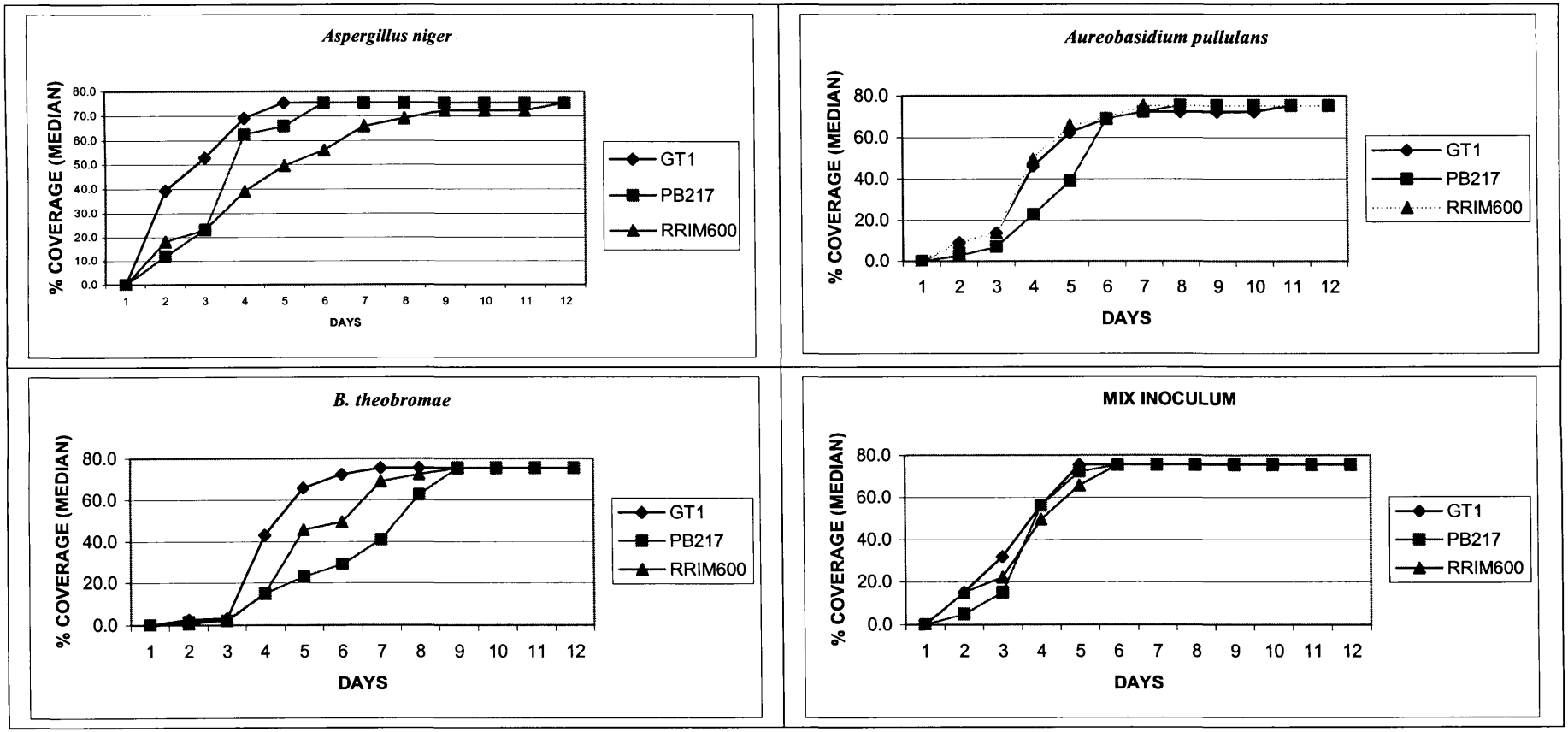


Figure 5-1. Four graphs to show FRIM rating by day for *A. niger, A. pullulans, B. theobromae* and a mixed inoculum (all these organisms) on the surface of rubberwood clones GT1, PB217 and RRIM600.

5.3.2.2 Effect of clone on rate of mycelial coverage of the fungi.

Figure 5-2 shows the daily FRIM rating on rubberwood surface of clones GT1, PB217 and RRIM600 for the different fungi *A. niger, A. pullulans, B. theobromae* and the mixed inocula. The graphs showed that in all the three clones the slowest fungus to cover the sample surface was *B. theobromae.* For clone GT1 the fastest to cover the surface was *A. niger (*$p<0.05$). For clone PB217 the fastest was the mixed inocula and *A. niger* ($p<0.05$). For clone RRIM600 the fastest was the mixed inocula and *A. pullulans* ($p<0.05$).

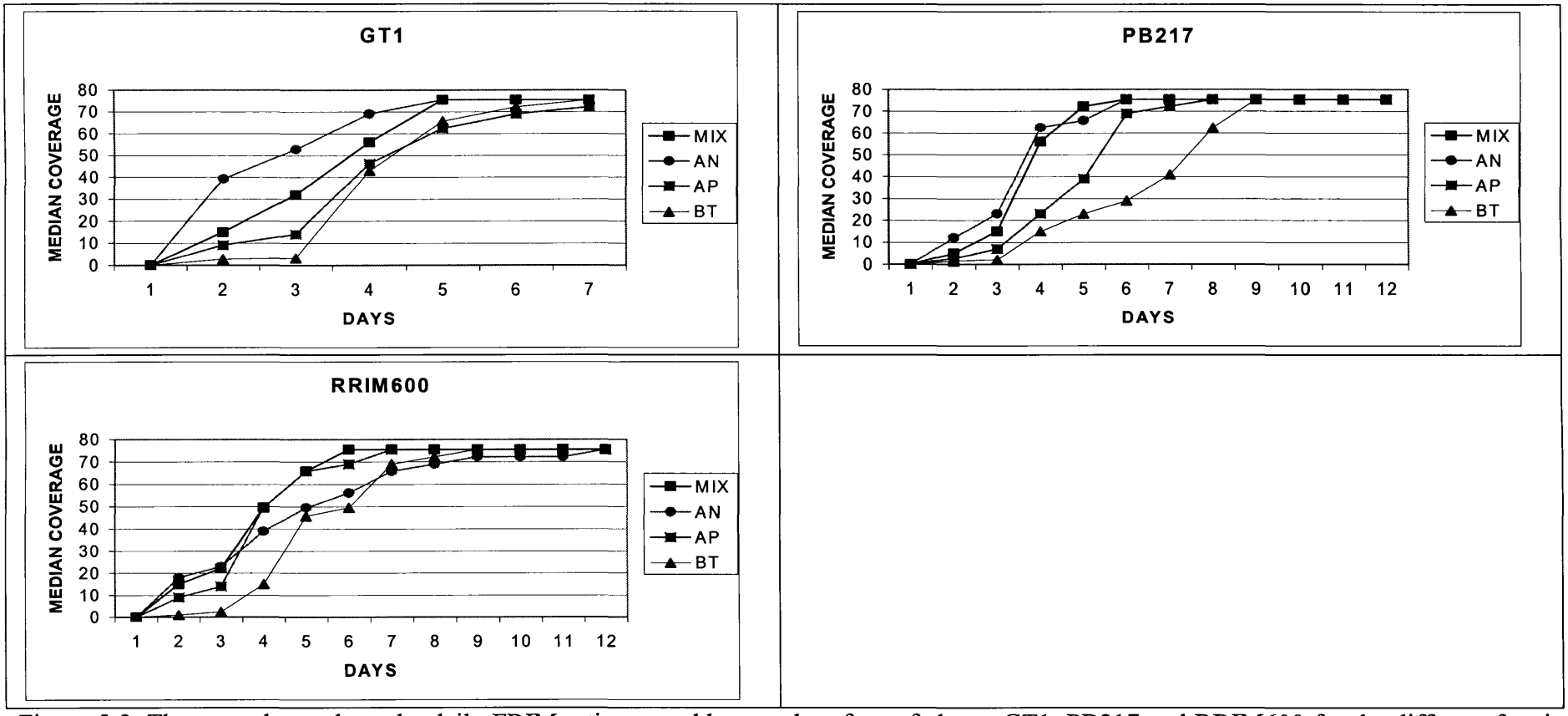


Figure 5-2. Three graphs to show the daily FRIM rating on rubberwood surface of clones GT1, PB217 and RRIM600 for the different fungi *A. niger, A. pullulans, B. theobromae* and mix inocula.

5.3.3 Laboratory sapstain test

5.3.4 Wood surface darkness rating after sanding

It is also evident from the results of the present study that all the three rubberwood clones treated with *B. theobromae* were darkly stained even after minimal sanding. In Sections 5.2.1.1 and 5.2.2.2 it was shown that *B. theobromae* had the slowest rate of mycelial coverage compared with either *A. niger* or *A. pullulans.* This shows that a rapid rate of mycelial coverage by a fungus does not necessarily result in a higher level of staining (darkening) by the same fungus. This is evident when growth of the true sapstain fungi (*B. theobromae*) were compared with mould fungi (*A. niger).*

5.3.5 Quantitative assessment and derived visual stain rating after sanding.

5.3.5.1 Statistical analysis

Table 5-3 shows the ANOVA of mean percent darkness (by spectrophotometry) and mean fungal coverage rating of rubberwood samples exposed to sapstain fungi treatments. It shows that clone, fungal treatments and clone and fungal treatments interaction all have highly significant effect ($p<0.01$) on percent darkness and percent fungal coverage of the rubberwood blocks exposed to various sapstain fungi treatments.

Table 5-3. Mean percent darkness (by spectrophotometry) and FRIM stain rating of rubberwood samples exposed to sapstain fungi treatments.

SOURCE	df	*DARKNESS (%)*	*COVERAGE (%)*
Clone	2	109.60*	574.49*
Fungi treatments	8	2022.54*	9212.48*
Clone x fungi treatment	16	40.29*	218.24*
Error	108	17.29	95.99

* denotes significant difference ($p<0.05$) and, NS denotes non-significant difference ($p>0.05$).

Table 5-3 shows the a) mean percent darkness (after sanding) and, b) stain coverage (after sanding) among rubberwood clones and the various fungal treatments. The mean percent coverage was derived from the median of the FRIM rating score of 1-5 as described in Section 2.4.3. The trend for between-clone mean percent darkness corresponded with their similar trend in percent stain coverage. However significant ($p<0.05$) variations were observed in the individual treatment assessments of the rubberwood samples for both percent darkness and percent stain coverage.

5.3.5.2 Clonal effects of percent darkness and percent stain coverage after sanding.

The overall effect of clone (mean across treatments) on mean percent darkness assessed by spectrophotometry is shown in Table 5-4a. Clone RRIM600 had significantly higher ($p<0.05$) overall mean darkness (39.7%) than clone PB217 (36.6%). The mean darkness of clone GT1 (38.2%) was not significantly different ($p>0.05$) from either PB217 or RRIM600. The percent coverage of RRIM600 (25.6%) was significantly higher ($p<0.05$) than clone GT1 (22.6%) and PB217 (18.4%).

Means across all clones (Table 5-4b) show that *B. theobromae* stains with the highest percent darkness (56.49%) and percent coverage (75.5%), significantly higher ($p<0.05$) than all the other fungi treatments. Compared with other treatments, NaPCP treated control was the lightest, with significantly ($p<0.05$) the lowest

fungal coverage (Table 5-4b). Samples treated with *A. niger* + *B. theobromae* and *A. pullulans* + *B. theobromae* showed significant ($p<0.05$) reduction in darkness. Further reduction in darkness was observed for wood samples treated with the tri-inocula mixture (*A. niger* + *A. pullulans* + *B. theobromae*) indicating possible synergy in biological control activity.

Table 5-4. Overall mean* percent darkness by spectrophotometry after sanding and derived % stain coverage for infected rubberwood samples a) between clones pooled across the fungal treatment regimes and b) between treatments pooled across clones.

a) CLONE	*DARKNESS (%)*	*COVERAGE (%)*
RRIM600	39.7 a	25.6a
GT1	38.2ab	22.6b
PB217	36.4 b	18.4b
b) FUNGAL TREATMENTS		
B. theobromae (BT)	56.5 a	75.5 a
A. niger + *B. theobromae*	44.6 b	39.3 b
A. pullulans + *B. theobromae*	42.7 bc	36.7 b
A. niger + *A. pullulans* + *B. theobromae*	39.1 cd	10.7 d
A. niger	38.2 d	11.0 d
A. niger + *A. pullulans*	36.4 d	22.7 c
A. pullulans	35.9 d	3.0 e
2% Na PCP + BT	28.6 e	1.0 e
Control (non-inoculated)	21.5 f	0.0 e

* = Same letter down the column for (between clones and between fungal treatments comparison of means) denotes non-significant difference ($p>0.05$)

Table 5-5 shows the mean percent darkness of infected rubberwood clones exposed to various treatment regimes. There is variability in the mean percent darkness of the samples among the three clones. Table 5-5 shows that for clone GT1,

rubberwood samples treated with *A. niger* + *B. theobromae* and *A. pullulans* + *B. theobromae* were not significantly different ($p>0.05$) from samples treated with the sapstain fungi *B. theobromae* alone. However rubberwood clone PB217 and RRIM600 samples exposed to the inoculum of sapstain fungi *B. theobromae* were significantly darker ($p<0.05$) compared with samples treated with inoculua mixture of *A. niger* + *B. theobromae* or *A. pullulans* + *B. theobromae* compared with samples of these clones. This indicates variability in biological control activity in the different rubberwood clones.

Table 5-5. Mean percent darkness of infected rubberwood samples for each fungal treatment by clone.

TREATMENT	% DARKNESS		
	GT1*	PB217*	RRIM600*
B. theobromae (BT)	52.9c	55.1d	61.5c
A. niger + *B. theobromae*	46.6c	42.52c	44.6b
A. pullulans + *B. theobromae*	46.9c	39.0bc	42.1b
A. niger + *A. pullulans* + *B. theobromae*	35.4bc	39.2bc	42.8b
A. niger	40.9bc	36.4bc	37.4b
A. niger + *A. pullulans*	37.3bc	37.34b	37.5b
A. pullulans	34.3b	33.9b	39.5b
2% Na PCP + BT	28.8ab	27.9a	29.0ab
Control (non-inoculated)	20.7a	21.2a	22.6a

* = Same letter down the column (between fungal treatments comparison of means) denotes non-significant difference ($p>0.05$)

Table 5-5 shows the mean percent coverage of infected rubberwood clones exposed to various treatment regimes. The table shows that there is variability but no specific trend in the percent stain coverage. However it shows that treatment with *B. theobromae* alone has the highest mean percent coverage compared to *A. niger, A. pullulans* and various other combinations of spore inocula. The use of two biological control fungi *A. niger* and *A. pullulans* in the mixed inocula significantly

($p<0.05$) reduced the percentage cover to (5-14%) as compared to the individual application of biological control fungus of either *A. niger* (28-47%) or *A. pullulans* (29-43%) in all the three clones. *A. niger* showed significantly the lowest ($p<0.05$) mean percentage of mycelial coverage especially on GT1 and PB217. The mycelial coverage of mixed inocula of *A. niger* and *A. pullulans* however showed differences in percent coverage among the three rubberwood clones. Mixed inocula of *A. niger* and *A. pullulans* showed lowest mean percent coverage in clone PB217 (9.0%). Despite that the percent darkness of PB217 (37.34%) was not significantly different ($p>0.05$) from GT1 (37.34) and RRIM600 (37.49%) as shown in Table 5-4.

Table 5-6. Mean percent stain coverage (derived from FRIM rating) of sapstain infected samples for each fungi treatment by clone.

TREATMENT	% COVERAGE		
	GT1	PB217	RRIM600
B. theobromae (BT)	75.5a	75.5 a	75.5 a
A. niger + BT	43.0 b	28.0 b	47.0 b
A. pullulans + BT	43.0 b	29.5 b	37.5 b
A. niger + *A. pullulans* + BT	5.0 c	13.0 c	14.0 d
A. niger	3.0 c	5.0 d	25.0 c
A. niger + *A. pullulans*	31.0 b	9.0 c	28.0 c
A. pullulans	3.0 c	3.0 d	3.0 d
2% Na PCP + BT	0.0 d	3.0 d	0.0 d
Control (non-inoculated)	0.0 d	0.0 d	0.0 d

* = Same letter down the column (between treatments comparison of means) denotes non-significant difference ($p>0.05$)

5.4 DISCUSSION

The main objective of this chapter was to examine whether clonal differences in physical, anatomical and chemical features affected a) sapstaining activity of *B. theobromae* and b) possible biological control activity of *A. niger* and *A. pullulans* on *B. theobromae*. The results will be discussed in relation to these objectives.

5.4.1 Sapstain assessment methodology

This study uses the general sapstain exposure protocol as described in Wong *et al.* (1997) and Wong and Sabri (2000) but with a different method of sterilisation. In the method of sterilization of the rubberwood blocks, Wong *et al.* (1997) and Wong and Sabri (2000) both used the standard autoclave method (120^0C steaming), however in the current study sterilisation was by means of gamma irradiation. Gamma irradiation was selected rather than the normal autoclave method in order to avoid exposure of the samples to high temperatures and to minimise degradation in the readily available sugars and starch in the rubberwood samples prior to sapstain exposure treatments. There are several sapstain exposure methods used in other countries with minor differences in sample size, method of sterilisation of the wood samples, incubation temperature and period of incubation modified to meet different assessment objectives as shown in Table 5-7.

Incubation temperature used ranges from 23 - 30°C. A temperature of 25°C, similar to FRIM protocol, was chosen for this study with the possible advantage of selecting potential biological control fungi that have optimal growth at about that temperature and may not grow well, or at all beyond 30°C.

Table 5-7. Examples of laboratory sapstain test protocols reported showing different methods of sterilization, temperature, assessment criteria and duration of incubation.

Wood species	Sample size (mm)	Method of sterilisation	Fungi (method of inoculation)	Incubation temperature	Assessment criteria	Duration of test	Reference
Betula verrucosa *Pinus caribbea* *Pinus sylvestris*	5 x 15 x 30	Autoclave	*B. theobromae* (spray to runoff)	30°C	Weight loss of wood sample due to *B. theobromae*	30, 60, 90, 120, 150 and 180 days.	Encinas & Daniel (1999)
B. verrucosa *P. caribbea* *P. sylvestris*	5 x 15 x 30	Autoclave	*bacteria* (spray to runoff)	30°C	Weight loss of wood sample due to *B. theobromae*	2, 30 and 60 days	Encinas & Daniel (1996)
Pinus pondorosa	3 x 15 x 30	Gamma radiation (Cobalt 60)	Target fungi & bacteria (spray to runoff)	28°C	a) Discoloration and b) Spatial distribution of bacteria on target fungi (SEM)	14 and 28 days	Kim & Morrell (1998)
H. brasiliensis	5 x 20 x 40	Autoclave	*B. theobromae* (dip for 5 s)	25°C	Cell wall degradation of *B. theobromae* (TEM)	28 days.	Wong & Singh (1997)
H. brasiliensis	5 x 20 x 40	Autoclave	*B. theobromae* (dip for 5 s)	25°C	Cell wall degradation of *B. theobromae* (TEM)	28 days.	Wong *et al.* (1997)
H. brasiliensis & 8 other hardwoods species.	5 x 20 x 40	Autoclave	*B. theobromae* (dip for 5 s)	25°C	Percent mycelial coverage of sapstain & mould fungi.	2, 7 and 21 days.	Wong & Sabri (2000)
H. brasiliensis Clones	5 x 20 x 40	Gamma radiation	*B. theobromae* (dip for 5 s)	25°C	a) % darkness b) % sapstain coverage and c) SEM of *B. theobromae* & other fungi	28 days.	Ashari *et al.* (2000)

5.4.2 Assessment of sub-surface sapstain

The normal method of assessment to evaluate sapstain uses the coverage of fungal mycelium before sanding. The assessment of mycelial coverage has the merit of being able to discriminate acceptable or unacceptable degrees of disfigurement as required by the industry. The scale rating of 0 to 5 is adjusted to the level acceptable by the industry. However to date there is no single standard scale for use in the different countries. With this method of assessment, maximum coverage defined as coverage with median greater than 75.5% was found after 4 weeks on all rubberwood samples. This method also assumes that the wood surface below the area covered with mycelium is stained without taking into account the amount of pigment present in the mycelium that penetrates the wood.

However after sanding, the exposed sub-surface sapstain coverage reveals considerable variation. Therefore besides mean percent stain coverage, darkness ratings of sample may be another important parameter, although there can be a negative relationship between the two (R^2 = -0.74) (Table 5-3). Percent darkness (after sanding) produced by the various fungi on the rubberwood clones is shown in Table 5-4. The effect of a single biological control or target fungus provides quantitative evaluation of the staining characteristics of the hyphae and is a useful selection criterion for identifying fungi with dark mycelium that stained the rubberwood. The effects of combination of biological control fungi and the target fungus provide information on biological control activity due to the resultant hyphal penetration of biological control fungi and *B. theobromae.*

5.4.3 Assessment of sub-surface darkness

In the sapstain exposure test of biological control agent against *B. theobromae* infection of wood, the ultimate objective was to prevent *B. theobromae* hyphae from penetrating the wood and darken it. If this were completely successful then the percent darkness after the experiment would not be significantly different ($p>0.05$) from the control unstained samples. This could be possible if the wood is exposed to non-staining fungus (with colourless spores) or if there is complete biological control of *B. theobromae* hyphal penetration into the wood surface. Results of the present study demonstrated the additional advantage of assessing the surface without the fungal mycelium (Figure 5-3). In the present study the samples were further analysed after drying the sapstained exposed samples by minimal sanding (0.5mm) as shown in Figure 5-3.

To date this is the first attempt in laboratory sapstain test, to evaluate sub-surface staining. Sub-surface sapstain evaluations have been commonly conducted in sapstain field-tests elsewhere (Wong and Woods, 1997). This is also the first attempt to evaluate differences in staining characteristics of rubberwood clones in the laboratory.

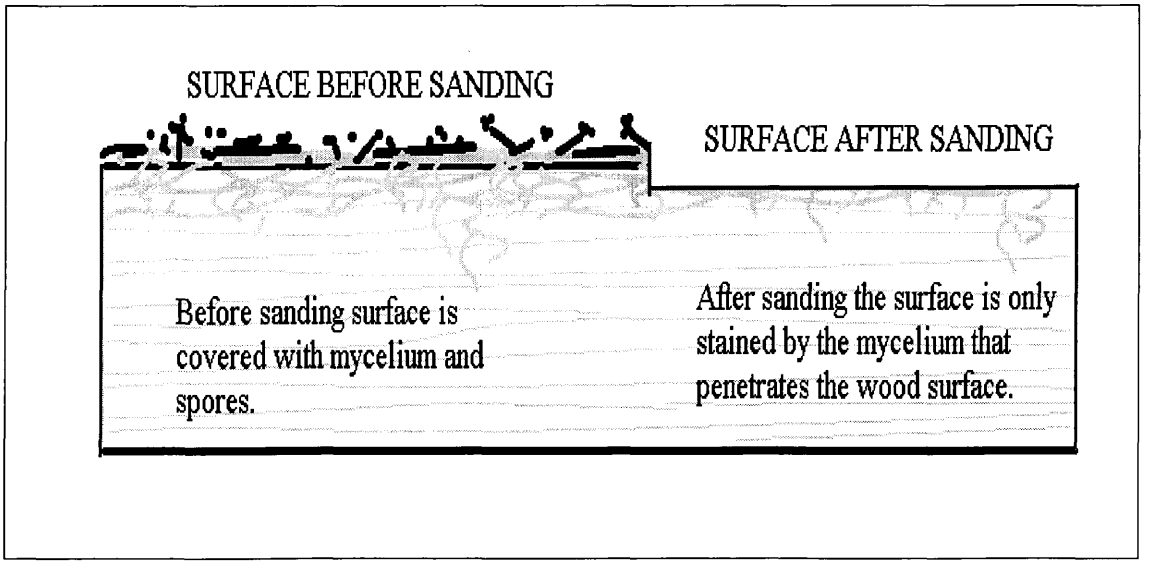


Figure 5-3. Figure to illustrate the mycelial coverage on the surfaces of the wood wafer before and after sanding.

5.4.4 Clonal differences (variation)

Another significant finding of the present study is that there was clonal variation in percent darkness of stain on the rubberwood samples exposed to different combinations of fungal treatment regime. This finding is consistent with the findings reported in Chapters 3 and 4 where the physical, anatomical and chemical characteristics of the three clones suggested that RRIM600 might be most susceptible to sapstain. Because starch is a rich source of nutrients, it was suggested that rubberwood is susceptible to *B. theobromae* because of the high content of starch (Simatupang, 1986; Azizol and Rahim, 1989). The importance of starch and the role of pH in rubberwood is discussed in Section 5.4.6.3.

5.4.5 Fungal inocula differences (variation)

Single organism treatments (present study) clearly showed that *B. theobromae* stained deeply into the sub-surface layer (exceeding 0.5 mm) for all clones. The degree of subsurface staining is dependent on the amount of pigment in the hyphae of the fungus that penetrates the wood. Therefore the relatively slower colonisation rate of *B. theobromae* indicated that it might well be susceptible to biological control by the faster growing organisms.

It is also evident in the current study that samples exposed to *A. niger* and *A. pullulans* showed staining after minimal sanding but the darkness ratings are not as high as those exposed to *B. theobromae*. The mixed fungal inocula (*B. theobromae* + *A. niger* + *A. pullulans*) incorporating *B. theobromae* in rubberwood clones resulted in further reduction in percent darkness after sanding suggesting possible synergism with application of more than one biological control fungi.

The effectiveness of the biological control fungi is shown to be related to the nutrient content of growth media (Srinivasan *et al.,* 1992, Score and Palfreyman, 1994). The results presented here further suggest that the effect of biological control can vary among rubberwood clones containing different nutrient levels. Biological control fungi were also reported to limit, but not completely inhibit, physiological activity of the target stain organisms (Florence and Sharma, 1990, Highley and Croan, 1991, Kreber and Morrell, 1993). Croan (1996) showed that non-pigmented isolates of

Ceratocystis spp. and *Ophiostoma* spp. could also be used for the biological control of sapstain on yellow pine logs. Florence (1991) reported that *Bacillus subtilis*, a bacterium isolated from rubberwood was found to be antagonistic against several stain and mould fungi. The efficacy of this bacterium was tested in the field and proved to be effective in controlling sapstain up to a level of 80% in green lumber. Several other reports also described potential bacteria and fungi for biological protection against stain fungi, but field performance of these organisms has been inconsistent (Florence and Sharma, 1990, Benko, 1989). These observations may again be related to the nutrient variability in the wood or the sensitivity of the bioprotectants to environmental factors.

Unlike other clones, the mean darkness of GT1 samples exposed to *A. niger* + *A. pullulans* + *B. theobromae* was not significantly different ($p>0.05$) from samples treated with 2% NaPCP+ *B. theobromae* (Table 5-5), suggesting an effective biological control of *B. theobromae*. However, both *A. pullulans* + *B. theobromae* and *A. niger* + *B. theobromae* combinations inoculated on wood of GT1 failed to reduce *B. theobromae* penetration into the wood surface, as such both treatments imparted significant ($p<0.05$) darkness compared with NaPCP + *B. theobromae*, suggesting that other, as yet unknown, factors influence biological control of *B. theobromae* growth on a rubberwood clone in the laboratory. The failure to attain significant ($p<0.05$) reduction in darkness using *A. niger* and *A. pullulans* could be due to pigments present in *A. niger* and *A. pullulans* that still caused significant darkness ($p<0.05$) as compared to the unstained control. The use of non-staining fungus or mutant colourless strain of *B. theobromae* could be an ideal alternative.

Dawson-Andoh (2000) reported that colonisation of wood by biological control fungi (*Gliocladium roseum*) could be enhanced by altering the wood substrate to stimulate spore germination and/or limit that of competing microflora. Dawson-Andoh (2000) showed that the presence of additional nutrients (amino acids) was able to benefit the biological control agent wihich are less competitive in initial establishment on wood with low nutrients on the surface in the biological control of *Ophiostoma picea* on western hemlock (*Tsuga heterophylla*). Difference in the fructose and glucose content of the rubberwood clones could therefore provide the same explanation for the observed differences in biological control activity. The failure of *A. niger* to

restrict growth of *B. theobromae* in GT1 is probably related to the lower contents of fructose and glucose in GT1 as compared to the other two clones. This is supported by Garraway and Evans (1984) as they rated that glucose is a good carbon source for production of conidia in *A. niger*. The same could be true for *A. pullulans*. However, in the current study, the use of both *A. niger* and *A. pullulans* has a synergistic effect on the control of *B. theobromae*. The positive biological control effect of *A. niger* and *A. pullulans* could be due to other mechanisms not related to the source of nutrients. One possible explanation is the secretion of metabolites from either or both *A. niger* and *A. pullalans* suppressed the growth of *B. theobromae*.

5.4.6 Physical, anatomical and chemical characteristics in relation to sapstain susceptibility

The differences in sapstain susceptibility in the three rubberwood clones is influenced by several factors. The results shown in Table 5-8 indicate that factors causing sapstain susceptibility of wood are most likely, vessel diameter and frequency, pH, starch contents and the content of soluble sugars. The table also provides circumstantial evidence on an association between sapstain susceptibility and basic density, green moisture content, carbon:nitrogen ratio, width and height of ray parenchyma cells.

Table 5-8. Differences in chemical constituents and sapstaining characteristics of clones GT1, PB217 and RRIM600

CHARACTERISTICS OBSERVED	GT1	PB217	RRIM600
Mean % darkness* (overall)	38.20b	36.64a	39.67b
Mean % darkness* (exposed to BT)	52.88a	55.10a	61.50b
Median % coverage* (overall)	22.61a	18.40b	25.57c
Days* to develop score 3	3.57b	3.75b	2.70a
Days* to develop score 5	5.80b	5.57b	4.30a
Basic density* (kg/m^3)	615.6b	582.2a	628.9b
Green MC* (%)	56.2a	64.1b	61.1b
pH*	5.8a	5.9a	6.2b
Carbon* (%)	46.2a	46.5a	45.2a
Nitrogen* (%) (stem)	0.106b	0.064a	0.078a
Nitrogen ratio (stem)	433a	713b	591b
After drying (sawn timber)	919b	574a	656a
Starch* (%)	4.3a	3.6a	5.8b
Fructose* (mg/g)	0.43a	1.43c	0.91b
Glucose* (mg/g)	0.27a	1.23b	0.56a
Sucrose* (mg/g)	8.48a	7.39a	7.47a
Total sugars* (mg/g)	9.19a	10.16a	8.94a
Vessels diameter (μm)	200-250	200-240	250-300
Vessels frequency (per field of view 40X)	12-15	10-12	16-18
Ray width	2-4 cells	3-6 cells	3-4 cells
Ray height	Shortest	Longest	Medium
Biocontrol activity of *A. niger*	ns	+	+
Biocontrol activity of *A. pullulans*	ns	+	++
Biocontrol activity of *A. niger* + *A. pullulans*	++	+	++
Initial rate of coverage *A. niger*	high	medium	low
Initial rate of coverage *B. theobromae*	high	low	medium

ns = Not significantly different ($p>0.05$)
\+ = Significant ($p<0.05$) reduction in darkness (20% reduction)
++ = Significant ($p<0.05$) reduction in darkness (30% reduction)
* = Same letter across rows (among 3 clones) denotes non-significant difference ($p>0.05$)

Higher levels on nutrients in PB217 (fructose, glucose and sucrose) and RRIM600 (starch) seem to facilitate growth of *B. theobromae* and thus causing biological control to be less effective in these two clones compared to GT1.

5.4.6.1 Vessel diameter and frequency

Vessel lumens provide uninterrupted pathways with little resistance for mycelial extension and growth of fungal mycelium during wood colonisation. This study has shown that clone RRIM600 has relatively larger vessel diameter and higher vessel frequencies than the other two clones, probably allowing *B. theobromae* to spread more readily in search of nutrients in the ray parenchyma cells which are normally accessible through pit openings in the case of RRIM600. This may also account for the darkly stained tissues of RRIM600.

5.4.6.2 Starch

Encinas and Daniel (1999) showed that *Lasiodiplodia theobromae* (synonymous to *B. theobromae*) is capable of utilising starch. They showed that *B. theobromae* could grow rapidly into wood and consume the readily available nutrients. After only 1 month both fructose and starch were almost completely consumed in both softwoods (Caribbean pine and Scots pine) and hardwoods (birch). Glucose consumption in the two softwoods was delayed until after 3 months incubation. Thus starch is one of the important sources of nutrient for the growth of *B. theobromae* and is consistent with the rapid colonization of RRIM600 found in this current study. RRIM600 had the highest content of starch (5.8%) than any remaining clones.

Starch consists of two polymers, a) amylose, a linear molecule of mainly α-1, 4-linked units, and b) amylopectin which makes up 75-85% of most starches and is highly branched with a α-1,6-links along the main α-1,4 chain (Cooke and Whipps, 1993). A range of enzymes α amylase (optimum pH of 3.0 - 6.0), amylo glucosidase (4.5 - 5.0), α-glucosidase (3.0-7.5) and isoamylase (5.0 – 6.5) commonly found in microorganisms are capable of breaking these linkages at various pH (Cooke and Whipps, 1993). The relationship between pH and enzymic degradation of starch is discussed in Section 5.4.6.4.

5.4.6.3 pH

As mentioned in Section 1.12, pH influences the growth and survival of fungi and this influence is likely to be complex. Fungi are characteristically tolerant of low pH and most have an optimum range of between 5.0 to 7.0. This acid tolerance has frequently been used to isolate fungi from mixed cultures of fungi and bacteria (Smith and Berry, 1975).

The internal pH of the cell is controlled independently of the medium at around 5.0-6.0 so the effects of the changes in the external pH are restricted to permeability and other surface phenomena (Smith and Berry, 1975). Uptake of amino acid is pH dependent and although the optimum pH varies between individual amino acids, in general uptake occurs most rapidly at pH 5 - 6 (Garraway and Evans, 1984). The pH of the substrate normally influences the uptake of amino acids by determining the charge on them and on their carrier (Garraway and Evans, 1984).

The pH of substrate is also important in the breakdown of starch or cellulose available nutrients in the wood. The role of isoamylase is probably important in the breakdown of starch in rubberwood and so is the pH of rubberwood clones as discussed in Section 5.3.7.2

5.4.6.4 Starch and enzymic reaction.

The role of isoamylase in degradation of starch has also been studied on *Talaromyces*. *Talaromyces* has been reported to increase in both mycelial growth and the concentration of isoamylase (a major starch-degrading enzyme) with increase in concentration of starch (Garraway and Evans, 1984). In this organism starch is the most effective inducer of amylase. However no amylase was detected when sucrose is the sole carbon source. The presence of amylase facilitates breakdown and assimilation of starch. Higher percentage of starch in RRIM600 (Table 5-8) could possibly induce isoamylase production and facilitate assimilation of starch by *B. theobromae*.

5.4.6.5 Soluble sugars

The role of soluble sugars as a source of carbon is more complex than that of starch. There have been several studies on the influence of sugars on the physiology and biochemistry of sugar utilisation of sapstain fungi. Garraway and Evans (1984) in their studies involving sapstain fungi, showed that fructose and glucose are good carbon sources for growth of fungi but not for the production of conidia. They demonstrated in vitro that concentrations of glucose and fructose as low as 10 – 20 mM (1.82– 3.64 mg/g) markedly stimulate growth but at 100 mM (18.2 mg/g) a plateau is reached where increasing concentration has little effect, whereas sucrose is a poor carbon source even at concentration of up to 100 mM. Rubberwood clone PB217 contains the highest levels of fructose and glucose (Table 5-8), although at level much lower than that found by Garraway and Evans (1984). It seems unlikely, therefore, that levels of fructose and glucose contribute to the differences in sapstain found between the clones.

5.4.6.6 Carbon/nitrogen ratios

In the previous chapter total organic carbon (Section 4.3.2) and total organic nitrogen (Section 4.3.3) were determined in order to examine the association of C:N ratio to mycelial growth and pigmentation. Table 5-8 shows that clonal variation in rate of sapstain may be associated with clonal variations in nitrogen levels. Levi and Cowling (1969) demonstrated on agar media that at C:N ratios of 4:1 to 400:1 the amount of mycelial growth was not limited by nitrogen but by the total carbon available, whereas at higher ratios growth was limited by the amount of nitrogen available. The C:N ratio of this study is within the scope of the findings of Levi and Cowling (1969). In a similar study, Aube and Gagnon (1969) tested the response of 3 isolates of two stains of *T. viridae* on differenct C:N ratios using glucose and L-arginine on agar. Their results show that optimum mycelial growth of *T. viridae* is at C:N ratio of 35 and 50. Lower dry mass of mycelium was obtained below the ratio of 35 and also if C:N ratio is above 50.

However both these studies were conducted on artificial media. The ratios obtained by Levi and Cowling (1969) and Aube and Gagnon (1969) therefore cannot be directly applied to the values obtained in this study. The general C:N ratios

obtained for rubberwood will be much lower if C:N ratio were based on the parenchyma cells where most of the nutrients are located i.e. if the structural components (cellulose) are excluded from the calculation. However both these studies demonstrated the significance of nitrogen and carbon for mycelial growth.

Ritchkoff *et al.* (1998) studied the effect of the carbon source and the amount of nitrogen on the melanisation and the production of mycelia mass of blue stain fungus *A. pullulans* by using solid-state cultivation. The carbon sources used varied from easily soluble sugars to structural polysaccharides existing in lignocellulosic material. The amount of melanin was evaluated by using partial purification and the conventional measurement methods. The production of melanin was dependent on the amount and quality of the carbon source as well as the amount of nitrogen. *A. pullulans* tends to produce melanin on nitrogen poor media supplemented with easily soluble sugars (e.g., glucose, sucrose, mannose, and xylose). However melanin production was restricted on nitrogen rich media. The production of melanin was totally inhibited on the media containing celluloses or lignin as sole carbon sources. The rubberwood samples treated with *A. pullulans* indicated that most staining is found in clone RRIM600 which contain significantly higher ($p<0.05$) nitrogen than GT1, indicating that there must be other factors which are more important in causing the differences in staining of the rubberwood clones.

5.5 CONCLUSION

The objective of this chapter was to examine the differences in sapstaining characteristics of the three rubberwood clones and to relate to the findings of chapters 3 and 4. The results indicate that there are differences in sapstain susceptibility in the rubberwood clones. The least and most susceptible to sapstain are, respectively, clones PB217 and RRIM600. The results also indicate possible biological control effect of inocula of *A. pullulans* and *A. niger* mixture on *B. theobromae* growth, especially for clones PB217 and RRIM600.

The results of the findings in this chapter also indicate that it was meaningful to examine the subsurface staining of the rubberwood samples in order to evaluate the darkening effect of the pigments present in the mycelium of the biological control agent and also the target fungi. The subsurface examination will also quantify the

presence or absence of darkening effect of the biological control fungi and target fungi mycelium.

In the next chapter the quantitative method of sapstain assessment as used in this chapter will be evaluated with two other quantitative methods of sapstain assessment. The selected method will be used to quantitatively screen potential non-pigmented biological control fungi that may possibly eliminate or minimise the sub-surface darkening (staining) effect of *B. theobromae*.

CHAPTER 6

6. COMPARISON OF THREE METHODS OF QUANTITATIVE EVALUATION OF SAPSTAIN IN RUBBERWOOD

6.1 INTRODUCTION

Laboratory trials using small wood specimens are widely used in the screening of biocides for preventing fungal stain. Several visual methods have been used for testing and assessing stain, but the procedures remain largely subjective (Sexton *et al.,* 1993). Many reports assign numbers to categories to provide ratings based on percentage area of fungal coverage (Laks *et al.,* 1991; Tsunoda and Nishimoto, 1988; Hong, 1989; Wong *et al.,* 1997; Wong *et al.,* 1999a, 1999b) or other criteria such as the percentage area of wood discoloured, as measures of biocide effectiveness (Morrell and Sexton, 1992). Although such ratings are no doubt practical, the reports rarely describe in detail the scales employed, the number of personnel making the evaluations, or whether the samples are coded to conceal the treatment groups. These parameters may bias ratings and thereby influence the results (Sexton *et al.,* 1993). Quantitative measurements on systematically assigned areas on the wood samples to be assessed might provide more precise and consistent estimate of the degree of discoloration as well as minimising operator subjectivity.

Assessing the extent of fungal stain on wood during laboratory trials is a subjective process and may be subject to considerable variation between individual evaluators and types of specimen. The purpose of the comparisons described in this Chapter was to explore the potential of three quantitative methods for the measurement of wood surface discolouration and to compare them with visual rating. Specimens degraded by sapstain and mould fungi *B. theobromae, A. pullulans* and *A. niger* were studied to identify the best possible method for quantitative assessment of sapstain on wood surfaces. The three methods evaluated were spectrophotometry (Spectroflash™ 500), colorimetry (Minolta™ Croma Meter CR200) and scanning densitometry (Hoefer GS300).

The first two methods (also used in Chapter 5) are based on CIELAB colour space. In both cases the relevant instrument is designed to measure light in a way equivalent to the way that the human eye perceives light (Minolta, 1994). The portable colorimeter (4 mm viewing aperture) is a colour-measuring instrument available at FRIM. Because it is portable, it allows more surface area of the wood samples to be measured. The third method estimates reflectance from the wood samples using a scanning densitometer.

6.2 METHODS

The sapstain-exposed rubberwood samples were dried to below 10% moisture content in an oven at 60°C for 48 hours to prevent any further fungal growth. A temperature greater than 60°C was avoided as it could cause darkening of the wood samples). After drying, the samples were sanded to remove approximately 0.5 mm of the surface layer, with the help of a thickness gauge. The relative darkness was then determined by: a) assessing percent reflectance of the surface using spectrophotometry (D65-daylight exposure or A-tungsten light exposure), colorimetry and scanning densitometry; and b) visual darkness assessment as described in Chapter 2, Section 2.5 and discussed in Chapter 5.

6.3 RESULTS AND DISCUSSION

6.3.1 Assessment Methods

Spectrophotometry and colorimetery describe colour numerically according to International Standards (Datacolor, 1992 and Minolta, 1994). Expressing colour in this way makes it possible for anyone to understand the colour being expressed. The two most widely known methods of expressing colour are the Yxy colour space (devised in 1931) and the La*b* (also referred to as CIELAB) colour space (devised in 1976) to provide a more uniform colour description in relation to visual differences (Minolta, 1994). The CIELAB colour space is widely used in virtually all fields. In this colour space L indicates lightness and a* and b* indicates colour directions. This study attempts to quantify darkness by examining the variation in L colour space, as L is the quantitative co-ordinate to measure the darkness or lightness of colour.

6.3.2 Assessment of clonal means

Table 6-1 shows the overall mean darkness percent obtained by the four methods of assessment. The table shows that all the quantitative methods give the same ranking for the rubberwood clones, RRIM600 being the darkest, followed by GT1 and finally PB217. Since the numerical scales of the four measurements are not the same a better means of comparison is to normalise all values relative to the lowest percent darkness of the unstained samples (values in parenthesis). Thus values for the unstained samples were standardized to 1.0 and the normalised values provide comparisons of each method relative to the same initial value of the control unstained sample.

Table 6-1. Overall mean[1] darkness (%) of rubberwood clones exposed to various fungi treatment regime for 4 weeks, determined by the four methods of assessment, spectrophotometry (Smeter), colorimetry (Cmeter), densitometry (Dmeter) and visual.

Clone	Smeter	Cmeter	Dmeter	Visual
RRIM600	41.85 (1.95)	38.23 (1.85)	67.5 (2.03)	62.47 (1.42)
GT1	39.67 (1.85)	36.02 (1.74)	66.5 (2.00)	47.48 (1.08)
PB217	38.49 (1.79)	35.82 (1.73)	62.8 (1.89)	45.1 (1.03)

Note: [1] Numbers in parenthesis denote values normalised to control value of respective method.

Figure 6-1 shows the normalised values (no units) derived from Table 6-1 for the three rubberwood clones exposed to sapstain fungi as determined by the four methods of assessment. From this graph it can be seen that the pattern of the mean results obtained by spectrophotometry, colorimetry resemble that from the visual method of assessment. However it is difficult to confirm the similarity without detail least square analysis of the assessment of the four methods.

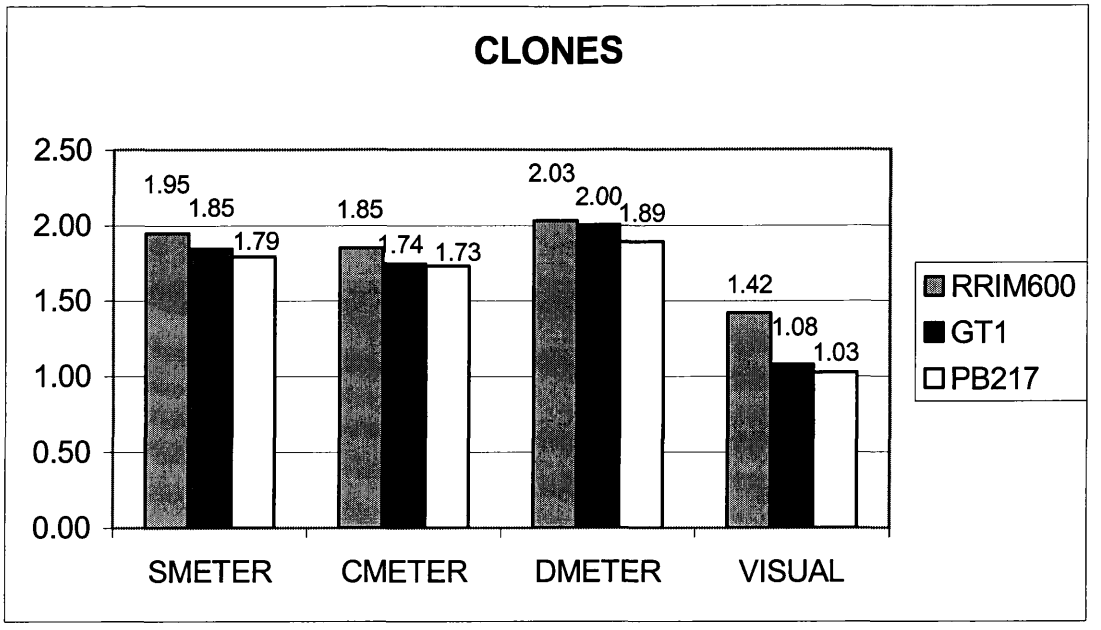


Figure 6-1. Normalised overall mean for rubberwood clones GT1, PB217 and RRIM600 exposed to sapstain fungi as determined by the four methods of assessment.

A series of 12 regression equations, as shown in Table 6-2, were derived from the raw data to predict the qualitative value of visual assessments from the three quantitative methods via least square analysis. Table 6-2 shows that all comparisons are highly significant ($p<0.01$). Based on the coefficient of determination (R^2 values) out of the three quantitative methods of darkness evaluation using colorimetry (83.7%) gave the best predictive estimates. This method also showed the best coefficient of determination ($R^2>80\%$) for the three rubberwood clones. The poorest coefficient of determination was for estimations using densitometry (R^2 <44%).

Table 6-2. Regression equations derived for predicting visual assessments rating (V) from the three quantitative methods of assessment spectrophotometry (Smeter), colorimetry (Cmeter), and densitometry (Dmeter).

Equation No,	Clone	Regression equation	F-ratio	R^2 (%)
Cmeter 1	All clones	V = 15.610 + 1.052 Cmeter	606.7*	83.7
2	GT1	V = 15.546 + 0.995 Cmeter	171.2*	81.4
3	PB217	V = 13.689 + 1.130 Cmeter	4500.8*	87.3
4	RRIM600	V = 17.374 + 1.038 Cmeter	5486.5*	88.0
Smeter 5	All clones	V = -21.209 + 0.922 Smeter	196.9*	62.2
6	GT1	V = -14.607 + 0.864 Smeter	62.7*	61.3
7	PB217	V = -30.662 + 1.047 Smeter	65.2*	58.6
8	RRIM600	V = 21.882 + 0.903 Smeter	100.5*	71.8
Dmeter 9	All clones	V = 41.983 + 0.678 Dmeter	53.9*	31.3
10	GT1	V = 33.349 + 0.844 Dmeter	29.2*	43.4
11	PB217	V = 35.273 + 0.938 Dmeter	17.5*	31.5
12	RRIM600	V = 49.364 + 0.509 Dmeter	13.6*	26.4

* denotes significant difference ($p<0.05$)

6.3.3 Assessment of treatment means

6.3.3.1 Comparison of fungi exposure treatments

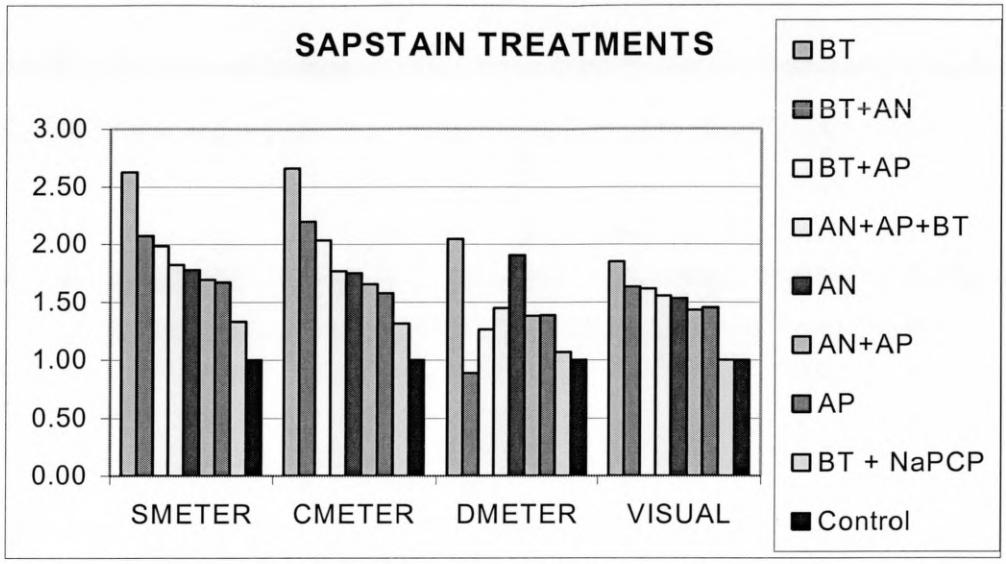
Table 6-3 shows that the mean darkness percent values for *B. theobromae* exposed samples and the control untreated samples were respectively the highest (darkest) and lowest (lightest). Based on percent coverage of stain, the visual method was unable to distinguish differences between the control NaPCP and the unstained rubberwood samples. All other quantitative methods could detect the difference (see also Figure 6-2).

Table 6-3. Mean darkness (%) of rubberwood samples exposed to various fungi treatment regime by the four methods of assessment, spectrophotometry (Smeter), colorimetry (Cmeter), densitometry (Dmeter) and visual method of assessment.

Fungi treatment[1]	Smeter	Cmeter	Dmeter	Visual
BT	56.5 (2.63)	55.1 (2.66)	68.04 (2.05)	81.33 (1.85)
BT + AN	44.6 (2.07)	45.47 (2.20)	29.51 (0.89)	71.67 (1.63)
BT + AP	42.67 (1.98)	42.14 (2.04)	41.91 (1.26)	71.00 (1.61)
AN + AP + BT	39.13 (1.82)	36.51 (1.76)	48.13 (1.45)	68.33 (1.55)
AN	38.24 (1.78)	36.16 (1.75)	63.33 (1.91)	67.33 (1.53)
AN + AP	36.45 (1.70)	34.26 (1.66)	45.81 (1.38)	63.00 (1.43)
AP	35.88 (1.67)	32.61 (1.58)	46.05 (1.39)	64.00 (1.45)
BT + NaPCP	28.57 (1.33)	27.21 (1.31)	35.4 (1.06)	44.00 (1.00)
Control	21.5 (1.00)	20.7 (1.00)	33.24 (1.00)	44.00 (1.00)

Note: [1] AP = *A. pullulans,* AN = *A. niger* and BT = *B. theobromae*
[2] Numbers in parenthesis denote values normalised to control value for respective method.

Figure 6-3 shows the normalised overall mean for each sapstain treatment derived from Table 6-3. All methods were in agreement that rubberwood samples exposed to *B. theobromae* were the most darkly stained. Samples assessed by spectophotometry and colorimetry showed significant differences ($p<0.05$) between the unstained samples and samples treated with 2% NaPCP + BT, however the differences were not detectable by visual assessment. The mean normalized values for densitometer were different when compared with values obtained by the other three methods. BT+AN, was rated the lighter (0.89) and AN (1.91) which was rated darker by densitometer as compared to the other three methods.



Note: BT = *B. theobromae*, AP = *A. pullulans* and AN = *A. niger*

Figure 6-2. Normalised mean darkness for rubberwood clones GT1, PB217 and RRIM600 after the various fungi treatments using the spectrophotometer (S'meter), colorimeter (C'meter), densitometer (D'meter) and visual assessment.

The Hoefer GS300 densitometer was designed for scanning protein after electrophoresis separations. The optical system was therefore designed to maximise the responses to the blue and the black dyes commonly used as protein stains. It is capable of scanning under transmitance (transparent medium) or reflectance (opaque medium) mode. Transmittance is generally used for scanning gels and films. Reflectance is used for scanning paper chromatograms. This instrument however was shown to have a specific disadvantage when used to scan the surfaces of wood samples, in that it is calibrated for a fixed height of the light source and detector from the base. Since the rubberwood samples were placed on the fixed horizontal base of the densitometer, the distance of the wood surface from the light source, and thus the amount of light reflected, was affected by the variation in sample thickness (Figure 6.3). The random variation in sample thickness resulted in inaccurate reading. This may explain the differences between the densitometer values and those from the other systems.

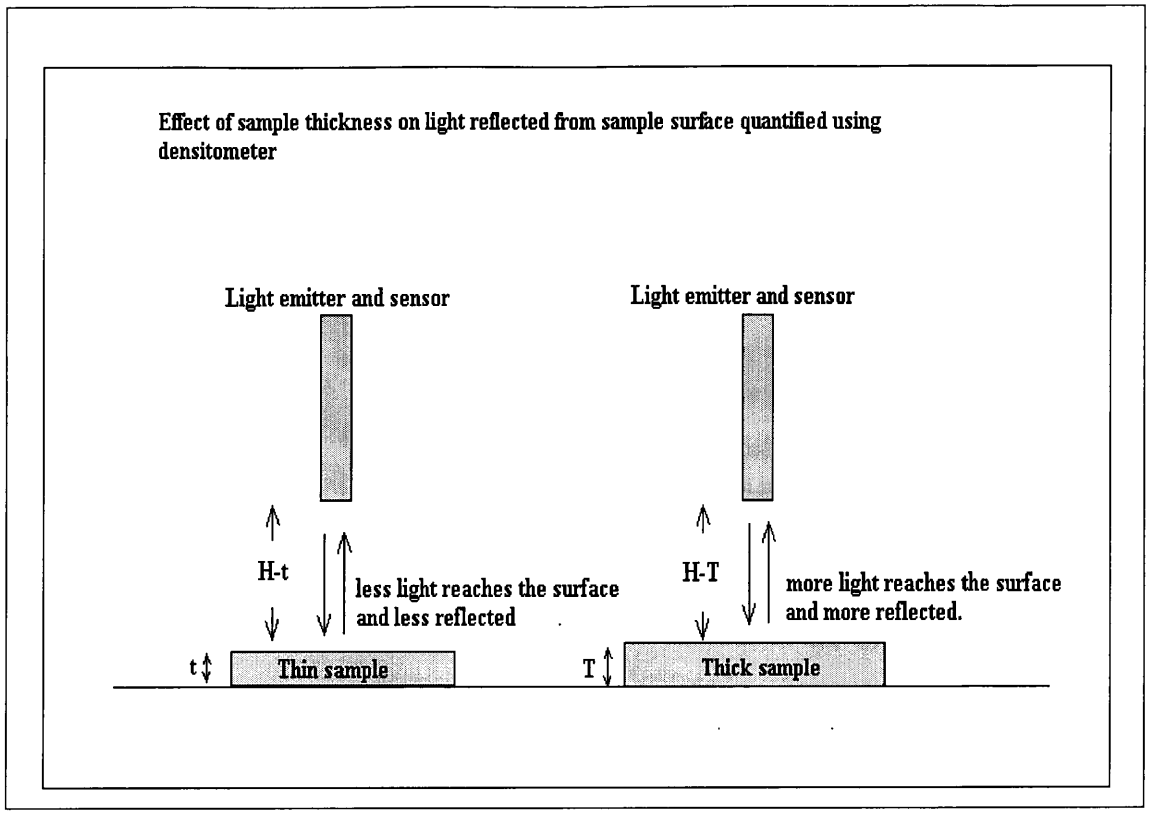


Figure 6-3. The effects of sample thickness on reflectance measurements of wood samples using densitometer.

6.3.3.2 Comparison of the sapstain assessments of fungi exposure treatments by clone

Since the densitometer showed the poorest coefficient of determination (Table 6-2), the remaining chapter will only discuss the detailed differences for the remaining three methods. Figure 6-4 (Clone GT1), Figure 6-5 (Clone PB217) and Figure 6-6 (Clone RRIM600) show the mean normalised value for each treatment as determined by the three methods of assessments, the spectrophotometer, colorimeter and the visual method of assessment. The data shown in this Figure suggests that after normalisation the ranking by visual method of sapstain assessment correlates better to the ranking by colorimetry measurements. This is confirmed by the analysis shown in Table 6-3. To assist the comparison, the results of the three quantitative methods were regressed with the results of visual assessment.

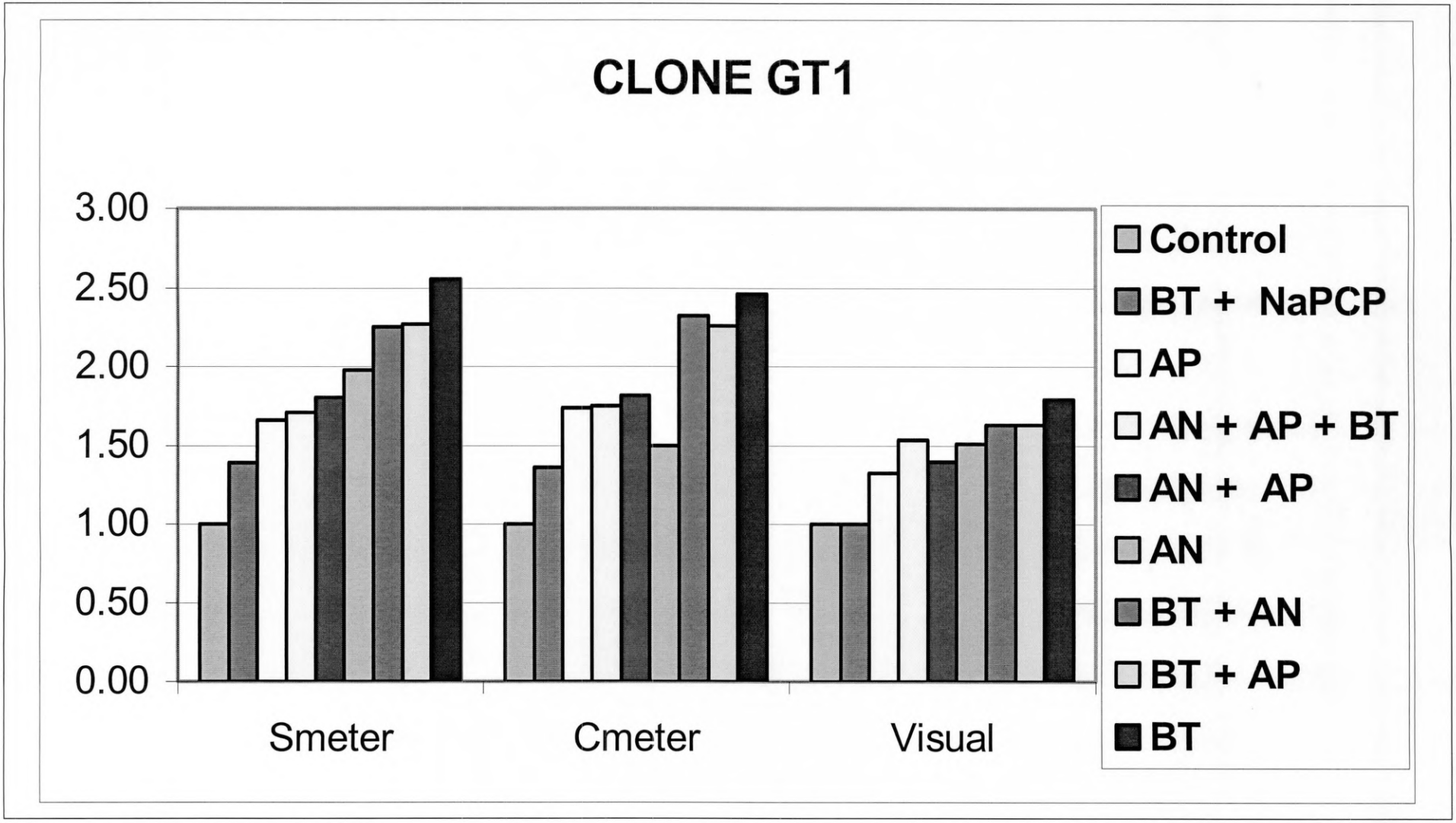


Figure 6-4. Mean normalized darkness value for clone GT1 after the various fungi treatments, assessed using the spectrophotometer (Smeter), colorimeter (Cmeter) and visual methods.

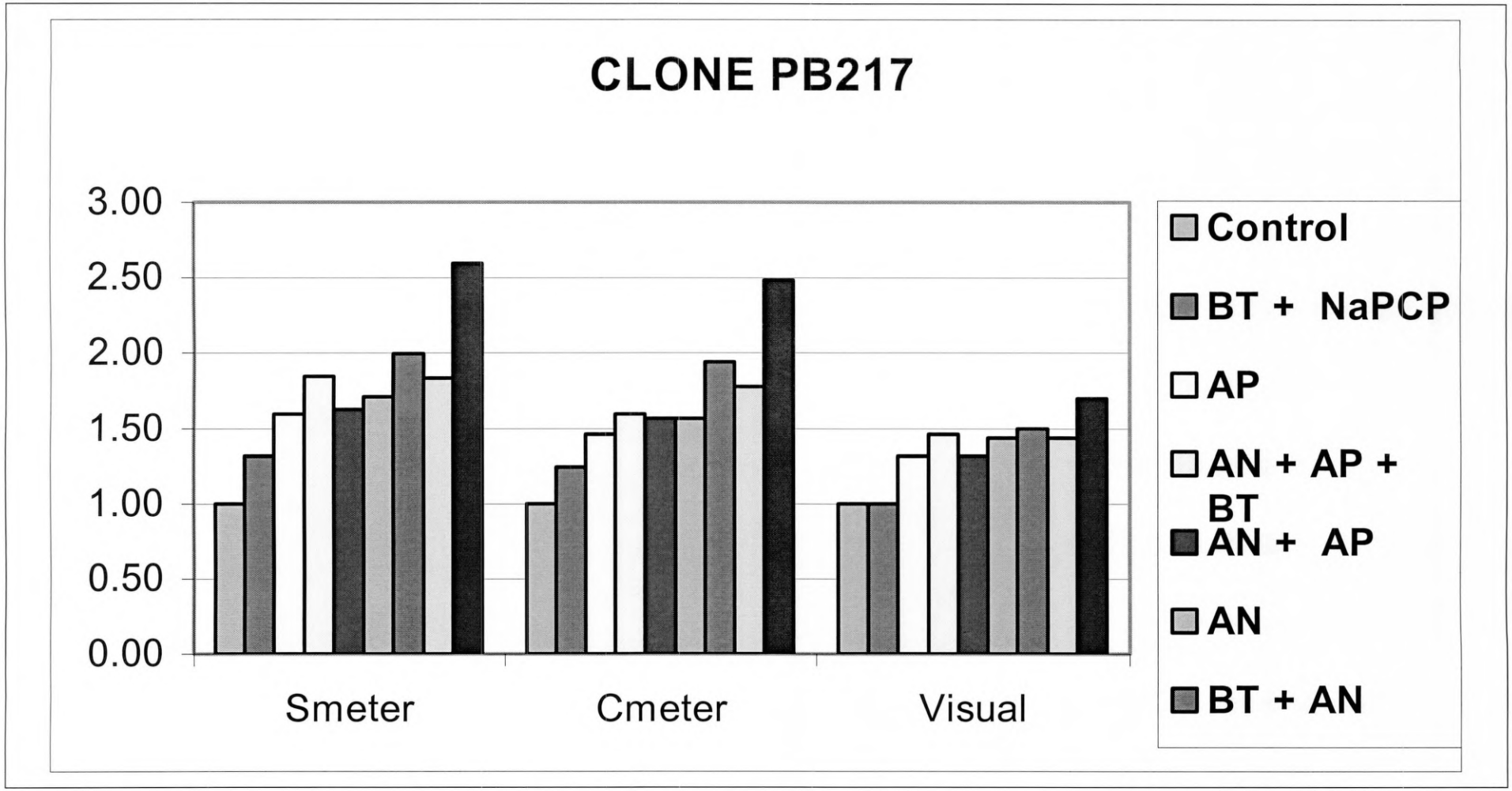


Figure 6-5. Mean normalized darkness value clone PB217 after the various fungi treatments, assessed using the spectrophotometer (Smeter), colorimeter (Cmeter) and visual methods.

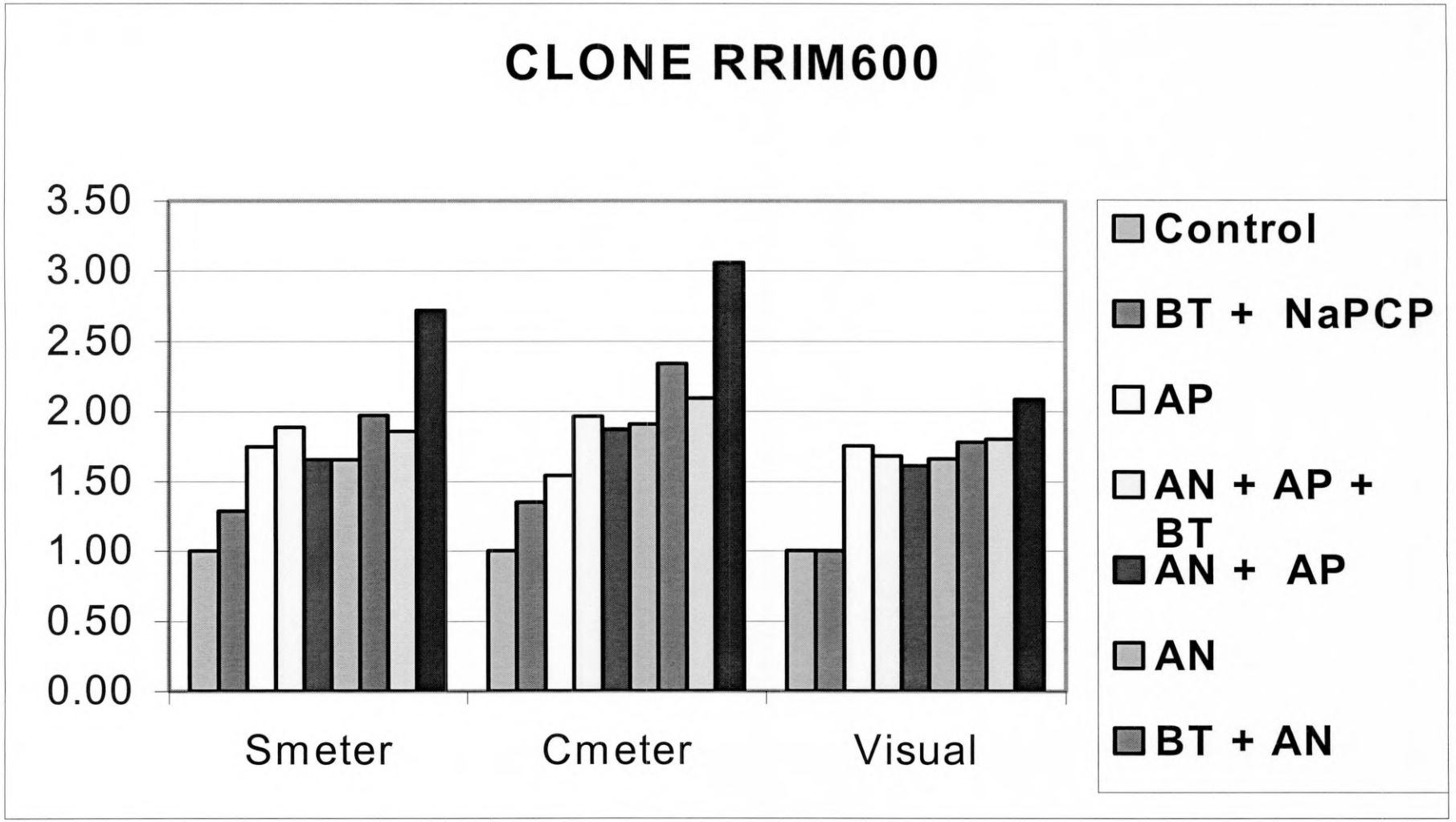


Figure 6-6. Mean normalized darkness value for clone RRIM600 after the various fungi treatments, assessed using the spectrophotometer (Smeter), colorimeter (Cmeter) and visual methods.

Another series of 12 regression equations as shown in Table 6-2, were derived from the normalized raw data to predict the qualitative value of normalized visual assessments via least square analysis. Similar to Table 6-2, Table 6-4 shows that all equations are highly significant ($p<0.01$). Again based on the R^2 values darkness evaluation using colorimetry (83.6%) shows the best predictive estimates compared to the other two quantitative methods of sapstain assessments. Similarly the colorimetry method showed the best correlation and also for overall prediction of darkness (R^2 greater than 81%). The poorest correlation were estimations using densitometry (R^2 less than 42%). There were differences in the R^2 values however, both the original and the normalized data analysis came to the same conclusions.

Table 6-4. Regression equations derived for predicting normalized visual assessments rating (V_n) from the three quantitative methods of assessment spectrophotometry ($Smeter_n$), colorimetry ($Cmeter_n$), and densitometry ($Dmeter_n$) data.

Equation No,		CLONE	Regression equation	F-ratio	R^2 (%)
Cmeter	1	All clones	$V_n = 0.520 + 0.859\ Cmeter_n$	606.7**	83.6
	2	GT1	$V_n = 0.518 + 0.812 Cmeter_n$	171.2**	81.4
	3	PB217	$V_n = 0.456 + 0.922\ Cmeter_n$	257.9**	87.3
	4	RRIM600	$V_n = 0.579 + 0.847\ Cmeter_n$	286.1**	88.0
Smeter	5	All clones	$V_n = 0.048 + 0.560\ Smeter_n$	214.0**	64.2
	6	GT1	$V_n = 0.918 + 0.516\ Smeter_n$	63.8**	61.7
	7	PB217	$V_n = 0.870 + 0.631\ Smeter_n$	86.2**	68.6
	8	RRIM600	$V_n = 0.970 + 0.574\ Smeter_n$	104.0**	72.5
Dmeter	9	All clones	$V_n = 1.399 + 0.416\ Dmeter_n$	53.9**	31.3
	10	GT1	$V_n = 1.112 + 0.518\ Dmeter_n$	29.2**	41.9
	11	PB217	$V_n = 1.176 + 0.576\ Dmeter_n$	17.5**	29.7
	12	RRIM600	$V_n = 1.645 + 0.312\ Dmeter_n$	13.6**	24.4

Note: ** denotes highly significant difference ($p<0.01$)

6.3.4 Previous attempts to quantitatively assess sapstain

Objective ratings for sapstain have been reported in several studies. Bjurman (1989) used ergosterol measurements to evaluate the degree of fungal mould and stain. Ergosterol is a prominent membrane component of most eumycotic fungi which has been frequently used as a fungal-index molecule in natural substrates. Although ergosterol levels were shown to correlate to the fungal biomass, which is useful in studies of fungal activity in wood decay, they did not necessarily relate to the degree of discoloration (Sexton *et al.,* 1993). Ergosterol levels also fluctuate with time and can decrease even after the wood has been stained as observed in the findings of Encinas and Daniel (1999). Therefore ergosterol is not suitable as a quantitative measure of sapstained samples. Wazny *et al.* (1989) and Grant (1973) used changes in the reflectance of the wood to directly measure discoloration. Reflectance has been used successfully to detect defects, including blue stain, in Douglas fir veneer (Maristany *et al.,* 1992). Sexton *et al.,* (1993) explored the use of image analysis to assess the extent of fungal discoloration and found that the results obtained were comparable to those produced by human evaluators. This method however requires high technical skill as compared to the methods described in the present study. The colorimetry quantitative method described in the present study is simple and rapid and could easily be extended to field application.

6.4 CONCLUSION

The quantitative measurement of effects 0.5 mm below the surface of sapstain-exposed rubberwood, was able to assess, in detail, the susceptibility of rubberwood clones to sapstain fungi. There is a statistically justifiable case for using the colorimeter to investigate the sapstaining activity and the cosmetic degrade caused by dominant sapstain fungi such as *B. theobromae.* The results obtained by this method could account for more than 81% of the variation made by visual assessment and the method is free of human bias.

CHAPTER 7

7. BIOLOGICAL CONTROL OF SAPSTAIN CAUSED BY *BOTRYODIPLODIA THEOBROMAE.*

7.1 INTRODUCTION

The problem of sapstain in rubberwood in tropical Malaysia could be overcome if it were logistically possible to dry timber within 24 hours at felling or sawing. Once the moisture content declines below that required for fungal growth the rubberwood is safe from discoloration unless it is wetted. However approximately 76% of the felling and sawing of rubberwood trees is in remote areas therefore delays are often unavoidable due to problems of logistics. Under these circumstances chemical treatments are used as temporary protection before the sawn timber can be properly stacked for air-drying or kiln drying.

Recent awareness of the industry and the general public of the environmental impacts of chemicals used as wood preservatives have led to the development and use of chemicals of lower impact and also to increase research into biological control as a non-chemical method of wood preservation. One area in which such bioprotection may have potential application is in the prevention of fungal stains. Biological protection is an appropriate alternative in this case since the period of temporary protection required (before drying) is relatively short (about 4-6 weeks) compared to the period of protection required for wood decay. This short-term protection would be delivered by surface coating with the biological control agent.

Freshly felled timbers do not contain high inocula of fungal microorganism so biological control agent can readily establish in the wood. Generally the wood will only require protection for a reasonably short period until the wood is further processed or dried. The only constraint is that the biological control organism itself must not stain the wood. Most of the current research focused on softwood and very limited research has been undertaken on hardwoods including rubberwood.

As primary colonisers of freshly felled lumber, sapstain fungi are ideal targets for bioprotection. Screening of fungi as potential biological control agents for wood sapstain and decay and study involved in antagonism have often used artificial media using agar plate systems, though some researchers used wood wafer screening systems for this purpose. (Freitag and Morrell, 1990). Agar based systems provide a fast, simple, cost effective method for screening. The major disadvantage is that they do not accurately reflect the nutritional status of the wood. Rubberwood has, as shown in Chapter 4, a C:N ratio ranging from 400:1 to 900:1. By contrast a common medium for the growth of fungi, such as malt extract agar has a much lower C:N ratio (50:1) (King *et al.*, 1981). Another common flaw of artificial media is that evaluation of potential biological control agents has often involved the use of mycelial inoculum rather than the spores. Spores are the primary source of infection leading to sapstain on wood. In this present study the spores were separated from the mycelium by filtering through cotton gauze as described in Section 2.4.

Results presented in Chapter 5 indicated that there is some control by primary fungi, as illustrated by the reduced amount of sapstain by a mixture of *A. niger* and *A. pullulans*. The experiments described in this chapter continue to explore the biological control activity of other primary fungi individually and in combinations to see whether there is any potential for long terms application. The study also examined differences in control of sapstaining activity of *B. theobromae* in the three clones. The specific objectives were to examine:

i. the staining on wood of potential biological control systems

ii. the potential of spores for inoculating wood in field applications

iii. the usage of media available for spore storage prior to field application and

iv. any possible synergistic effects between test fungi in their ability to inhibit target fungi.

7.2 MATERIALS AND METHODS

Tests on biological control activity were carried out on a usage of selected primary mould fungi. 14 fungi were tested on agar-based tests and six fungi were tested

on wood blocks. Sixteen combinations (Figure 7-3) of these fungal inocula were used as the treatment culture regimes comprising of single inoculum, dual and tri-culture mixed inocula. Control blocks were either treated with 2% NaPCP, then inoculated with *B. theobromae* or blocks untreated and not inoculated. The sapstain exposure test protocols were as described in Chapter 2, Section 2.4.

7.3 RESULTS

7.3.1 Agar screening of potential biological control fungi

7.3.1.1 **Test A.** *Paired plugs on MEA (Sensitivity Test)*

Table 7-1 shows the results of the sensitivity test on MEA agar. After 4 weeks it was found that 5 of the test fungi in TEST A showed positive biological control activity. These were *T. viridae*, *Trichoderma* spp., *Trychosporon* spp., *A. niger* and *A. oryzae*. After 3 weeks all the plates of the sensitivity test for *T. viridae*, *Trichoderma* spp., *Trychosporon* spp., *A. niger* and *A. oryzae*. were covered with these test fungi and were stained black. However out of these five isolates *T. viridae* showed a zone of clear yellowish agar after 2 weeks as shown in Plate 7-1.

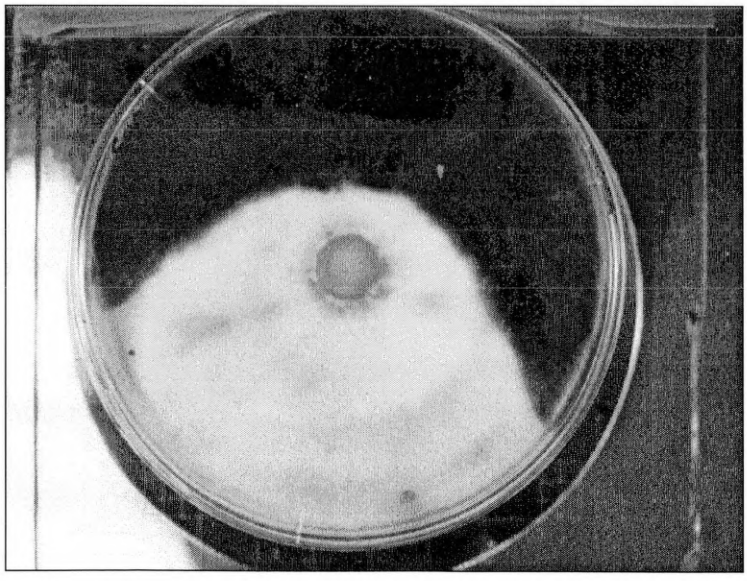


Plate 7-1. Plate showing zone of reduced staining of *B. theobromae* for test fungi *Trichoderma* spp. (F06).

7.3.1.2 Diameter growth criteria (Test B, Test C, Test D, Test E, Test F and Test G)

Further screening of the test fungi were carried out using the agar plug test B, Test C, Test D, Test E, Test F and Test G as described in Section 2.3.1.

Table 7-1. Summary of results on performance of various test fungi (TF) against target fungi *B. theobromae* (BT).

	TEST FUNGI	Days to cover petri-dish On MEA	Days to cover petri-dish On PDA	A. PAIRED TF & BT plugs	B. TF (spores in MEB) (BT plug)	C BT (spores in MEB) (TF plug)	D. TF (spore in H_2O) (BT plug)	E. BT (spore in H_2O) (TF plug)	F. BT seeded PDA (TF plug)	G BT seeded MEA (TF plug)
F02	*Paecilomyces variotii*	nt	nt	nt	+ (2.40)	± (4.20)	nt	nt	nt	nt
F03	*T. reesei*	4	4	++	± (5.37)	++ (8.50)	++ (1.90)	± (4.07)	± (4.47)	++ (8.50)
F04	*T. viridae.*	4	4	++	++ (1.60)	++ (8.50)	++ (1.60)	++ (8.50)	± (4.57)	++ (8.50)
F06	*Trichoderma* spp.	4	4	- -	++ (1.77)	-- (2.33)	++ (1.77)	- (2.43)	± (4.85)	- - (0.23)
F11	*Unknown*	nt	nt	- -	± (3.83)	-- (1.43)	+ (2.30)	- - (1.33)	± (5.15)	- (2.30)
F13	*Trychosporon* spp.	5	7	++	++ (1.57)	-- (1.90)	+ (2.07)	- - (1.67)	± (4.47)	- (2.80)
F14	*A. niger*	6	5	++	+ (2.00)	-- (1.93)	± (4.10)	- - (1.50)	++ (8.17)	-- (0.14)
F15	*Unknown*	nt	nt	- -	± (3.50)	± (3.60)	+ (2.63)	-- (1.73)	± (4.50)	-- (0.19)
F16	*Unknown*	nt	nt	- -	+ (2.60)	- (1.50)	± (3.50)	-- (1.43)	-- (3.20)	± (5.27)
F17	*P. chrysogenum*	nt	nt	- -	± (3.40)	± (4.00)	nt	nt	nt	nt
F18	*A. oryzae*	5	7	++	+ (2.40)	± (3.20)	nt	nt	± (3.5)	- (0.19)
F30	*Fusarium monoliforme*	6	5	- -	± (3.33)	± (4.90)	+ (2.27)	± (4.70)	++ (8.50)	nt
F31	*Aspergillus flavus*	5	8	- -	++ (1.47)	-- (1.70)	+ (2.43	-- (1.53)	± (5.37)	- (0.17)
F32	*Aspergillus fumigatus*	5	5	- -	+ (2.39)	++ (8.50)	± (3.40)	-- (1.03)	-- (3.00)	± (5.97)
F33	*Mucor* spp.	5	5	- -	± (3.97)	-- (2.00)	± (3.97)	-- (1.53)	± (3.93)	- (0.20)
BT	*B. theobromae*	4	3							

Note: Numbers in parenthesis denotes perpendicular diameter growth (average of 3 plates). nt – not tested.

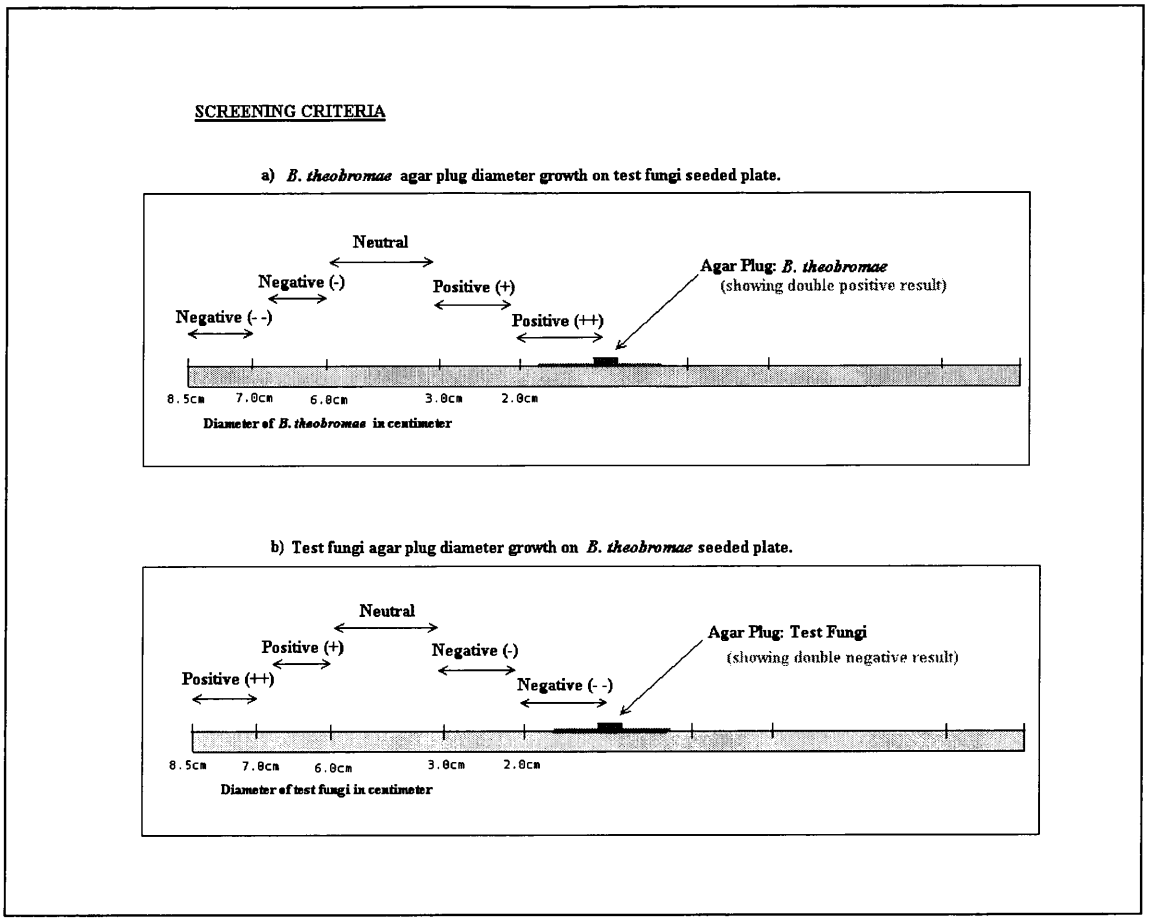


Figure 7-1. Screening criteria based on diameter growth of test plug.

The data shown in Table 7-1 indicate that the most positive biocontrol results organism was *T. viridae*, which showed six double positive test results. Only in Test F, where the score was neutral (diameter growth of *T. viridae* test plug is about 4.8 cm) showed that there was no biological control seen. The next best result was with *T. reesei* with 4 double positives, *A. niger* with 2 double positive and one positive and *Trychosporon* spp. both with 2 double positive. *T. viridae* and *T. reesei* were selected to be tested on wood. Data for *Penicillium* spp., *A. niger* and *A. oryzae* tested on wood during preliminary testing were included for comparative purposes.

7.3.1.3 Comparing tests B, C, D and E.

The survival of spores in different types of media, viz. malt extract broth (MEB) and distilled water was evaluated in tests B to E. Fungal spores were incubated in

MEB or distilled water in tests B and D respectively and BT spores incubated in MEB or distilled water in tests C and E respectively.

For 4 test fungi, *T. viridae*, *Trichoderma* spp. (F06), *Trychosporon* spp. and *A. falvus* each was more successful against *B. thoebromae* after incubation in MEB. However for 3 fungi, *T. reesei, T. viridae* and *Trichoderma* spp. (F06) each was more successful against *B. thoebromae* after incubation in distilled water. When *B. theobromae* spores were incubated in distilled water (Test E) only *T. viridae* showed double positive biological control. However, when *B. thoebromae* spores were incubated in MEB (Test C) two test fungi, *T. reesei* and *A. fumigatus,* were more successful and each showed 'double positive' biological control results.

Table 7-1 also shows that in test F isolate F14 (*A. niger)* and F30 (*Fusarium monoliformae*) had the largest diameter when seeded on to target fungus *B. theobromae* on PDA. By contrast *T. viridae* showed neither positive nor negative biological control as shown in Plate 7-2. However in test G when placed on malt extract agar seeded with *B. theobromae, T. viridae* showed the maximum growth as shown in Plate 7-3.

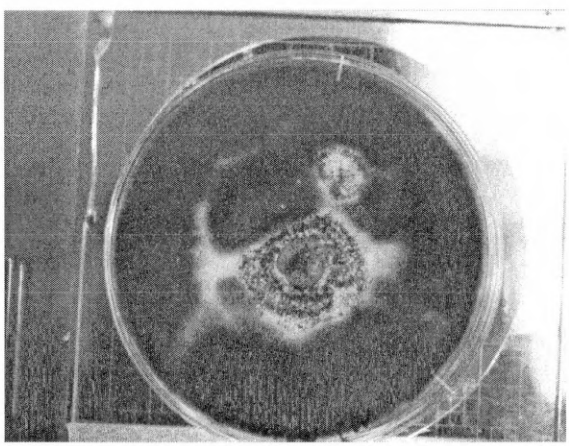


Plate 7-2. Photo showing the outcome of interaction between *B. theobromae* and *Trichoderma reesei* on potato dextrose agar after 7 days.

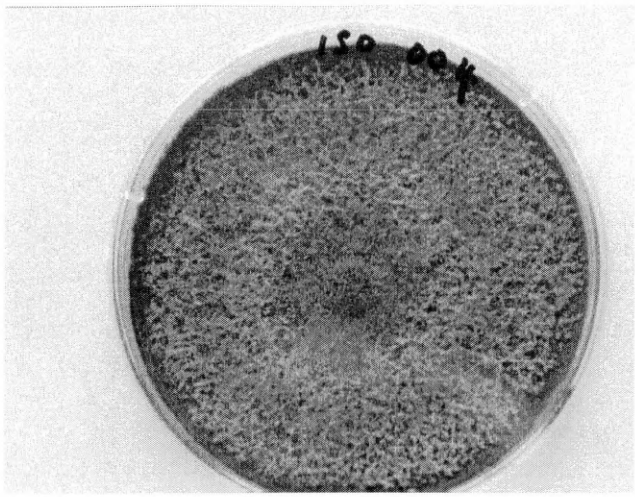


Plate 7-3. Diameter of test fungi (plug) of *T. viridae* as observed on malt extract agar (MEA) seeded with *B. theobromae* spores after 7 days.

7.3.2 Sapstain exposure tests of rubberwood samples.

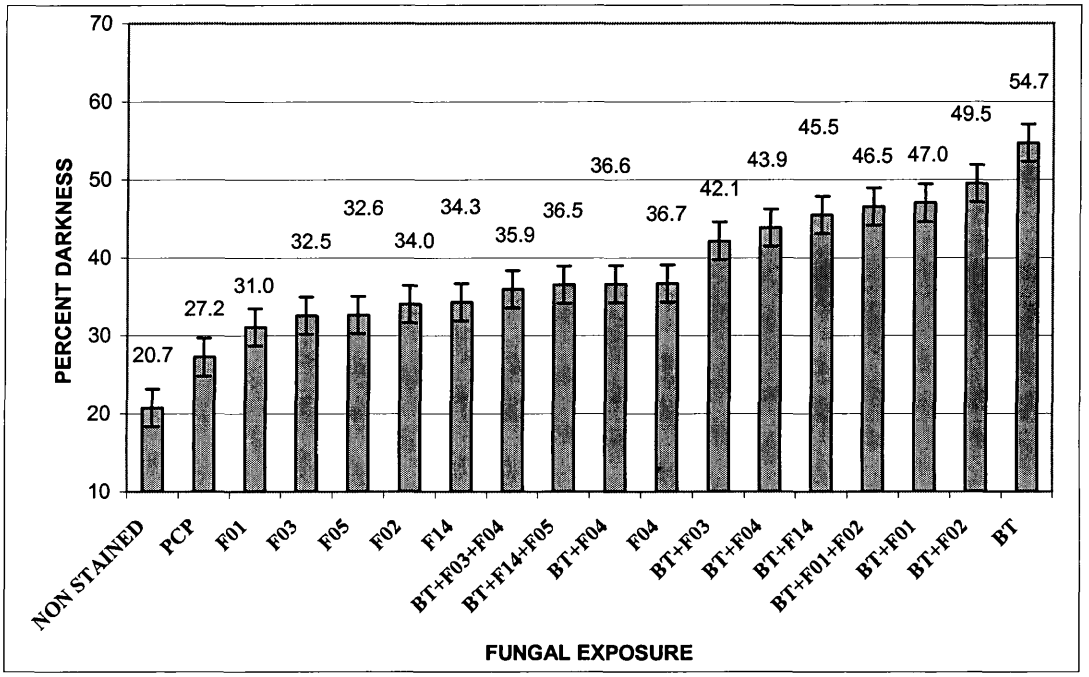
The results of darkness measurements on rubberwood samples, using the FRIM sapstain exposure protocol, are presented in graphical form in the following section to help visualise differences between treatments and clones. In cases where significant differences ($p<0.05$) exist between treatments and between clones the results are further illustrated with a) photographs to illustrate the different degrees of darkness observed and b) scanning electron micrograph (SEM) images to illustrate the condition of the starch grains after fungal treatment, general characteristics of the fungal hyphae, fungal interaction with presence of *B. theobromae* and the mode of growth and colonisation inside the wood (Section 7.3.3).

7.3.2.1 Overall effect of fungal treatments

The overall mean percent darkness of the 16 fungal treatment regimes and 2 selected controls with all rubberwood samples pooled across clones are presented in Figure 7-2 in ascending order of darkness. The darkest sample is the rubberwood sample exposed *to B. theobromae* (54.7%) fungi for 4 weeks. The mean for the control unstained rubberwood sample is at the other extreme (20.7%). The percent darkness of the other samples treated with other fungal treatment regimes are within

these values. These sapstain-exposed samples can be arbitrarily grouped into 3 categories, a) more than 40% darkness, b) between 35-40% darkness and, c) less than 35% darkness.

Figure 7-2 shows that 7 fungi treatments attained mean percent darkness significantly higher ($p<0.05$) than 40% (first category). These are rubberwood samples treated with BT, (BT+ *P. variotii*), (BT+ *Penicillium* spp. + *P. variotii*), (BT + *Penicillium* spp.), (BT + *A. niger*), (BT + *T. reesei*) and (BT + *A. pullulans*). All these treatments contained spores of *B. theobromae*. All treatments in this category showed significantly darker ($p<0.05$) stain that the other two categories indicating insignificant ($p<0.05$) biological control activity.



Legend: F01 (*Penicillium* spp.), F02 (*P. variotii*), F03 (*T. reesei*), F04 (*T. viridae*), F05 (*A. pullulans*) F14 (*A. niger)*, and BT *(B. theobromae).*

Figure 7-2. Mean darkness of fungal treatments pooled across clones of rubberwood after 4 weeks exposure to various fungal treatment regimes.

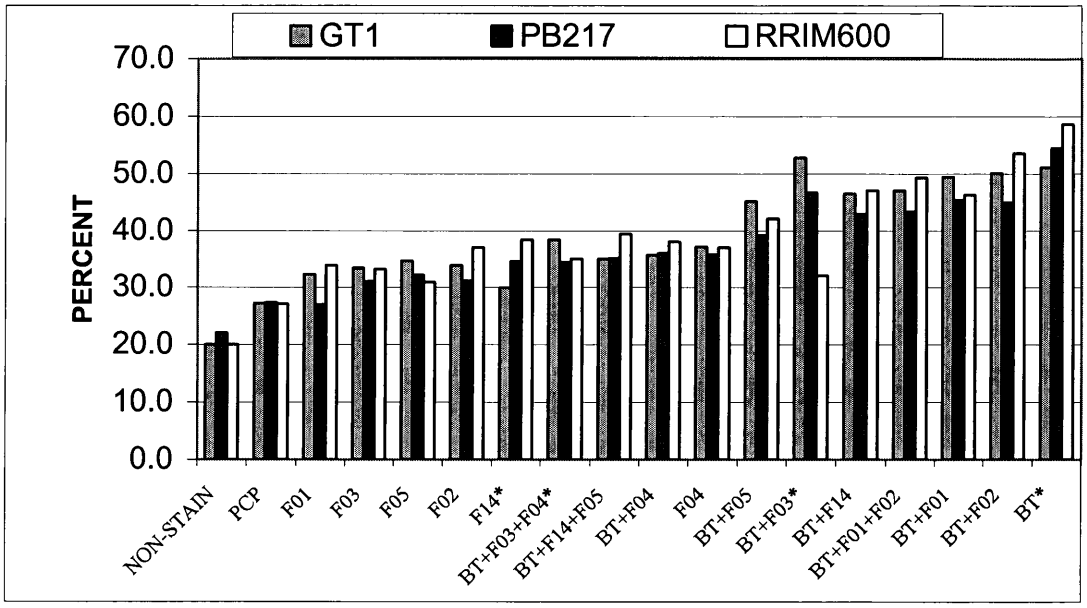
A second group of treatments attained mean percent darkness between 35 - 40%. There were four treatments in this group. Only one treatment *T. viridae* (F04) was a single fungus treatment without the staining fungi *B. theobromae*. The three other treatments were treatments of BT+ *T. viridae* (36.6%), BT + *A. niger* + *A. pullulans*

(36.5%) and BT + *T. reesei* + *T. viridae* (35.9%). These three treatments showed significantly lower ($p<0.05$) darkness than the BT treated sample. All the three treatments however were significantly different ($p<0.05$) from the PCP control.

All treatments in the third category (less than 35% darkness) are single fungi treatments. However only one treatment in the third category was not significantly different ($p<0.05$) from control samples treated with NaPCP + *B. theobromae* (PCP). That was rubberwood samples exposed to *Penicillium* spp (F01). This shows that *Penicillium* species does not significantly ($p>0.05$) stain the wood below the mycelium after minimal sanding. The other fungi *P. variotii* (F02), *T. ressei* (F03), *A. pullulans* (F05), *and A. niger* (F14) were significantly darker ($p<0.05$) than the control samples treated with NaPCP + *B. theobromae* (PCP).

7.3.2.2 Treatment effects on clones

The means for each treatment by clone is shown in Figure 7-3. The analysis of variance (ANOVA) to test differences between the rubberwood clones for each individual treatment is listed in Table 7-2. The table shows that only four treatments i.e. (BT + *T. reesei* (F03)) , (BT + *T. reesei* (F03) + *T. viridae* (F04)), *A. niger* (F14), and the control BT showed significant difference ($p<0.05$) between clones. This will be examined in detail with illustrations in the discussion section.



Legend: **F01 (*Penicillium* spp.), F02 (*P. variotii*), F03 (*T. reesei*), F04 (*T. viridae*), F05 (*A. pullulans*) F14 (*A. niger*), and BT (*B. theobromae*). * denotes significant differences between clones.**

Figure 7-3. Mean darkness of clones GT1, PB217 and RRIM600 after 4 weeks exposure to various fungal treatment regimes and minimal surface sanding.

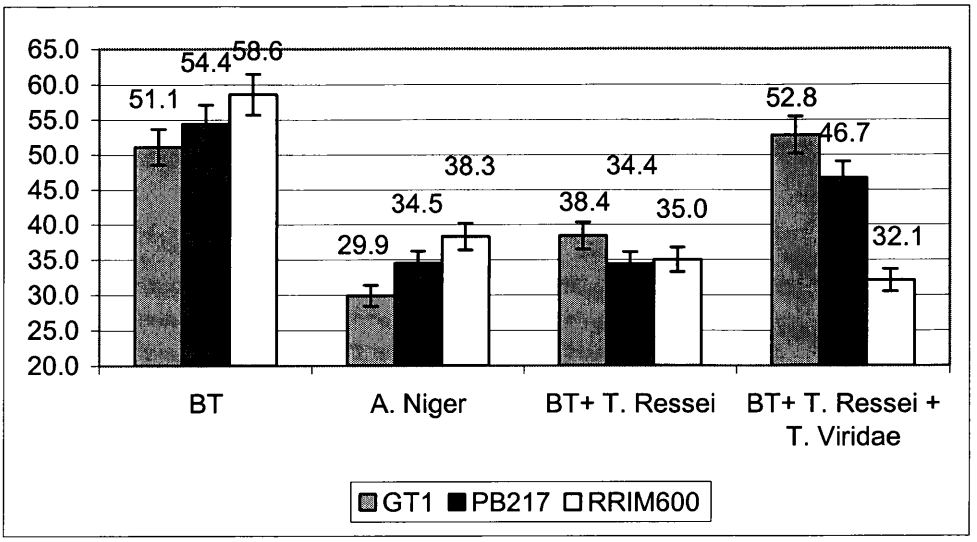
Table 7-2. Summary of the Analysis of variance (ANOVA) of the fungal treatment regimes on clones GT1, PB217 and RRIM600.

Source of variation	df	F01	F02	F03	F04	F05	F14	BT	BT + F01	BT + F02	BT + F03	BT + F04	BT + F05	BT + F14	BT + F01 + F02	BT + F14 + F05	BT + F03 + F04
Mean square clone	2	65.017 ns	41.979 ns	8.593 ns	2.825 ns	18.598 ns	88.097 **	216.422 **	23.162 ns	97.026 ns	567.744 **	8.073 ns	45.241 ns	26.681 ns	44.716 ns	32.014 ns	23.208 *
Mean square error	12	32.934	27.546	17.325	14.958	6.994	10.772	17.984	63.338	67.991	25.000	6.651	14.704	36.950	26.918	13.064	3.523

Note: F1 (*Penicillium* spp.), F2 (*P. variotii*), F03 (*T. reesei*), F04 (*T. viridae*), F05 (*A. pullulans*) and F14 (*A. niger)*

ns = non significance difference ($p>0.05$), * = significance difference ($0.1<p<0.05$) and ** = highly significance difference ($p<0.01$)

The four fungal treatments (*A. niger*, BT, BT + *T. ressei* and BT + *T. reesei* + *T. viridae*), which showed significant clonal variation, are presented in Figure 7-4



(derived from Figure 7-3) to display the mean values of individual treatments.

Figure 7-4. Mean darkness of clones GT1, PB217 and RRIM600 samples after exposure to *B. theobromae* (BT), *A.* niger (F14), BT + *T. reesei* (F03) and BT + *T. reesei* (F03) + *T. viridae* (F04).

For the first two fungal treatment means, i.e. *B. theobromae* and *A. niger* (single fungal inoculum) clone RRIM has significantly ($p<0.05$) the highest mean percent darkness. The mean percent darkness of RRIM600 wood samples when exposed to the sapstain fungus *B. thoebromae* (58.6%) were significantly ($p<0.05$) the darkest (Plate 7-4) and similarly when exposed to mould fungus *A. niger* (38.3%) as illustrated in Plate 7-5. Both these results show that the rubberwood clones RRIM600, PB217 and GT1 differ in susceptibility to staining when exposed to the two fungi *B. theobromae* and *A. niger*.

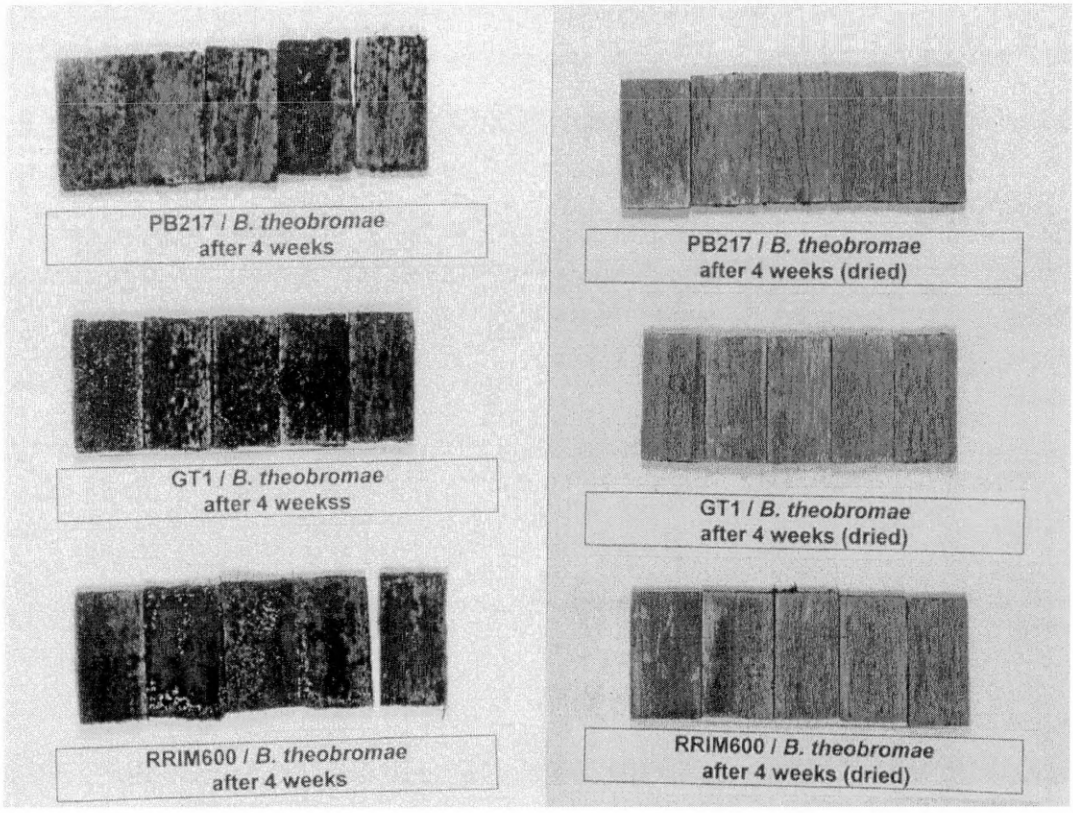


Plate 7-4. The relative darkness of clones GT1, PB217 and RRIM600 after 4 weeks exposure to *B. theobromae* (before and after sanding).

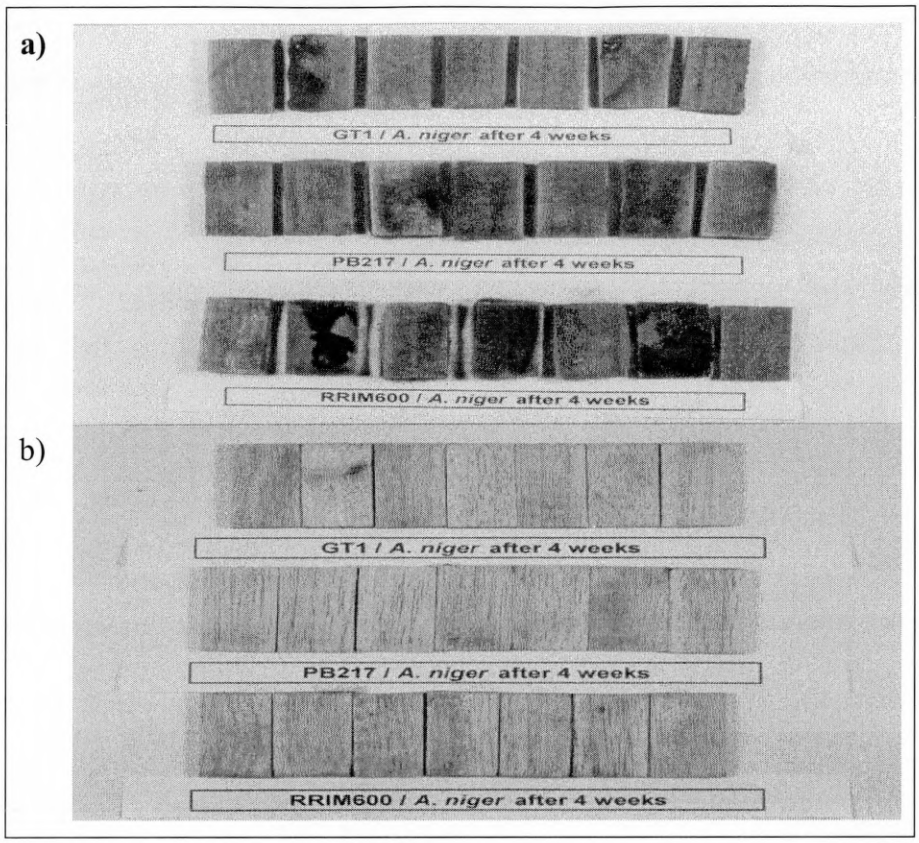


Plate 7-5. The relative darkness of clones GT1, PB217 and RRIM600 after 4 weeks exposure to *A. niger* a)before and b) after sanding.

The other two fungal treatments that showed clonal differences were BT+ *T. reesi* (F03) and BT+ *T. reesei* (F03) + *T. viridae* (F04). These treatments were designed to test the potential bicontrol fungi *T. ressei* against the sapstain fungi *B. thoebromae.* In both these two treatments GT1 was significantly ($p<0.05$) the darkest. The differences in darkness of the three clones are illustrated in Plate 7-6. Similarly for treatment BT+ *T. reesi* (F03) + *T. viridae* (F04), the mean darkness of GT1 of 38.4% was also significantly higher ($p<0.05$) than the other two clones (Plate 7-7). This results illustrates that biological control is least active in clone GT1.

Comparison of Plate 7-6 and Plate 7-7 confirmed that addition of both *T. viridae* and *T. reesei* resulted in further reduction in staining on the rubberwood samples. This illustrates the synergism of the two control fungi *T. reesi* and *T. viridae*.

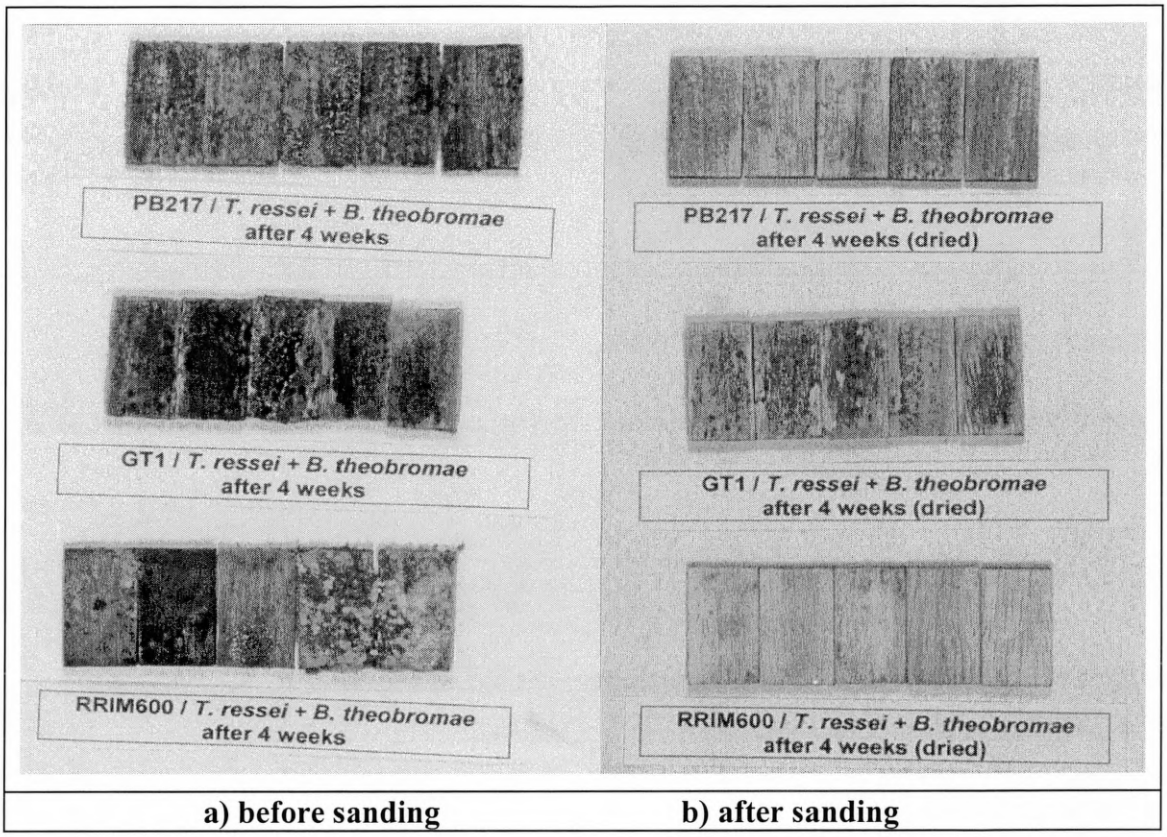


Plate 7-6. The relative darkness of clones GT1, PB217 and RRIM600 after 4 weeks exposure to *B. theobromae* and *T. viridae* (before and after sanding).

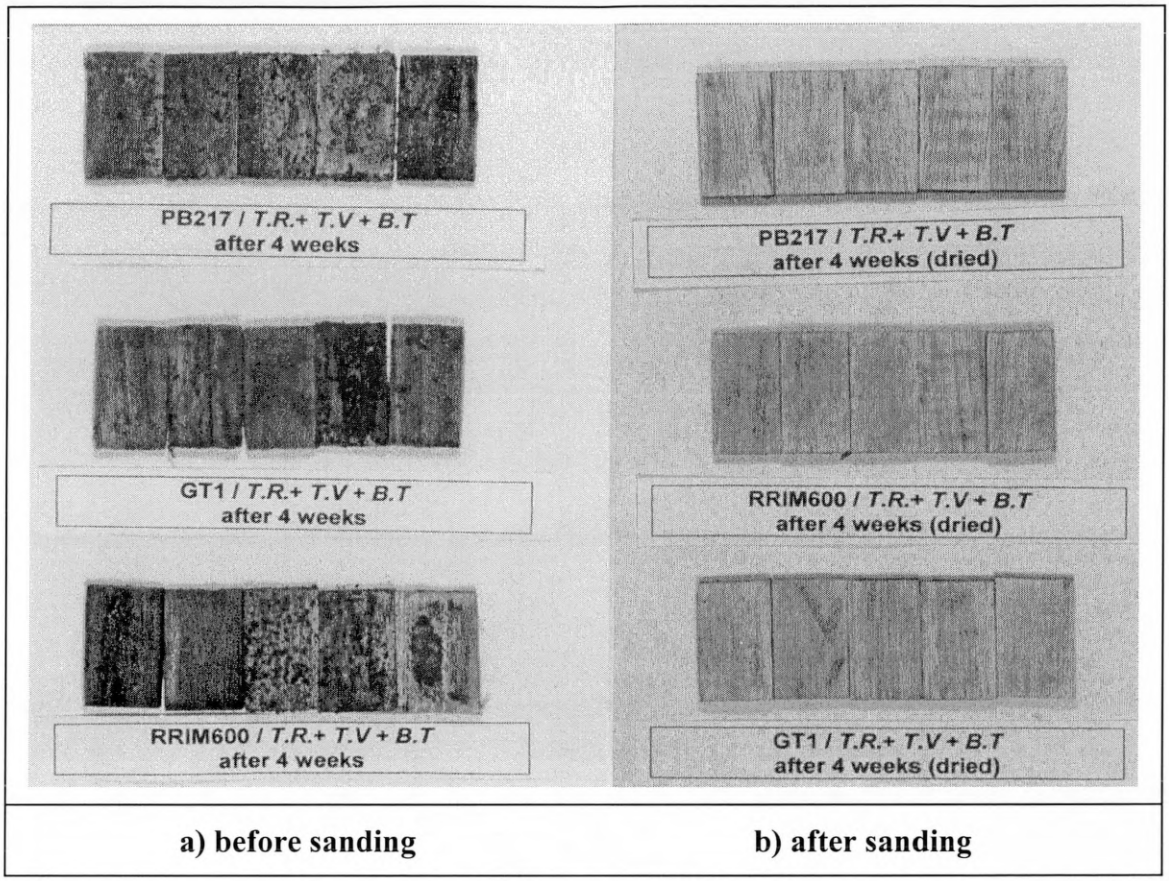


Plate 7-7. The relative darkness of clones GT1, PB217 and RRIM600 after 4 weeks exposure to *B. theobromae, T. reesei (F03)* and *T. viridae (F04)* (before and after sanding).

7.3.3 SEM examination of unstained and sapstained rubberwood samples

Rubberwood samples exposed to *B. theobromae* (BT), *A. niger* (F14), BT + *A. niger*, BT + *T. viridae* and *T. viridae* were further examined by SEM to examine a) the general characteristics of the fungi b) the mode of colonisation of the wood samples by the fungal hyphae c) interaction of the control and sapstaining fungi and d) the effect of 4 weeks fungal treatment on starch grains.

Plate 7-8 shows the view of the vessel of GT1 treated with *B. theobromae.* The *B. theobromae* hyphae are about 3-5 μm in diameter. Hyphae are also present in the parenchyma cells (H).

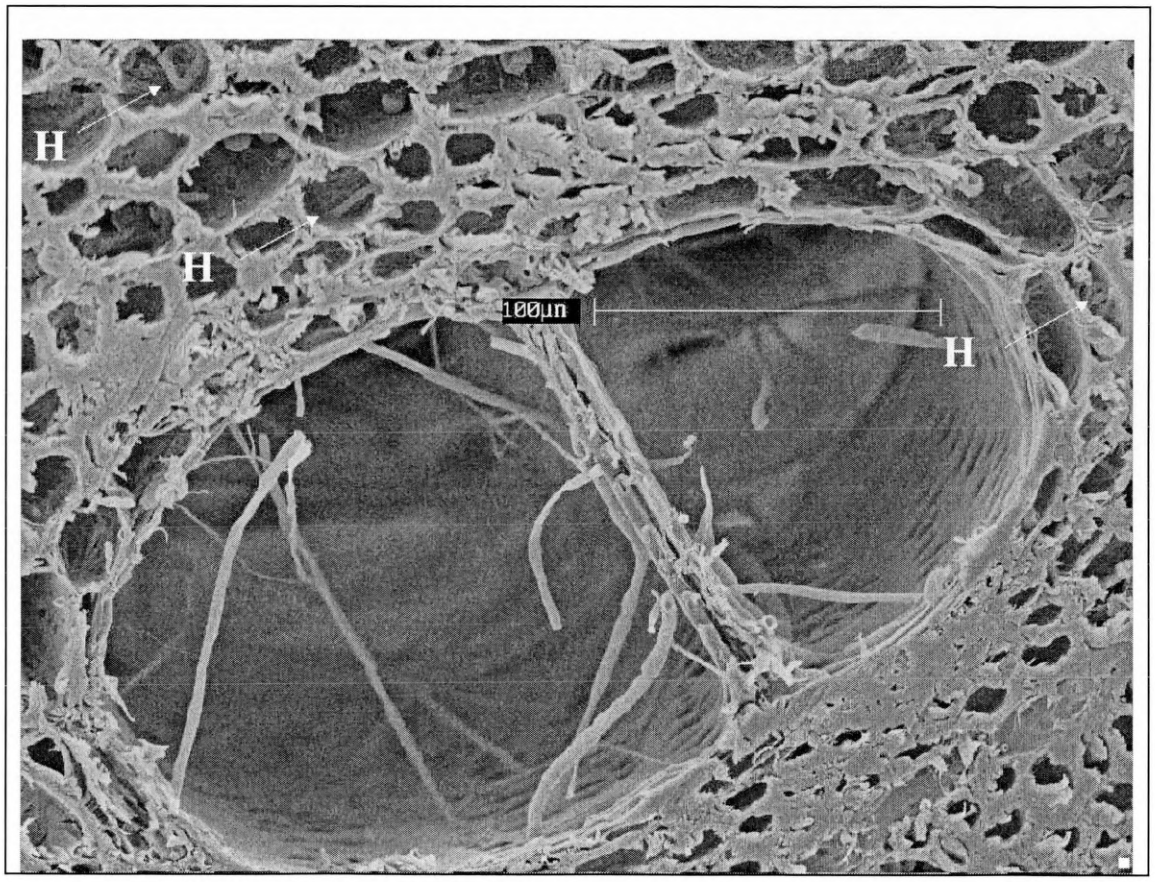


Plate 7-8. SEM image showing the hyphae of *B. theobromae* in vessel pores of clone GT1.

Plate 7-9 shows the view inside the vessel of clone GT1 exposed to sapstain treatments of BT + *A. niger*. The diameter of *A. niger* hyphae is about 1 μm in diameter. The hyphae of *A. niger* are thin and form a thick mass of mycelium below the pit membrane layer and the cell wall layer. There is noticeable absence of the thicker hyphae of *B. theobromae.*

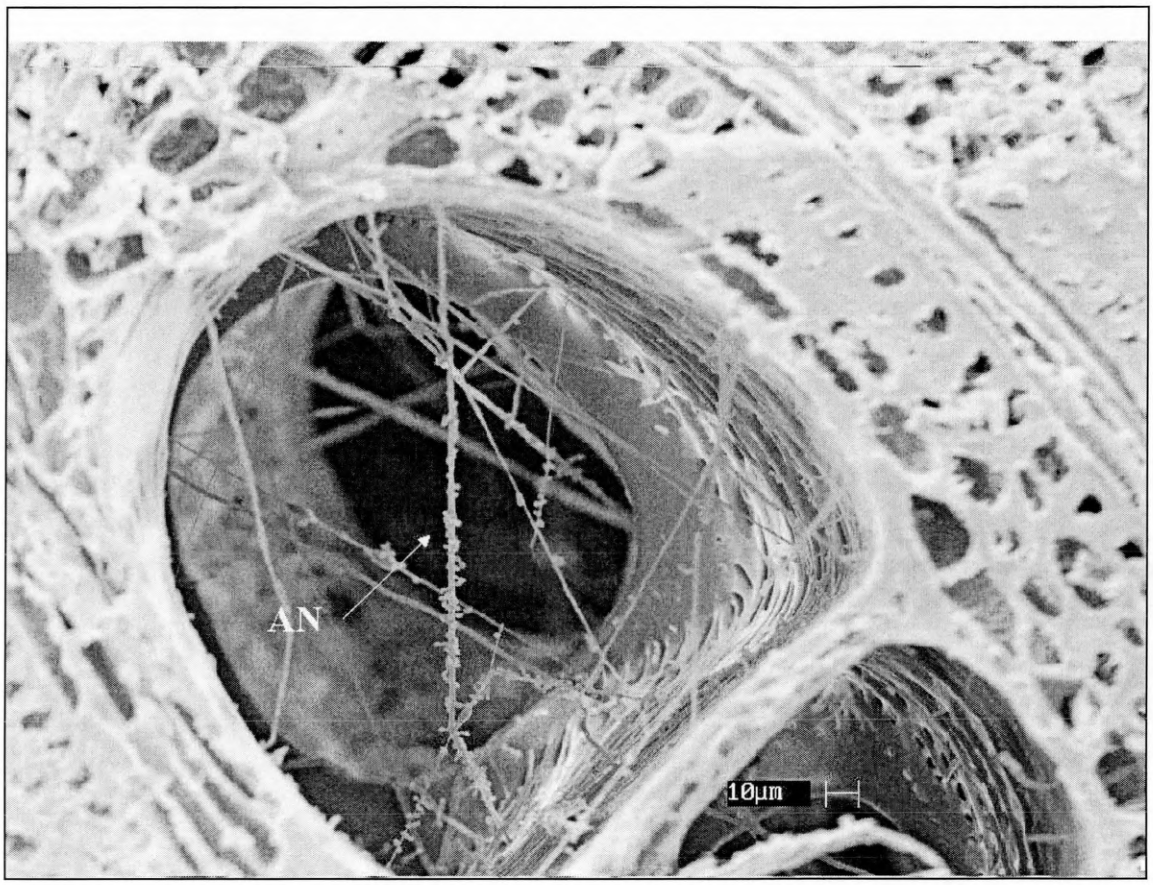


Plate 7-9. SEM image showing fine hyphae of *A. niger* (AN) in a sample of clone PB217 exposed to sapstain fungi *A. niger*

Plate 7-10 shows a closer view of the hyphae of *B. theobromae* and *A. niger*. Note the difference in diameter. It also shows the passive penetration of *A. niger* through the pit opening of the vessel wall and that not all hyphae of *B. theobromae* or *A. niger* cling to the wall of the vessel. The contraction on certain portions of the hyphae could be the results of drying of cytoplasm or the mechanism used to increase cytoplasmic pressure at the tip of the hyphae for the extra pressure required in penetrating the pit membrane. It was also observed that there was no sign of serious degradation of cell walls.

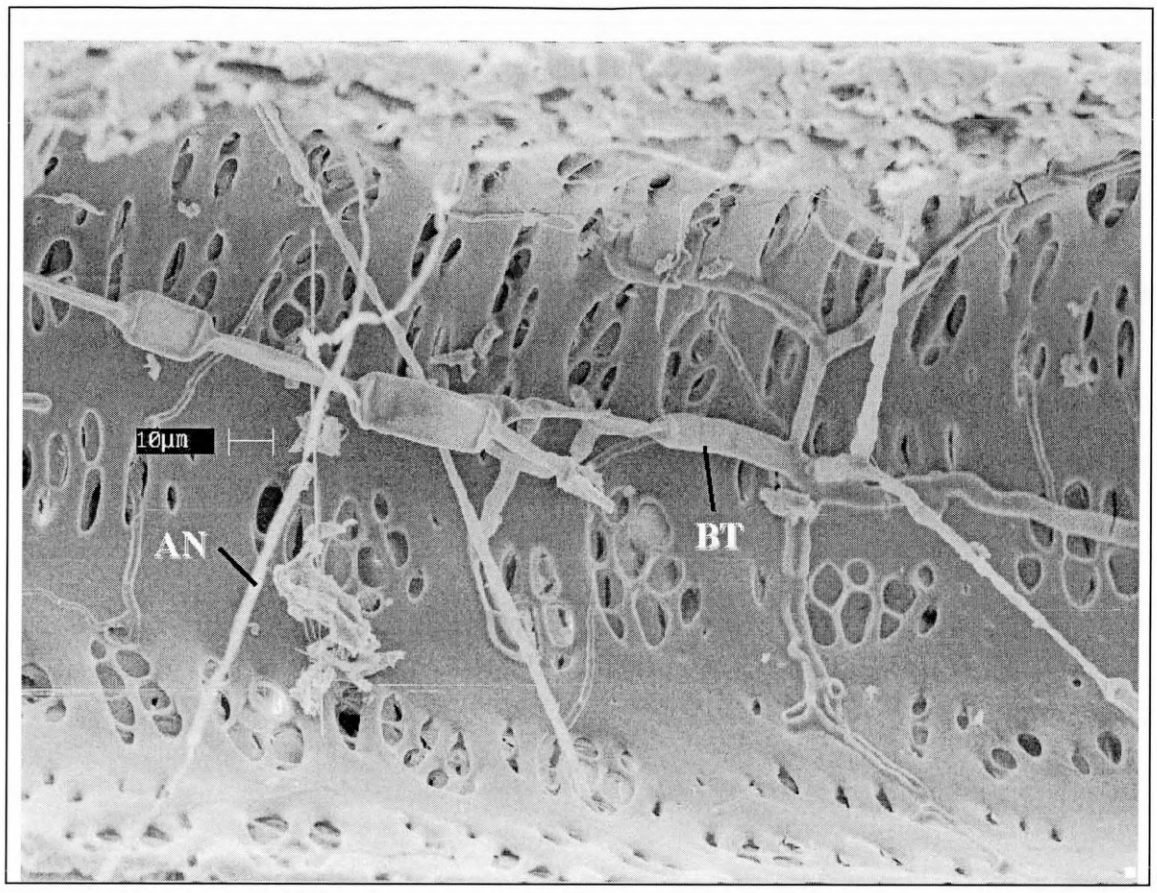


Plate 7-10. SEM image showing hyphae of *B. theobromae* (BT) and *A. niger* (AN) inside the vessel of clone GT1 treated with BT + *A. niger*.

Plate 7-11 shows that the hyphae of both *T. viridae* and *B. theobromae* were found inside the vessels. The hyphae of *B. theobromae* are relatively larger (4-5 µm in diameter) while hyphae of *T. viridae* are about half this size (1.5 – 2.0 µm in diameter) . The SEM image also shows that the pit openings on the vessel wall are between 3-8 µm. It also shows that both the hyphae *of B. theobromae* grow via the pit openings of the vessel walls. The diameter of the hyphae is smaller at the point of entry into the pit. This plate also shows budding of the hyphae as it appears on the pit opening from the vessel/parenchyma cells below (B).

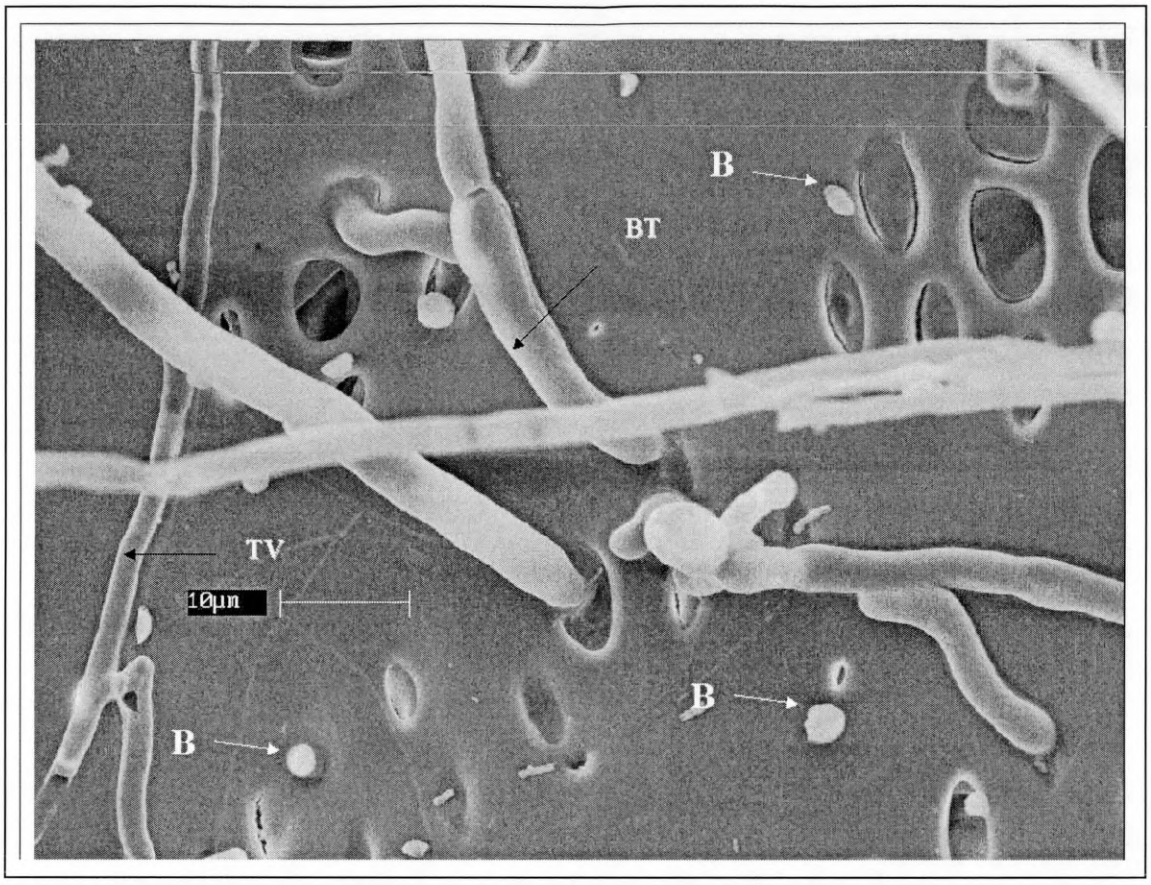
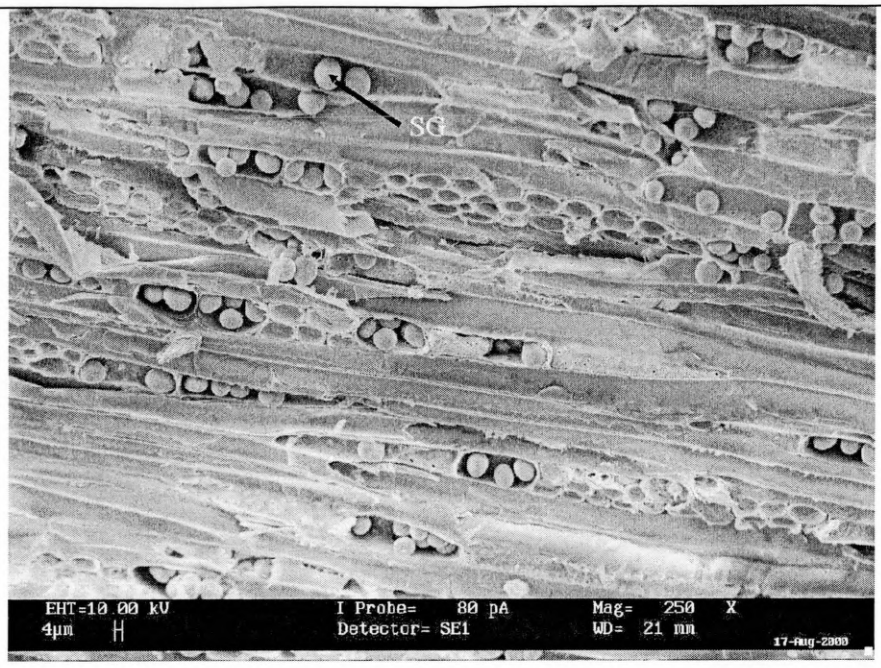
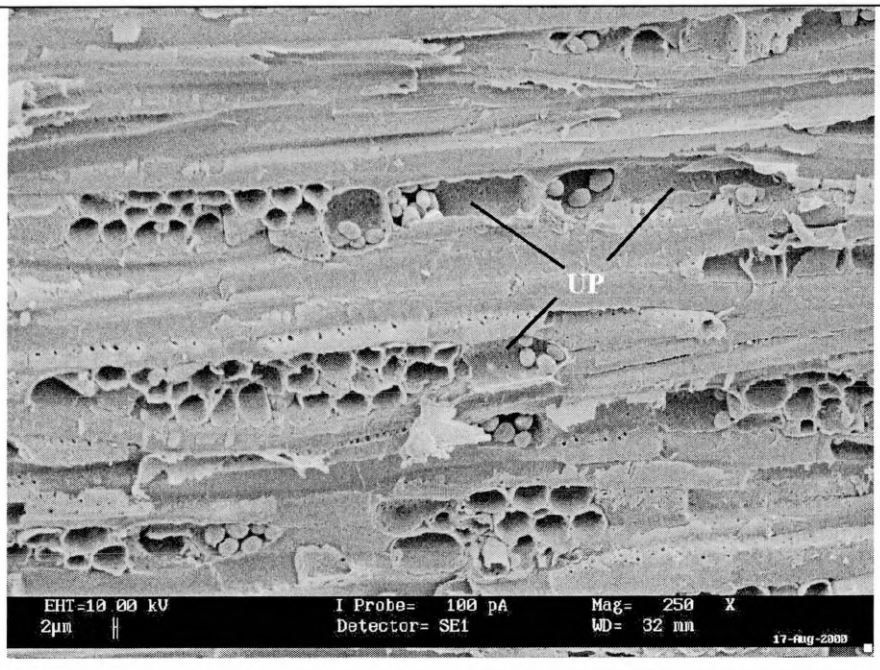


Plate 7-11. SEM image showing hyphae of both *T. viridae* (TV) and *B. theobromae* (BT) as observed in *B. theobromae* + *T. viridae* treated PB217 samples.

Observation of tangential sections untreated or treated with *B. theobromae* showed a reduction in starch grain numbers. However no attempt was made to quantify this effect. Plate 7-12 shows a typical view of the SEM images of tangential section of the rubberwood clone GT1 a) untreated and b) those treated with *B. theobromae* after 4 weeks showing reduced starch grains in the upright parenchyma cells.



a) Untreated sample of GT1



b) GT1 treated with *B. theobromae*

Plate 7-12. SEM image of tangential section of rubberwood clone GT1 showing the starch granules (SG) and almost empty upright parenchyma cells.

7.4 DISCUSSION

Rubberwood is rich in total available sugars with variable amounts of fructose, glucose, sucrose and starch in the selected clones. Kreber and Morrell (1993) observed that competition for nutrients and space, the territory that a fungus occupies and the area surrounding them are the dominant mode of action in bioprotection using bacterial and fungal bioprotectants tested against target fungal stains in pondorosa pine sapwood. This observation is sensible because the amount of nutrients available in the surrounding territory will be modified and changed depending on the type and species of organism. Although Kreber and Morrell (1993) tested with fungal isolates of *Gliocladium aurifilum, G. virens, Penicillium. thomii, Saccharomyces cerevisiae* and *Trichoderma harzianum,* similar observations of competition for nutrients and territory was observed in the present study using a different set of fungi. This section will discuss the mode of competition of the target fungus *B. theobromae* and the biocontrol test fungi as observed on agar-based systems and in the later section (Section 7.4.1.7) on the wood samples.

7.4.1.1 Comparison of wood-based and agar-based systems

Basically screening test on agar media is a relatively more rapid test of competition for nutrients and territory when compared to screening test on wood wafers. The purpose of agar-based screening requires about 7 days incubation as compared to 30 days when wood wafers were used. Although it allows more test fungi to be tested in the same period it has disadvantages. Agar cannot represent the a) exact microenvironment in wood, and b) the composition of available nutrients in wood.

The initial screening test involved placing two organisms on the same agar medium and observing their growth during a period of incubation. Under this standard condition their behaviour and interaction were influenced by the growth medium. In order to draw useful conclusions from the screening process Benko and Higlhey (1990a, 1990b) suggested that the medium must be selected so as to permit equal opportunity for the growth of the potential biocontrol fungi and the target fungi. Benko and Higlhey (1990a, 1990b) suggested that the media should be selected on

the basis of their ability to a) support both target and control organisms, and b) promote production of secondary metabolites by potential bioprotectant. Presumably the main objective was to expose both the potential biocontrol fungi and the target fungi under the best possible nutrient environment and to test the growth rate on the basis of equal competition for nutrient and territory. Results of the present study showed that screening of the potential biocontrol test fungi on malt agar (Section 7.3.1.2) resulted in *Trichoderma* spp., *T. viridae* , *Trychosporon* spp., *Aspergillus niger* and *A. oryzae* showing biological control activity. While on potato dextrose agar only *Aspergillus niger* and *Fusarium monoliforme* showed potential biological control activity. When tested on wood (Section 7.3.2.2) only spores of *T. viridae* (percent darkness 36.6%), spore mixture of *A. niger* + *Aureobasidium. pullulans* (36.5%) and spore mixture of *Trichoderma reesei* + *T. viridae* (35.9%) showed lower percentage darkness and positive biological control activity.

This simplistic general conclusion of Benko and Highley (1990a, 1990b) was based solely on tests with limited combinations of nutrient levels in agar-based system. Nutrient levels and distribution in agar cannot simulate the true nature and composition of nutrients in wood. In wood the distribution of nutrients is more complex. In rubberwood as used in the present study the starch and presumably sugars are mainly found in the parenchyma cells. Thus the nutrients in wood are not as evenly distributed as in the agar. Access to nutrients in wood is therefore not as simple as in agar medium. Due to the concentration of starch and nutrients in the parenchyma cells, access to these nutrients requires active extension of hyphae through intercellular pathways that is through pit openings and in some cases require penetration of the pit membrane.

The present study therefore focused on the relative accessibility of nutrients in the rubberwood samples relative to the agar nutrient. The objective of which was to test the biocontrol fungi and the target fungi similar to the conditions as found in the real wood substrate. Although the focus is on relative accessibility of nutrients in the wood the objective of the present study is not totally in agreement with Benko & Highley (1990a, 1990b). Both the recommended PDA sporulation agar (more

suitable for the screening of bacteria) and MEA (more suitable for screening of fungi) as tested in the present study were to provide two extreme nutrient environments for territorial competition in the process of initial screening. In the present agar based screening tests none of the screening exceeded 7 days. Apart from the advantage of the short screening period, the agar-based tests provide a state of competition with even distribution of nutrient both in term of amount and accessibility to the potential biocontrol and the target fungi *B. theobromae* unlike in actual wood samples. However, being aware of the heterogeneous nature of wood and its anatomical structure, the agar-based tests must not replace the required testing on small wood substrate in order to evaluate a more realistic environment for wood protection, as demonstrated by Payne and Bruce (1999).

The secondary screening on actual wood samples is mandatory because the level of nutrients in wood is very complex and almost impossible to reproduce in the agar-based systems as demonstrated in recent study by Payne and Bruce (1999). Payne and Bruce (1999) screened bacteria and yeast and *Trichoderma* isolates for antagonism towards stain and mould fungi on agar media and wood (Scots pine). In the study two types of low nutrient media prepared to simulate nutrient contents of Scots pine sapwood showed good inhibition but the two bacteria which gave the best biological control on the agar system did not behave in the same way as on wood. This study by Payne and Bruce (1999) concluded that effort to imitate level of nutrients in wood is not straightforward as the composition of carbon and nitrogen sources in wood is complex. Nevertheless Payne and Bruce (1999) demonstrated that the use standard malt extract agar provided the best screening condition for both the test and the target fungi as compared to the agar with the simulated nutrients levels of wood.

The sapstain tests on rubberwood samples illustrate the true competition between the potential biocontrol fungi and *B. theobromae* in wood. Biocontrol fungi are not only competing for nutrients but also in locating the relatively rich nutrient resources stored in the parenchyma cells; as a result a varying degree of darkness was observed. As described in Chapter 5, the varying degree of darkness may be due to a) amount of pigment in the fungal mycelium, and b) any metabolites that the

staining agent produces. After 4 weeks incubation the fungi that survived and won in the competition for territory and nutrients will determine the relative reduction in percent darkness of the wood samples. Further tests could also be conducted using incubated spores of *B. theobromae* in distilled water as a good simulation of *B. theobromae* spores invasion of wet rubberwood surfaces, and the darkness percentage measured accordingly.

The incubation of *B. theobromae* spores in distilled water before inoculation is a good simulation of the spread of *B. theobromae* spores invasion on very wet rubberwood. The results of the present agar-based screening indicated that when potential biocontrol test fungi were tested as agar plugs, the biocontrol fungi (*Trichoderma* spp., *T. reesei* and *T. viridae*) were found to control sporulation of the target fungi (*B. theobromae*). This is possibly due to biocontrol fungal mycelium being placed as agar plug was at the most active stage of growth and thus only needed to extend its mycelium in order to colonise the malt agar, however the *B. theobromae* (inoculated as incubated spores) will require time to initiate spore germination and the extension of hyphae on the agar plate. Similar reasoning could be used to explain why out of the three potential control test fungi tested as agar plugs (Table 7-1) only *T. viridae* showed biological control activity, probably due to its spores taking less time to germinate and were able to compete with the mycelium of *B. theobromae.*

The results of four agar-based tests (E, G, C and D) indicated that *Trichoderma* spp. and *T. viridae* could be potential biocontrol fungi. This result was confirmed for *Trichoderma viridae* when it was finally tested on wood samples. Therefore these four screening tests correspond well with the results on rubberwood samples and these tests are suggested for future screening of potential biocontrol fungi against *B. theobromae.*

7.4.1.2 Comparison of clonal results

Kreber and Morrell (1993) observed the competition for nutrients and space in *Pinus pondorosa* sapwood and found that the territory that a fungus occupies and the area surrounding them are the dominant mode of action in bioprotection of

bacteria and fungal bioprotectants tested against fungal stain.

Fungal growth and metabolism as observed in this study are influenced by rubberwood clonal factors such as wood pH and the availability of micronutrients. The staining is due to the production of dark coloured mycelium that penetrates into the wood surface. The overall staining effect of the fungal mycelium that penetrates into the rubberwood will depend on the amount of pigments (in the hyphae of *B. theobromae* and/or the biocontrol fungi). The lower darkness rating on the rubberwood samples is probably due to the suppressed biomass of pigmented hyphae of *B. theobromae*. The mechanisms by which biocontrol agent most likely function in reducing sapstain as shown in the earlier sections (Chapter 5) are related to competition for nutrients and territory. Within a territory the test fungus could exert a dominant influence for example by antibiosis which will ultimately determine the degree of staining as shown by Croan and Highley (1995). Croan and Highley (1995) investigated *in vivo* test fungi *Bjerkandera* and *Talaromyces flavus* on existing growth of stain fungi *Ceratocystis coerulescens*. They also found that the combinations of the metabolites also killed the existing fungal growth and removed the sapstain after 2 months on Southern pine veneer.

Mori and Takahashi (1997) examined and characterized pigments found in *A. pullulans* and *Ceratocystis picea* melanin. Mori and Takahashi (1997) found that the fungal pigments were similar to those of synthetic melanin. The H/C ratio of fungal melanins was however higher than that of the synthetic melanin, suggesting that both *A. pullulans* and *C. picea* melanin have high amount of aliphatic structure. They also reported that radial growth of *A. pullulans* was slightly faster than *C. picea*. *A. pullulans* mycelium was pigmented in a day while *C. picea* was coloured only after 2 or 3 days. The mechanism of stain production was not examined in *B. theobromae* of the present study because it is more practical to quantify the outcome of all possible mechanisms and only report the final darkness value caused or controlled by the biocontrol fungi as established by White-McDougall *et al* (1998).

Clonal differences in degree of staining could be explained by the difference in composition of wood sugars and other available nutrients. The amount and type

of nutrients have been reported to affect the degree of staining in temperate wood. Lin and Morrell (1997) studied the influence of types and amount of carbon source on the degree of discoloration on *P. pondorosa* wood wafers (5x10x30mm). They reported that the average degree of stain was found lowest in chitin (32%) followed by glucose (67%), glycerol (79%) and xylose (80%) but the fungi were able to use the remaining carbon sources in the wood for colonisation. This was confirmed by greatest inhibition of stain in agar media supplemented with chitin. Lin and Morrell (1997) also disputed the idea that protease could inhibit and prevent sapstain as they did not observe a consistent relationship between protease and stain inhibition.

7.4.1.3 Biological control activity of selected fungi on the selected clones

The clonal differences in anatomical features and chemical nutrients do not only cause differences in degree of susceptibility to sapstain but also showed differences in biological control activity. These differences in biological control activity in the different clones are probably related to the amount of starch as explained in Chapter 6. The differences in these clones showed possible correlation of sugars and starch contents to sapstain susceptibility.

Successful biological control application of mutant strains has been reported for temperate timber. The principle of territorial competition was shown to be successful in the biological control of sapstain fungi *Ophiostoma piliforme* (White-McDougall *et al.*, 1998; Morrell and Dawson-Andoh, 1998). Similarly Croan (1996) reported that non-pigmented isolates of sapstain fungi *O. pilliera* SC-46 and *Ceratocystis adipose* SC56 aggressively attacked sapstain fungi. The growth of the non-pigmented isolates are similar to those of the wild sapstain fungi as characterised by fungal growth on the surface and penetration into the sapwood of southern yellow pine. Field test results showed that green southern pine log sections treated with living cells of non-pigmented isolates of *O. pilliera* SC-46 were protected from discoloration. The non-pigmented isolates prevented the growth of the wild species and did not discolour the wood.

Successes of sapstain control on temperate woods had been documented (White-McDougall *et al*, 1998; Morrell and Dawson-Andoh, 1998; Croan, 1996), however

to date no work has been reported on the use colourless mutant to control sapstain fungi in hardwoods. The results of the present study showed that the use of non-staining mould fungi to compete with *B. theobromae* in rubberwood (occupying the same ecological niche) would be of considerable potential. The screening of numerous other non-staining primary fungi should continue and at the same time should be made to explore and exploit the potentials of colourless mutant of *B. theobromae* as has shown to be successful for temperate wood.

7.4.1.4 Clonal variation introduced by standard breeding protocols

One factor that may affect competitive outcome as has been shown in the present study is the nutritional quality of the rubberwood substrate. *B. theobromae* and some other secondary sapstain fungi are probably very well adapted to the nutrients components in rubberwood. These organisms rely on their ability to utilise the nutrients stored mainly in the ray parenchyma cells. Other studies indicated that these compounds include lipids, proteins, carbohydrated and resin acids (Abraham and Brueil, 1993; Gao and Brueil, 1995). It was suggested that selection of nutrients that are preferentially used by the biocontrol agent might improve stain prevention (Morrell and Dawson-Andoh, 1998). Results of the present study indicated that this could be made possible through selection of clones with the desired level of starch or other nutrients to the advantage of the biocontrol fungi. There are advantages of clonal selection to enhance biological control because a) it is very difficult to supply the nutrients that are selectively used by the biocontrol agent in their natural form, b) in nutrient enhanced biological control there is great potential for the depletion of the added nutrients during incubation and prior to biological control application, and c) to supplement a nutrient immediately before biological control application is again not practical as it will incur additional handling and cost.

Intensive research by Malaysian Rubber Board (LGM) has been very successful in selecting fast growing clones with good latex yield as well as high timber yield. In the most recent LGM planting recommendation for latex and timber production (the RRIM2000 series) showed further improvement in timber yield ranging from 1.02 m^3/tree (RRIM908) to 2.79 m^3/tree (PB355) (Anon., 1998). With such track record of LGM there is great potential for selection based on new wood characteristics

such as resistance to sapstain and lower starch and sugar contents. The results of the present study therefore indicates the potential of screening the newly recommended RRIM2000 series rubberwood clones in term of sugars and starch contents, for potential clones that could be less susceptible to sapstain fungi. Timbers from these RRIM2000 series rubberwood clones introduced in the late 1990's would be available for chemical analysis and sapstain susceptibility screening in 10 -15 years time. In the interim initial screening studies of sapstain in these rubberwood clones at 5 - 10 years of age could commence shortly.

7.4.1.5 Effect of incorporating two control fungi.

Synergism, in the context of bioactivity against wood destroying or sapstaining organism is when the activity observed by combining two or more components is greater than what would be predicted from each component individual activity (Richer, 1987). This concept of synergism was used by Schultz and Nicholas (1995) to develop wood preservatives using a combination of biocides but at lower concentrations. Thus a similar concept can be used to explain synergism as observed in the present study.

The easiest way to show synergism in a biological control mixture is to test each biocontrol organism individually and then these results are compared to the result obtained when a mixture of the two biocontrol agents are used at the same time. In the present study a mixture of *T. viridae* and *T. reesei* together significantly ($p<0.05$) reduced the percent darkness and percent stain coverage caused by *B. theobromae* (Plate 7-5). Unlike the single biocontrol treatment with *T. reesei* described above, the two fungi together were more effective in all the rubberwood clones. *A* possible explanation is that *T. viridae* and *T reesi* utilised different sugars in rubberwood clones and mixing them together posed a greater competition for *B. theobromae*. When present in the same niche the two biocontrol organisms might react by producing antibiotics against each other which are also effective against the target fungi *B. theobromae*.

Another example of synergism is the simultaneous application of low levels of biocide followed by the control agent. Diluted levels of biocide or compounds such

as boron and fluoride have been explored for this purpose (Morrell and Dawson-Andoh, 1998). Fluoride markedly enhances the growth of *Trichoderma* spp. many of which can inhibit stain and decay. Trials with a number of commercially used anti-sapstain chemical and biocontrol organisms such as *Bacillus subtilis* suggest that fungal stain can be inhibited to a greater extent when combinations of biocide and biocontrol agent are employed (Monkowski *et al.*, 1997).

In view of the added advantage of synergistic effect of the use of more than one bicontrol fungi or simultaneous application of low levels of biocides, it is evident from the results of the present study that the biological control of stain fungi on freshly felled and sawn rubberwood require further research.

7.4.1.6 Interpreting *SEM images of B. theobromae*

Observation of the SEM images of tests samples treated with both test and target organisms in the present study showed that competition is an important mechanism for biological control at least in rubberwood. This confirmed that the test organism at least in the time framed observed, did not kill the *B. theobromae* but merely competed for nutrients and territory. The difference in activity among the biocontrol fungi could be due to differences in mechanisms.

Encinas and Daniel (1995) observed that after 30 days exposure to *B. theobromae* the starch granules, a common feature of Carribean pine were absent. Both sugars and starch were also depleted in 30 days. This was probably due to the lower amount of sugar and starch reported in Carribean pine than as reported in rubberwood (Chapter 4). Encinas and Daniel (1995) also reported depletion of starch in samples of birch incubated with *B. theobromae* after 60 days. The depletion of starch in birch after 60 days further confirmed that *B. theobromae* uses starch as an alternative nutritional source after depletion of fructose, glucose and sucrose.

SEM images *of B. theobromae* incubated for 60 days as reported by Encinas and Daniel (1995) showed the presence of *B. theobromae* with hyphal diameter of about 4 – 5 μm and severe degradation of the cell wall. Severe degradation of the cell wall

and active penetration of the *B. theobromae* hyphae into the cell wall was however not observed after 28 days in the rubber clones treated with *B. theobromae* in the present study. Probably it would only occur after the readily available nutrients in the rubberwood were depleted.

Mumanis *et al.* (1988) used SEM images of hyphal interaction of *Trichoderma harzianum* and *T. polysporum* against a wood decay fungus to show that most often, their hyphae are longitudinally attached to the hyphae of the decay fungus. Hyphal coiling, hooks and appressoria-like structures were observed, but were infrequent. Spores of the *Trichoderma* were found attached to the hyphae of the decay fungus in a similar fashion to that of the aprasitic hyphae. The SEM images of rubberwood samples exposed to *B. theobromae and T. viridae* in this study did not show observations similar to Mumanis *et al.* (1988). However both hyphae of *B. theobromae* and *T. viridae* were found in the rubberwood exposed to *B. theobromae* and *T. viridae*, indicating possibly that *T. viridae* did not kill *B. theobromae* but merely compete for nutrients and territory.

7.4.1.7 Biological control applications on wood

Bruce (1998) and Schoeman *et al.* (1999) provided a comprehensive review of biological control applications of forest products highlighting previous research undertaken to evaluate the mechanisms by which biological control agents protect wood. The antagonistic ability of *Trichoderma* was discovered and suggested to serve as a biocontrol agent more than 50 years ago (Weindling, 1934). However only during the last two decades has there been a worldwide effort in utilising the fungus for commercial use. Attempts to use fungi or bacteria as control agents to limit or prevent colonisation of wood by moulds and stain fungi have been previously reported (Bernier *et al.,* 1986; Benko, 1988; 1989; Highley, 1991; Croan and Highley, 1991; Krebber and Morell, 1993; Seifert *et al.*, 1987, 1988). Most of these studies were on artificial media and using stain fungi other than *B. theobromae.*

Several other reports have indicated successful biological control by bacteria isolated from the surface of timber. Bernier *et al.* (1986) reported that *Bacillus*

subtilis was able to prevent colonisation of *Picea* spp. blocks by three sapstain or mould fungi. Seifert *et al.* (1987) rated *B. subtilis* as highly effective to control mould and sapstain growing on surface of unseasoned wood. Initial work on rubberwood in India was by Florence and Sharma (1990) and they reported biological control potentials of *B. subtilis.* Florence and Sharma (1990) showed that field test of *B. subtilis* was effective in preventing the fungal growth on the rubberwood up to 80%. However Kim and Morrell (1998) showed that *B. subtilis* reduced but did not completely inhibit sapstain due to *Ophiostoma perfectum* on sapwood of *Pinus ponderosa.* SEM examination by Kim and Morrell (1998) revealed that bacteria and fungi were often intimately associated on the wood surface and bacterial population was generally lower on wood wafers where stain fungi were present. This indicated that bacteria only inhibit the fungi through competition for nutrients or space but did not cause antibiosis. Successful *in vitro* biological control by bacteria has often been unsuccessful on scaled up field trial. This could be due to the stain fungi occupying a different niche in colonizing wood than bacteria (Benko, 1989).

Sapstain fungi colonise wood by growing along tracheid (in softwoods), vessels and fibres (in hardwoods) and through ray cells. Bacteria cannot colonise in a similar fashion because they lack the filamentous growth habit and cannot easily derive nutrients from parenchyma cells deep below the wood surface. Recent success in field trials showed that there is hope in the use of filamentous fungi in control of sapstain and decay. For example an isolate of *T. viridae* T60 had been shown in previous laboratory tests to be particularly effective against certain basidiomycete decay fungi. A recent field trial had shown some success in sapstain control using pressure impregnation of *T. viridae* isolate in cellar trials (Brown and Bruce, 1999). Schoeman *et al.* (1994) also showed that addition of *Trichoderma* spores to chain-saw oil resulted in significantly ($p<0.05$) reduced sapstain colonisation and was associated with lower levels of decay fungi in the air-seasoned timber.

The various tests conducted in this chapter suggest that although the *T. viridae* used in the present study is not the same isolate as those of Schoeman *et al.* (1994) it demonstrated effective biological control activity in all the rubberwood clones. *T.*

viridae was most active in clone RRIM600, and least active in clone GT. These results complement results of earlier findings by Bruce and Highley (1991) and Score and Palfreyman (1994) where they demonstrated the biological control activity and potentials of *Trichoderma* spp. in controlling wood decay fungi. Field experiments and genetic finger printing of wood extracts also indicated that *T. viridae* could inhibit invasion of sound wood by *Serpula lacrymans* (Palfreyman *et al.,* 1995).

7.5 CONCLUSIONS

The objectives of this chapter were to examine the interaction of *B. theobromae* and potential biocontrol fungi on agar based studies and to compare results with those found on the wood samples of the three rubberwood clones. The observed differences in staining activities of *B. theobromae* and biocontrol fungi were then reexamined in relation to the differences in physical properties, anatomical features, C:N ratio, starch contents and sugar contents of the 3 rubberwood clones.

The difference between biological control of sapstain as compared to control of decay is that the protection of wood from decay requires long term protection whereas protection from sapstain is a much shorter term. Therefore it should be easier to develop a practical biological control system against sapstain than wood decay. The relatively short period of protection required will mean that reapplication of biocontrol agent may not be necessary. The other difference is that in a sapstain control system it is more likely that the biocontrol agent is applied on a freshly cut surface thus reducing the possibility of the biocontrol agent to be exposed to other competing organisms. Therefore, a simple method of application, either by spraying or dipping could provide adequate biological protection for the short period of protection required. Any logistic delays however will greatly increase the exposure to other invading organisms.

The conclusions from this study on sapstaining activity of *B. theobromae* and biocontrol agents on rubber wood clones are:

- Clone RRIM600 is the most susceptible to *B. theobromae.*
- Starch contents and pH are important factors that contribute to the darker stain observed in clone RRIM600.
- None of the primary mould fungi showed better biological control potential than *T. viridae* and *T. reesei* as tested on media and wood specimen.
- Incorporation of *T. viridae* and *T. reesei* could further improve sapstain control.

CHAPTER 8

8. GENERAL DISCUSSION AND RECOMMENDATIONS

8.1 SUMMARY

The main objective of this study was to examine clonal differences in the wood characteristics of clones GT1, PB217 and RRIM600 in relation to its susceptibility to sapstain. It is evident from this study that the three rubberwood clones show differences in basic physical properties, anatomical characteristics, selected wood chemistry and the darkness rating of staining when exposed to *B. theobromae* and other primary fungal clonisers.

Clone RRIM600 seems to show the most variation in wood characateristics among the three clones. For example clone RRIM600 has the highest pH (6.17) of cold water extract, the highest starch content (5.81%) but significantly ($p<0.05$) lower in total sugar content (8.93%) than clone PB217. Among the three clones RRIM600 is the most susceptible to sapstain. It was also found that there were differences in degree of biological control activity among the rubberwood clones investigated. In conclusion it could be said that the rubberwood clones differ in its susceptibility to *B. theobromae* which could be associated with variation in pH and starch content.

Although qualitative visual assessments are used in most reported evaluations of sapstain severity on wood quantitative method of sapstain evaluation could be more objective and consistent, the quantitative method of sapstain assessment used in the present study indicated that the use of handheld colorometer shows better coefficient of determination compared to the use of densitometer and spectrophotometer. It could be applied to stain assessment of larger samples.

8.2 DISCUSSION

It is a well-known fact to timber manufacturers and rubberwood users that drying of timber and keeping it below the fibre saturation point will immediately stop any sapstain attack on rubberwood. This is the most preferred short-term approach and

should always be the topmost priority in sapstain prevention. However in practice delay in delivery of logs or freshly sawn lumber sometimes occur. Stockpiling of logs during the wet rainy season is unavoidable in order to keep the sawmills and other processing plants going. To minimize sapstain attack during storage of green rubberwood, the most effective method is to use chemical biocides. With increasing public concern for the environment, the industry is looking for environmentally friendly preservatives.

At present the short–term solution is still chemical biocides. Use of chemicals is not only hazardous to workers and the environment but also expensive. There is still hope to seek alternative, and more cost effective, bioprotectants that have minimal environmental impact as shown by recent advancements and success in bioprotection of sapstain and decay (Chapter 7). It poses a greater challenge for rubberwood protection because there is still a serious lack of information in this area. There is a great challenge in the long term to look into the other alternative options namely, a) biological control of stapstain, b) selection of naturally less sapstain susceptible rubberwood material, c) breeding and selection of rubberwood clones of lesser sapstain susceptibility and, finally d) genetic engineering to produce albino sapstaining fungi for sapstain control.

The problem of sapstain in rubberwood will improve with better understanding of the various other methods available to overcome it apart from the use of chemical biocides because it has been shown that chemical treatment is not the only solution. There are other non-chemical short-term and long-term strategies in minimizing the sapstain problem. Immediate processing and drying of rubberwood to overcome sapstain is of course the choice approach so far.

The advantage of biological control of sapstain as compared to biological control of decay is that the period of protection required is very much shorter while awaiting processing. The biological control of sapstain is also applied to freshly cut surface predisposed to low degrees of possible competing organisms. Also, the routine simple chemical spraying and dipping could provide adequate protection for the short period of protection required until the timber is fully dried.

The role of biocontrol agents in sapstain control has been extensively studied for a variety of applications (Bruce and King, 1983; Dawson-Andoh *et al.*, 1994.. One factor that could affect competitive outcome as has been shown in the present study is the nutritional quality of the rubberwood substrate. *B. thobromae* and some other secondary sapstain fungi are probably well adapted to the nutrient components in rubberwood. These organisms rely on their ability to utilise the nutrients stored mainly in the ray parenchyma cells. The present study indicates that these compounds include lipids, proteins, carbohydrated and resin acids (Abraham and Breuil, 1993; Gao and Breuil, 1995). It has been suggested that selection and enhancement of nutrients that are preferentially used by the biocontrol agents might improve anti-stain effectiveness (Morrell and Dawson-Andoh, 1998). Results of the present study indicates that this could be made possible through selection of clones with the desired level of starch or other nutrient to the advantage of the control agent. This could be an advantage to nutrient-enhanced biocontol because a) it is difficult to supply the nutrients that are selectively used by the biocontrol agent in its natural form, b) in nutrient-enhanced biological control there is potential depletion of the added nutrients during incubation or prior to application and, c) to supplement nutrient immediately before field application is not practical as it will incur additional treatment costs.

Another alternative to nutrient-enhanced biological control is the simultaneous application of low levels of biocide followed by the control agent. Dilute levels of biocide or compounds such as boron and fluoride have been explored for this purpose (Morrell and Dawson-Andoh, 1998). Fluoride furthermore was shown to markedly enhance the growth of *Trichoderma* spp. many of which can inhibit stain and decay. Trial with a number of commercially used anti-sapstain chemical and bacterial biocontrol organism *Bacillus subtilis* suggests that fungal stain can be inhibited to a greater extent when combinations of biocide and biocontrol agent are employed (Mankowski *et al.*, 1997) than was found with either bocides or the biocontrol agent alone. Similar synergism was observed in the present study when a combination of two biocontrol organisms were used to control sapstain in rubberwood caused by *B. theobromae.*

The present breeding and propagation of rubber trees is similar to clonal reforestation, which is practised operationally with both temperate and tropical species worldwide (Hartment *et al.,* 1990). Interest in clonal reforestation has increased dramatically within the past 20 - 30 years in response to awareness of the benefits of such systems (Foster and Bertolucci, 1994). Malaysia is very fortunate that rubber trees improvement programme has been initiated since 1928. The provenance trials have established the pattern of genetic variability available especially in term of latex production, latex and timber production, growth characteristics, resistance to disease, resistance to wind damage etc. Large number of trees and several superior clones have been selected and planted throughout the country based on these recommendations. The experience and benefit with rubberwood has been described in Section 1.2. There is great potential to include additional selection criteria based on characteristics identified in the present study such as starch content or other characteristics associated with lower level of sapstain susceptibility quantitatively assessed. The method developed in this study to quantitatively assess the wood subsurface darkness of rubberwood samples exposed to sapstain fungi could be modified or adapted to test newly recommended clones and relate the degree of sapstain to potential clonal traits. These added information is useful for rubberwood tree breeders as well as sawmillers concerned with timber grading. It is worthwhile to examine the possibility of reviewing the selection criteria for planting recommendations. It might be possible to include other clonal traits for example susceptibility to sapstain that shows clonal variation. Before then a detailed study has to be undertaken on other newly recommended and planted clones. The focus should be on: a) starch and sugar contents and, b) quantity and proportion of the two main types of parenchyma cells (upright and procumbent cells), as discussed in Section 5.4. With these information additional progress and improvement of rubberwood clones could materialise.

The approval for large scale 'NoGall' in Australia in February 1989 was the first world commercial biopesticide based on a live, genetically engineered strain of *Agrobacterium tumefacens.* The modified strain has a small piece of DNA removed so that it is no longer able to cross with pathogenic strain of *Agrobacterium tumefaciens* in nature (Kerr, 1989; Wright, 1989). Similar modification and

genetic engineering of starch in plants has also been reported. Several such examples have been cited in Glaun and Breiman (1997). An interesting approach to affect starch was by Dutch team (Pen *et al.,* 1992). The idea was to liquify starch *in situ*. For that they used the α-amylase gene from *Bacillus licheniformis*, which is the common bacterium for the production of industrial liquified α-amylase. They used *Agrobacterium*-mediated transformation to obtain transgenic tobacco. The heterologeous genes as well as its respective enzymes were expressed in transgenic tobacco seeds and the starch of the latter was liquified. Another example of modification of starch in transgenic plants was provided by Shewmaker *et al.* (1994). The investigators used a bacterial (*E. coli*) gene for glycogen synthesis (*glg*A). The cDNA of the *glg*A gene was engineered into cassette for expression in potato-tuber amyloplasts. After genetic transformation of potato the heterologous enzyme was expressed as intended and caused a vast change in the starch content in the transgenic tubers: a 30 - 50% reduction in starch and a decrease in the amylose/amylopectin ratio. Against this background , there is thus a great potential for the development of a novel rubberwood clone with minimal starch content because the outcome of the present study suggested that starch contributes towards sapstain susceptibility. Similarly these successful reports of novel transgenic plants will perhaps also stimulate interest in genetic engineering of mutant *B. theobromae* which could live and compete with the wild strain and other sapstain fungi found on rubberwood without causing stain degradation.

There are prospects to progressively develop protocol for generating transgenic *H. brasiliensis* trees from somatic embryos. Positive development of transformed rubber tree from somatic embryos will lead the way to the possibility of insertion of foreign genes, which will enable the introduction of superior characteristics (genes) into *H. brasiliensis* clones and the prospect of genetically engineered naturally durable transgenic *H. brasiliensis* tree.

As rubberwood is used to produce furniture for the export markets, a clean, cream coloured appearance is essential prerequisite. Such strict requirements however, must not deter further investigation into the possibility of biological control of sapstain. Several bacterial-based and fungal-based research on softwoods have

shown promising results (Table 7-3). The use of current and new biotechnology and genetic engineering techniques should be capitalised to speed up breeding and selection of rubberwood clones to produce in a near future a naturally durable rubberwood of the new millennium.

8.3 CONCLUSIONS AND RECOMMENDATIONS

It cannot be denied that drying is the best possible preservative treatment against sapstain fungi. Perhaps with the reduced starch content, presumably the rubberwood would become less susceptible to sapstain and there will be no need for biological control. A better management in scheduling harvesting and extraction plus additional drying facilities would solve the sapstain problem of the rubberwood furniture industry.

To overcome the problem of sapstain in rubberwood with minimal damage to the environment would require implementation of integrated planning strategies (both short term and long term). There are several possible options posed here to face the challenges of sapstain prevention without chemicals (via drying and biological protection):

a) Immediate processing and drying of logs and lumber after tree felling (kiln drying).

b) Potential biocontrol systems/organisms for freshly felled logs and timber which cannot be immediately dried.

c) Selection of sapstain resistant rubberwood clones (to delay the onset of stain)

d) Breeding and selection of sapstain resistant rubberwood clones

e) Genetic engineering of mutant (non-pigmented) strain sapstain fungi or sapstain control fungi.

REFERENCES

ABRAHAM, L.D. and BREUIL, C. 1993. *Organic nitrogen in wood: Growth substrates for a sapstain fungus.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/93-10019.

ALI SUJAN, TAN, A.G. and ASHRAF, M.S. 1980. *Some studies on fungal deterioration of rubberwood,* The International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/2140.

ANANTHANARAYANAN, S. 1971. Investigations on the cause of discoloration of some rosewood (*Dalbergia latifolia*) veneers. *J. Ind. Acad. Wood Sc.* **2(1):** 27-31.

ANG, B.B. and SHEPHERD, R. 1979. Promising new Prang Besar rubber clones. Report on the *Proceeding of The Rubber Research Institute of Malaysia Planters' Conference 1979*, Kuala Lumpur. 15-17 October 1979. Rubber Research Institute of Malaysia. Kuala Lumpur, pp. 219-225.

ANI SULAIMAN and LIM S.C. 1992. Wood quality study of rubberwood at different ages and clones. In: *Report on properties and tilisation of rubberwood from trees of diffeerent age groups.* Forest Research Institute Malaysia, Kuala Lumpur, pp 39-50.

ARENAS, C.V., MENDOZA, E.M., and BANATIN, C.C.1967. Studies on the effect of the staining fungus *Diplodia theobromae* (Pat.) Nowell on the toughness and bending strength properties of Duklitan (*Pouteria duclitan* (Blanco) Baehni) *Wood Preservation Report*, University of Phillipines, Laguna.

ASANO, I. and CHOO, K.T. 1990. Radiofrequency-vacuum drying of rubberwood (*Hevea brasiliensis*). In: *Proceedings of International Rubberwood Seminar.* 21-22 May 1990. Kuala Lumpur. Forest Research Institute Malaysia, Kuala Lumpur. Pp. 141- 147

ASHARI, A.J., PALFREYMAN, J.W. and WONG, A.A.H. 1999. *Association of contents of nitrogen and sugars in rubberwood (Hevea brasiliensis) clones with susceptibility to sapstain by B. theobromae, Aureobasidium pullulans and Aspergillus niger.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP 99-10307.

ASHARI, A.J., PALFREYMAN, J.W. and WONG, A.A.H. 2000. Sectrophotometric analysis of sapstain caused by *B. theobromae, Aureobasidium pullulans and Aspergillus niger* on three rubberwood clones. *Journal of Tropical Forest Science,* **6(2):** 152-164.

AUBE, C. and GAGNON, C. 1969. Effect of carbon and nitrogen nutrition on growth and sporulation of *T. viridae* Pers. Ex Fries. *Canadian Journal of Microbiology,* **15**: 703-706.

AZIZOL, A.K. and RAHIM S. 1989. Carbohydrates in rubberwood. (*Hevea brasilensis* Muller Arg.). *Holzforschung,* **43(3):** 173-178.

BAKER, K.F. and COOK, R.J. 1974. *Biological control of plant pathogens.* In: Kelman, A. and L. Sequeria (Eds.), San Francisco: Freeman & Co, 433pp.

BAKSHI, B.K. 1951. Studies on four species of *Ceratocystis* with a discussion on fungi causing sapstain in Britain. *Mycological Paper* No. 35. Commenwealth Mycological Institute, UK.

BALANSUNDARAN, M. AND GNANAHARAN, R. 1990. Laboratory evaluation of preservative treated rubberwood against fungi. *Journal of Tropical Forest Science,* **2(4):** 303-306.

BALLARD, R.G. WALSH, M.A.and COLE, W.E. 1982. Blue-stain fungi in xylem of lodgepole pine: a light-microscope study on extent of hyphal distribution. *Canadian Journal of Botany* **60**: 2334-2341.

BARLOW, C. 1978. *The Natural Rubber Industry, Its Development Technology and Economy in Malaysia.* Oxford University press. Kuala Lumpur. 500 pp.

BARNES, H.M. 1993. *Wood protecting chemicals for the 21st century,* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP 93-30018.

BENKO, R. 1983. *The study of blue stain found in coniferous timber in SR Slovenia (Yugoslavia).* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/117.

BENKO, R. 1988. *Bacteria as possible organisms for biological control of blue stain.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/1339.

BENKO, R. 1989. *Biological control of blue stain on wood with Pseudomonas capacia 6253: laboratory and field test.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/1380.

BENKO, R., and T.L. HIGHLEY. 1990a. Selection of media for screening interactions of wood attacking fungi and antagonistic bacteria.(i). interaction on agar (ii) interaction on wood. *Material und Organismen* **25(3)**: 172-180.

BENKO, R., and T.L. HIGHLEY. 1990b. *Evaluation of Bacteria for Biological Control of Wood Decay.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/1426.

BERNIER, R., M. DESROCHER and L. JURASEK. 1986. Antagonistic effects between *Baccillus subtilis* and wood staining fungi. *Journal of the Institute of Wood Science,* **10**: 214-216.

BJURMAN, J. 1989. *Influence of storage on mould susceptibility of wood at RH values lower than 100%.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/1413.

BLANK, L.M. and TALLEY, P.J. 1941. American Journal of. Botany 28:564-569. In: Chap 3. Carbon Nutrition. Cochrane, V.W. 1958. *Physiology of Fungi.* London: John Wiley & Son, Inc., pp. 55-98.

BRAY, A.R. 1981. *Hazard warning. Pentachlorophenol.* Ottawa: Canada Safety Council.

BREUIL, C. 1998. Wood as a nutritional resource for staining fungi. In: J.J. Morrell and D.J. Dickinson, (Eds.), *Proceedings of The Conference on "Biology and Prevention of Sapstain",* 1998 May 25, 1997, Whistler, Canada: Forest Products Society Publication No. 7273, pp. 1-5.

BRISSON, A., GHANIBIAN, S., EAGEN, R., LECLERC, D.F. and BREUIL., C. **V** 1996. Localization and characterization of the melanin granules produced by the sap-staining fungus *Ophiostoma piceae. Material und Organismen,* **30**: 23-32.

BROWN, H.L. and BRUCE, A. 1999. Assessment of the biocontrol potential of a *Trichoderma viridae* isolate- Part I: Establishment of field and fungal cellar trials. *Intern. Biodeterioration and Biodegradation,* **44(4):** 219-223.

BRUCE, A. 1992. *Biological control of wood decay.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/93-1531.

BRUCE, A. 1998. Biological control of wood decay. In: A. Bruce and J.W. Palfreyman (Eds.), *Forest Products Biotechnology*, London: Taylor & Francis, pp 251-267.

BRUCE, A. and KING, B. 1983. Biological control of wood decay by *Lentinus lepideus* (Fr.) produced by *Scytalidium* and *Trichoderma* residues. *Material und Organismen,* **18:** 171-181.

BRUCE, A., KING, B and HEIGHLEY, T.L.. 1991. Decay resistance of wood removed from poles biologically treated with *Trichoderma. Holzforschung,* **45:** 307-311.

BUTCHER, J.A. 1974. *A Practical Guides to Fungal Damage of Timber And Wood Products*. Wellington: New Zealand Forest Service, Information Series No. **65:** 8-10.

CAMPBELL, R. 1989. *Biological Control of Microbial Plant Pathogens*. Cambridge: Cambridge Univiveristy Press, 218pp.

CARLILE, M.J. and WATKINSON. S.C. 1994. *The Fungi.* Academic Press. London.

CARTWRIGHT, K. ST. G. and FINDLAY, W.P.K. 1958. *Decay of Timber and Its Prevention. 2nd edn.* London: Her Majesty's Stationery Office. 332 pp.

CASSEN, D.L., FEIST, W.C., JOHNSON, B.R. and DEGROOT, R.C. 1995. *Selection And Use Of Preservative-Treated Wood.* Madison: Forest Product Society, 104pp.

CHOO, K.T. and HASHIM, W.S. 1994. Seasoning of rubberwood. Pp. 105-120. In: L.T. Hong and H.C. Sim, (Eds.), *Rubberwood Processing and Utilisation*, Forest Research Record No 39. Forest Research Institute Malaysia, Kuala Lumpur.

COCHRANE, V.W. 1958. *Physiology of Fungi*. John Wiley & Son, Inc. London.

COLLEY, R.H. and RUMBOLD, C.T. 1930. Relation between moisture content of the wood and blue stain in Loblolly pine. *Journal of Agricultural Research* **41**:389-399.

COOKE, R.C. and WHIPPS, J.M. 1993. *Ecophysiology of Fungi.* London: Blackwell Scientific Publications.

COWLING, F.B. (1970). Nitrogen in forest trees and its role in wood deterioration. *Abstract of Uppsala Dissertations in Science,* **164:** 1 – 19.

CROAN S. C. 1996. *Biological control of sapstain fungi in wood.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/96-10158.

CROAN S.C. and T.L. HIGHLEY. 1991. *Control of sapwood inhabiting fungi by living bacterial cells of fractionated extra cellular metabolites from Coniophora putenana.* International Research Group on Wood Preservation. Stockholm, Sweden. Document No: IRG/WP/1494.

CROAN S.C. and T.L. HIGHLEY. 1994. Biological control of sapwood inhibiting fungi by metabolites from *streptomyces risosus*. Pp. 243-256 In: G.C. Llewellyn *et al.* (Eds.), *International Biodeterioration Research 4*, New York: Plenum Press.

CROAN, S.C. and HIGHLEY, T.L. 1995. Fungal removal of wood sapstain caused by *Ceratocystis coerulesceus. Material und Organismen,* **30 (1):** 46-56.

DATACOLOR. 1992. *Spectroflash 500 Operations.* Datacolor International, Singapore.

DAWSON-ANDOH, B.E., CHAN, M. MCAFEE, B. and LOVELL, R. 1994. Extracellular enzymes produced by potential bioprotectants and sapstain fungi during colonization of western hemlock. *Technical forum, Forest Products Scientiest 48th Annual Meeting*, Portland, Maine.

DOIMO, L. 1984. What is wood? A review of the major chemical components. Department of Forestry Queensland. *Technical Paper No 38*, ISSN 0155-9664.

EATON, R. A. and HALE, C.D. 1993. *Wood – Decay, Pests And Protection.* London: Chapman and Hall, 546 pp.

ENCINAS, O. and DANIEL, G. 1995. Wood cell wall degradation by the blue stain fungus *Botryodiplodia theobromae* Pat. *Material und Organismen,* **29:** 255-272.

ENCINAS, O. and DANIEL, G. 1996. Decay capacity of the different strains of the blue stain fungus *Botryodiplodia theobromae* Pat. on various wood species. *Material und Organismen* **30:** 237-259.

ENCINAS, O. and DANIEL, G. 1997. Degradation of the gelatinous layer in aspen and rubberwood by the blue stain fungus *Lasidiplodia theobromae. International Aassociation of Wood Anatomist Journal,* **18:** 107-115.

ENCINAS, O. and DANIEL, G. 1999. Depletion of non-structural compounds during attack of pine and birch wood by the blue stain fungus *Lasidiploida theobromae. Journal of Forest Products,* **5(2):**184-196.

FDM. 2000. Special report – Malaysia improves wood export performence. *Furniture Design and Manufacaturing Asia.* July 2000.

FENGEL, D. and WEGENER, G. 1984. *Wood – Chemistry, Ultrastructure, Reactions,* New York: Walter de Gruyter.

FINDLAY, W.K.P. 1959. *Sapstain of timber.* For. Abstr. **20**:1-14.

FINDLAY, W.K.P. 1985. *Preservation of Timber in the Tropics.* Boston, Lancaster: MartinusNijhoff/Dr.W. Junk Puibl. Dordrecht.

FISCHER, C. and W. HÖLL. 1992. Food reserves of Scots pine (*Pinus sylvestris*). II. Seasonal changes and radial distribution of carbohydrate and fat reserves in pine wood. *Trees,* **6:** 147-155. pp. 77-85.

FLORENCE, E. J. M. 1991. *Sapstain Fungi of Some Commercially Important Timbers and Their Chemical Control.* KFRI Research Report No. 80. Kerala Forest Research Institute. India.

FLORENCE, E. J. M., GNANAHARAN, R. and SHARMA J. K. 1993. *Influence of Moisture Content of Rubberwood on the Growth of Botryodiplodia theobromae.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/93-10029.

FLORENCE, E.J. SHARMA J.K. and GNANAHARAN, R. 1998. *Sapstain fungi associated with softwood species in Kerala, India.* The International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/98-10260.

FLORENCE, E.J.M. and SHARMA J.K. 1990. *Botrydiplodia theobromae* associated with blue staining in commercially important timbers of Kerala and its possible biological control. *Material und Organismen,* **25**: 193-199.

FREITAG, M. and MORRELL, J.J. 1990. Wood sandwich tests of potential biological control agents for basidiomycetous decay fungi. *Material und Organismen* **25:** 63-70.

FRIM. 1985. *Rubberwood - Processing and Utilisation.* Forest Research Institute Malaysia. Kuala Lumpur.

GAN, L.T., HO, C. and CHEW, O.K. 1987a. Rubberwood sawn timber production and recovery studies. In: Daljeet Singh, K., Choo, K.T. and Hong, L.T. (Eds.), *Proceedings of The Second Rubberwood Seminar* 19-20, November 1985. Kuala Lumpur. Forest Research Institute Malaysia, Kuala Lumpur, pp, 97-122

GAN, L.T., HO, C.Y. and CHEW, O.K. 1987b. Rubberwood sawntimber production and recovery studies. In: K. Daljeet Singh, K.T. Choo and L.T. Hong (Eds.), *Proceding of the Second Rubberwood Seminar*, 1985, Kuala Lumpur.

GAO, Y. and BREUIL, C. 1995. *Wood extractives as carbon source for staining fungi in the sapwood of lodgepole pine and trembling aspen*. International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/95-10098.

GAO, Y., BREUIL, C. and CHEN, T. 1994. Utilisation of triglycerides, fatty acids and resins in lodgepole pine wood by a sapstaining fungus *Ophiostoma picceae. Material und Organismen,* **28(2):** 105-118.

GARRAWAY, M.O. and EVANS, R.C. 1984. *Fungal nutrition and Physiology.* USA: Wiley Interscience Publication. John Wiley & Son.

GARRET, S.D. 1951. Ecological groups of soil fungi: A Survey of substrate relationship. *New Phytologist*, **50 (2):** 149-166.

GLAUN, E. and BREIMAN, A. 1997. *Transgenic plants*. Imperial College Press, London.

GNANAHARAN, R 1982. A simplified boron diffusion treatment for rubberwood. *International Wood Preservation.* **2:** 169-172.

GNANAHARAN, R. and DHAMODARAN, T.K. 1992. Mechanical properties of rubberwood from a 35-year old plantation in central Kerala, India. *Journal of Tropical Forest Science,* **6(2):** 136-140.

GRANT, C. 1973. The use of a reflectance method for estimating surface mould growth on chipboard. *International. Biodeterioration Bulletin*, **8(4):** 139-140.

GRANT, D.F. 1952. Exploitation of timber from rubber trees, *The Malayan Forester* **15(3):** 137-139.

GREWELING, T. and PEECH. 1960. *Chemical soil tests*. Cornell University Agricultural Experimental Station Bulletin No. 960.

GRIFFIN, D.H. 1981. *Fungal physiology.* New York: John Wiley, 389 pp.

GUHA, E.R.D. and LEE, T.W. 1972. Structure of hemicellulose isolated from rubberwood. *Journal of the Indian Academy of Wood Science,* **3(2):** 49-52.

HARTMANN, H.T., KESTER, D.E., DAVIES, F.T. and GENEVE, R.L. 1990. *Plant propagation: Principles and practices*. London: Prentice Hall International.

HIGHLEY, T and CROAN, C. S. 1991. *Laboratory studies on control of sapstain and mould on unseasoned wood by bacteria.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/1493.

HILLIS, W.E. 1987. *Heartwood and Tree Exudates*. Berlin: SpringerVedag.

HO, K.S. 1994. Harvesting and log characteristics of rubberwood. Pp. 51-60, In: Hong, L.T. and Sim, H.C. (Eds.), *Rubberwood Processing and Utilisation.* Forest Research Record No 39. Forest Research Institute Malaysia, Kuala Lumpur.

HO, K.S. and CHOO, K.T. 1982. Processing of rubberwood. *The Malaysian Forester,* **45(3):** 290-298.

HO, K.S. and CHOO, K.T. 1983. Processing of rubberwood. Pp 11-19. In L.T. Hong (Ed.), *Processing of rubberwood utilisation seminar.* FRI report No 28, Forest Research Institute Malaysia, Kuala Lumpur,

HO, K.S. and ROSLAN, A. 1994. Primary processing: sawing and peeling of rubberwood. Pp. 61-84. In: L.T. Hong and H.C. Sim (Eds.), *Rubberwood Processing and Utilisation.* Forest Research Record No 39. Forest Research Institute Malaysia, Kuala Lumpur.

HO, K.S. and SAID AHMAD. 1990. Moulding properties of some Malaysian timbers. *Journal of Tropical Forest Science,* **3(4):** 361-366.

HONG, L.T. 1976. A blue stain organism of jelutong (*Dyera costulata* Hk.f.) *The Malaysian Forester,* **39:** 177-188.

HONG, L.T. 1980. Temperature tolerance and its significance in the control of sap-stain caused by the blue stain fungus *Lasidiplodia theobromae. The Malaysian Forester,* **43:** 528-531.

HONG, L.T. 1981. Screening of preservatives against spstain and mould fungi from tropical woods. I – Laboratory tests against isolates from rubberwood (*Heve brasiliensis*). *The Malaysian Forester,* **44(1):** 116-121.

HONG, L.T. 1983. Screening of preservatives against sap-stain and mould fungi from tropical hardwoods. II. First assessment of four proprietary formulations on rubberwood. *The Malaysian Forester*, **46:** 478-480.

HONG, L.T. 1989. Perlindungan kayu getah dengan bahan awet. Forest Research Institute Malaysia, Kuala Lumpur. *FRIM Technical Report No.* 12. 8pp.

HONG, L.T. 1994. Introduction. In: H.L Hong and H.C. Sim (Eds.), *Rubberwood Processing and Utilisation.* Forest Research Record No 39. Forest Research Institute Malaysia, Kuala Lumpur. Pp 1-15.

HONG, L.T. and GAN, L.T. 1995. Suitablility of young rubber tree for the wood based industry. In: *Proceedings of Rubber Growers' Conference*, 17-19 July, Rubber Research Institute Malaysia, Kuala Lumpur.

HONG, L.T. and WONG, A.H.H. 1994. *Biodeterioration and preservation of rubberwood (Hevea brasiliensis).* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/94-10084.

HONG, L.T., MOHD ALI SUJAN, TAN, A.G. and DALJEET SINGH, K. 1982. Preservation and protection of rubberwood against biodeteriorating organisms for more efficient utilization. *The Malaysian Forester,* **45:** 229-315.

HONG, L.T., TAM, M.K., DALJEET SINGH, K. and ARSHAD OMAR. 1980. ThE effectiveness of preservatives in the control of sapstain in rubberwood (*Hevea brasiliensis*) logs. *The Malaysian Forester,* **43(4):** 522-527.

HONG, L.T., TAM, M.K., DALJEET SINGH, K. and SALAMIAH, S. 1987. The protection operations for rubberwood sawn timber ande finished products, In K. Daljeet Singh, K.T. Choo and L.T. Hong (Eds.), *Proceedings of the second rubberwood utilisation seminar,* 19-20 November 1985. Forest Research Institute Malaysia, Kuala Lumpur.

HONG, L.T., WONG, A.H.H. and HO, Y.F. 1994. Durability of rubberwood. Pp. 37-49. In: L.T. Hong and H.C. Sim (Eds.), *Rubberwood Processing and Utilisation,* Forest Research Record No 39. Forest Research Institute Malaysia, Kuala Lumpur.

HUDSON, H.J. 1986. *Fungal Biology,* London: Edward Arnold Ltd.

HUGHES, F.E. 1965. Tension wood - a review of literature. *Forestry Abstracts,* **26(1)**: 2-9.

HUMPHREYS, F.R. and KELLY, J. 1961. *Analytical Chemistry Acta,* **24,** pp 66-70.

JONES, P.A. 1981. *Chlorophenols and their impurities in the Canadian environment.* Environmental Protection Service. Environment Canada, E.P.S.-3EC-81.2.

KAARIK, A.A. 1974. Decomposition of Wood. *Biology of Plant Litter Decomposition.* Academic Press. London.

KAARIK, A.A. 1980. *Fungi Causing Sapstain in Wood.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/199.

KADIR, A.A.S.A. 1998. Viability of rubber plantations for the production of timbers. *Proceedings of the Colloquium on Rubberwood Resources and Technologies,* Forest Research Institute Malaysia, Kuala Lumpur. pp. 6-21.

KERR, A. 1989. Commercial release of a genetically engineered bacterium for the control of crown gall. *Agriculture Science,* **41:** 4.

KIM, G.H. and MORRELL, J.J. 1998. Biological protection against fungal discoloration. Spatial distribution of the bacterial bioprotectant and target fungi on pondorosa pine sapwood. *Material und Organismen,* **32(1):** 17-27.

KING, B., OXLEY, T.A. and LONG, K.D. 1981. Soluble nitrogen in wood and its redistribution on drying. *Material und Organismen,* **16:** 241-254.

KING, B., OXLEY, T.A. and LONG, K.D. 1983. Some biological effects of redistribution of soluble nutrients during drying of wood. *Material und Organismen,* **18:** 268-276.

KJELDAHL, J. (1883). A new method for determining nitrogen in organic materials. *Journal Analytical Chemistry,* **22:** 366-369.

KOLLMANN, F.P.K. and COTE, W.A. 1968. *Principles of wood Science and Technology I – Solid Wood,* New York: Springer-Verlag Berlin Heidelberg, 592pp.

KRAMER, P. and KOZLOWSKI, T.T. 1979. *Physiology of woody plants.* New York/San Francisco/London: Acad. Press.

KREBER, B and MORRELL J.J. 1993. Ability of selected bacterial and fungal protectants to limit fungal stain in Ponderosa pine sapwood. *Wood and Fibre Science,* **25(1):** 23-34.

LAKS, P.E., PICKENS, J.B., WOODS, T.L. BELL, J.P., and NOLF, J.M. 1991. Performance of chlorothalonil and chlorthalonil/biocide combinations in anti-sapstain tests. *Forest Product Journal,* **41(5):** 23-30.

LANGRISH, J.M. and WALKER, J.F. 1993. Timber preservation. Pp. 285-320. In: J.C.F. Walker, B.G. Butterfield, T.A.G. Langrish, J.M. Harris and J.M. Uprichard (Eds.), *Primary wood processing: Principles and practice,* Chapman & Hall, London:

LEVI, M. P. and COWLING E.B. 1969. Role of nitrogen in wood deterioration. VII. Physiological adaptation of wood-destroying and other fungi to substrate deficient in nitrogen. *Phytopathology,* **59:** 460-468.

LEVI, M.P. 1973. Control methods. In: D.D. Nicholas, (Ed.), *Wood Deterioration and Its Prevention by Preservative Treatments. Vol I. Degradation and Protection of Wood,* New York: Syracuse University Press, pp. 183-216

LEVY, J.F. 1982. The place of basidiomycetes in the decay of wood in contact with the ground. In: J.C. Frankland, J.N Hedger and M.J. Smith, (Eds.), *Decomposer Basidiomycetes: Their Biology and Ecology,* Cambridge:Cambridge University Press.

LEVY, J.F. and DICKINSON D.J. 1981. Microbial biodeterioration of wood. Pp.19-60. In: A. H. Rose (Ed.), *Economic Microbial. Volume 6.* Academic Press, New York.

LGM. 1995. LGM Planting recommendations 1995-1997. *Planters' Bulletin*, Malaysian Rubber Board, **224-225**: 51-83.

LGM. 1998. LGM Planting recommendations 1998-2000. *Planters' Bulletin*, Malaysian Rubber Board. **3:** 1-30.

LIM, S.C. 1985. Observations on the anatomical structures of some Malaysian woods by SEM. In: *Report on the Training Course on Wood Technology at the State University of Ghent,* F.R.I. Report No. 42. Forest Research Institute Malaysia, Kuala Lumpur. Pp. 11-28.

LIM, S.C. 1996. Density and some anatomical features of the stem and branch woods of rubber trees. *Journal of Tropical Forest Products,* **2(1):** 52-58.

LIM, S.C. and ANI SULAIMAN. 1994. Structure and characteristics of rubberwood. Pp. 17-25. In: H.L. Hong and H.C. Sim, (Eds.), *Rubberwood Processing and Utilisation.* Forest Research Record No 39. Forest Research Institute Malaysia, Kuala Lumpur.

LIM, S.C. and FUJIWARA. 1995. Wood density variation in two clones of rubberwood tree planted at three different spacing. *Journal of Tropical Forest Products,* **3(2):** 151-157.

LIM, S.C. and MOHD. NADZRI, Y. 1995. Tension wood in the stem and branches of rubberwood. *Journal of Tropical Forest Products,* **3(2):** 222-225.

LIN, J. and MORRELL, J.J. 1997. Effect of biocontrol inoculum growth condition on subsequent chitinase and protease levels in wood exposed to biocontrol and sapstain fungi. *Material und Organismen,* **31(4):** 265-279.

LINDGREN, R.M. 1942. Temperature, moisture and penetration studies of wood staining *Ceratostomellae* in relation to their control. U.S.D.A. **Tehnical Bulletin** 807. Washington, D.C.

LINDGREN, R.M. and HARVEY, G.M. 1952. Decay control and increased permeability in southern pine sprayed with fluoride solutions. *Forest Produts Journal,* **2(5):**250-256.

MAGEL, E.A. and HOLL, W. 1993. Storage carbohydrates and adeline nucleotides in trunks of *Fagus sylvatica* L. in relation to discoloured wood. *Holzforschung,* **47:** 19-24.

MANKOWSKI, M.M., ANDERSON and MORELL, J.J. 1997. *Integrated protection of freshly sawn lumber using Bacillus subtilis and selected fungicides.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/97-70235.

MARISTANY, A.G., LEBOW, P.K., BRUNNER, C.C., BUTLER, A.D. and FUNCK, J.W. 1992. *Classifying Wood-Surface Features Using Dichromatic Reflection.* Vol. 1936. SPIE (International Society for Optical Engineering) Press Bellingham, WA. USA.

MARTAWIDJAJA, A. 1971. Keawetan dan pengawetan kaju karet (*Hevea brasiliensis* Muell. Arg.). *Rimba Indonesia,* **3 & 4:** 176-187.

MENON, P.K.B. 1993, *Structure and Identification of Malaysian Woods* (Revised edn.) Ani Sulaiman and Lim, S. C. (Eds.). Forest Research Intitute Malaysia, Kuala Lumpur.

MERRILL, W. and COWLING, E.B. 1966. Role of nitrogen in wood deterioration. IV. Relationship of natural variation in nitrogen content of wood to its susceptibility to decay. *Phytopathology,* **56:** 1324-1325.

MINEMURA, N, LIM, S.C. and HONG, L.T. 1999. Photo-induced discoloration of rubberwood (*Hevea brasiliensis*) and its control by *polyethyleneglycol. Journal of Forest Products,* **5(1):** 9-16.

MINOLTA. 1994. *Precise Color Communication – Color Control from Feeling to Instrumentation.* Minolta Co. Ltd. Osaka, Japan.

MOHD DAHLAN, J. and TAM, M.K. 1987. Natural durability of some Malaysian timbers by stake test. *The Malaysain Forester,* **48(2):** 154-159.

MOHD. DAHLAN, J., HONG. L.T. AZLAN, M., and WONG, A.H.H. 1994. preservation of rubberwood. Pp. 85-103. In: L.T. Hong and H.C. Sim, (Eds.), *Rubberwood Processing and Utilisation,* Forest Research Record No 39, Forest Research Institute Malaysia, Kuala Lumpur.

MONKOWSKI, M., ANDERSON, M. and MORRELL, J.J. 1997. *Integrated protection of freshly sawn lumber using Bacillus subtilis and selected fungicides.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/97-702335.

MORI, M. and TAKAHASHI, M. 1997. *Characateristics of the pigments produce by sapstain fungi.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/97-10223.

MORRELL, J.J. and DAWSON-ANDOH, B.E. 1998. Biological control: Panaceae or Boodnoggle. In: J.J. Morrell and D. J. Dickinson (Eds.), *Biology and prevention of sapstain*, Medison: Oregon State University, Forest Products society.

MORRELL, J.J. and SEXTON, C.M. 1992. *Effect of nutrient regimes, temperature, pH and wood sterilisation method on performance of selected bioprotectants against wood staining fungi.* International Research Group on Wood Preservation. Stockholm, Sweden. Document No: IRG/WP/229.

MURMANIS, L.L., HIGHLEY, T.L. and PALMER, J.G. 1988. The action of isolated brown rot cell free culture filtrates, H_2O_2 - Fe^{2+}, and the combination of both on wood. *Wood Science Technology,* **22:** 59-66.

NAJIB LOTFI, A. and RAMLI, O. 1996. Rubber Plantations as a timber resource. *Planter,* Rubber Research Institute Malaya, **72:** 483-500.

NAYAGAM, S. 1987. Studies on the soluble nutrient components in wood and their influence on decay susceptibility of preservative efficacy. Ph D thesis. Dundee Institute of Technology.

OLDFIELD, M.L. 1984. *The value of Conserving Genetic resources.* Massachusetts: Sinauer Associates Inc. Publishers, 379 pp.

OLOFINBOBA, M.O. 1974. Sapstain in *Antiaris africana* an economically important tropical white wood. *Nature,* **249:** 860.

ONG, S.H., RAMLI, O., OTHMAN, H., MASAHULING, B. and NAIMAH I. 1994. Rubber breeding, progress and strategies to meet future needs of the plantation industry. *International Planters' Conference.* Kuala Lumpur.

ONIONS, A.H.S. 1975. Organisms for biodeterioration testing – moulds and fungi. *Microbial aspects of the deterioration of materials.* Academic Press, New York.

OXLEY, T.A., KING, B. and LONG, K.D. 1976. Some effects of decay of wood caused by redistribution of nutrients during drying. Record of Annual Convention of. British Wood Preservation Association. pp 87-96.

PALFREYMAN, J.W., GEORGE, M.S. and BRUCE, A. 1996. Timber preservation; current status and future trends. *Journal of the Institute of Wood Science,* **14(1):** 3-8.

PALFREYMAN, J.W., WHITE, N.A., BUULTJENS, T.E.J. and GLANCY, H. 1995. The impact of current research on the treatment of infestations by the dry rot fungus *Serpula lacrymans. Int. Biodeterioration and Biodegradation,* **35:** 369-395. Elsevier Science Ltd. Great Britain.

PANIKKAR, A.O.N. 1971. Occurrence of tension wood in Hevea. *Rubber Board Bulletin,* **11(2):** 55-58.

PAYNE, C and BRUCE, A. 1999. *Screening of bacteria, yeast and Trichoderma isolates for antagonism toward stain and mould fungi on agar medium.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/99-20159.

PEECH, M., DEAN, L.A. and REED, J. 1947. *Methods of soil analysis for soil fertility investigation.* U.S. Department of. Agriculture, Circular 757.

PEEL, J.D. 1958. The suitability of rubberwood as a raw material for fibreboard. *The Malayan Forester,* **21:** 112-115.

PEEL, J.D. and PEH, T.B. 1960. *Para rubberwood (Hevea brasiliensis muell-Arg) for pulp and paper manufacture: An account of laboratory experiments.* Malayan Forest Research Pamphlet No. 34. Forest Research Institute, Kuala Lumpur. 20pp.

PEN, J., MOLENDIJK, L., QUAX, W.J., SIJMONS, P.C., VAN OOYEN, A.J.J., VN DEN ELZEN, P.J.M., RIETVELD, K. and HOEKEMA, A. 1992. Production of active *Bacillus licheniformis* alpha-amylase in tobacco and its application in starch liquefiction. *Bio/Technology*, **10:** 292-296.

PINHEIRO, A.C.A. 1971. Blue-stain in poplar wood. *Material und Organismen,* **6(2):** 93-100.

POMERANZ, Y. and MOORE, R.B. 1975. Reliability of several methods for protein determination in wheat. *Bakers Digest,* **49:** 44-49.

RA0, V.R., DAYA.L, R. and SHARMA, B. 1983. Studies on the nature and pattern of distribution of ten wood in *Hevea brasiliensis*. *Indian Forester,* **109(4)**: 286-291.

RAMLI, O., ARSHAD, N.L., ONG, S.H., OTHMAN, H., BENONG, M., WAN CHIK, M.G., MOHD ZAIN, A.Z., ZAWAWI, A.G. and MOHD NOOR, A.G. 1995. Potential Hevea genotypes for timber production. In: *Proceedings of Rubber Growers' Conference.* 17-19 July, Rubber Research Institute Malaysia, Kuala Lumpur, pp 340-359.

RAYNER, A.D.M. and BODDY, L. 1988. *Fungal Decomposition of Wood. Its Biology and Ecology.* John Wiley & Sons, New York.

RENGHU, C.P., PREMA KUMARI, D. and PANII, A.O.N. 1989. Wood anatomy of *Hevea brasiliensis* 1: distribution pattern of tension wood and dimensional variation of wood fibre. *Indian Journal of Natural Rubber,* **2(1)**: 27-37.

RICHARDSON, B.A. 1993. *Wood Preservation (2nd ed).* Chapman & Hall, London. 226 pp.

RICHER, D.L. 1987. Synergism – A potent review. *Pesticide Science,* **19**:309-315.

RITSCHKOFF, A., RÄTTÖ, M. and THOMASSIN, F. 1998. Influence of the nutritional elements on pigmentation and production of biomass of bluestain fungus *Aureobasidium pullulans*. In: J.J. Morrell and D.J. Dickinson, (Eds.), *Biology and Prevention of Sapstain.* Oregon State University. Forest Products Society. Madison, WI.

RRIM. 1959. Foreign exchange clones – 1954 Collection. *Planters' Bulletin.* Rubber Research Institute of Malaya, **42**: 59.

RRIM. 1963. Planting recommendations 1963-64. *Planters' Bulletin.* Rubber Research Institute of Malaya, **66:** 54-55.

RRIM. 1965. RRIM600 . RRIM 700 series clone trials. *Planters' Bulletin,* Rubber Research Institute of Malaya, **76**: 24-25.

RRIM. 1970. Review of modern Hevea Clones: Clone GT1. *Planters' Bulletin,* Rubber Research Institute of Malaya, **109**: 113-114.

SCHEFFER, T.C. and LINDGREN, R.M. 1940. Stains of sapwood products and their control. US Department of Agriculture, Washington, D.C. *Technical Bulletin No. 714.*

SCHEFFER, T.C. and COWLING, E.B. 1966. Natural resistance of wood to microbial deterioration. *Annual Review Phytopathology,* **4**: 147-170.

SCHMIDT, O. and GANG, W.K. 1989. Microbial degradation of natural materials. In: *Biotechnology. Vol. 8.* H.J. Rehn and G. Reed, (Eds.), Germany: VCH, pp. 559-582.

SCHOEMAN, M.W., WEBBER J.F. and DICKINSON D.J. 1994. Chain-saw application of *Trichoderma harzianum Rifai* to reduce fungal deterioration of freshly felled pine logs. *Material und Organismen,* **28(4):** 243-250 1994.

SCHOEMAN, M.W., WEBBER, J.F. and DICKINSON, D.J. 1999. The development of ideas in biological control applied to forest products. *International Biodeterioration & Biodegradation*, **43**:109-123.

SCHULTZ, T.P and NICHOLAS, D.D. 1995. Utilising synergism to develop new wood preservatives. Wood preservation in the 90's and beyond. *Proceedings from the conference sponsored by Forest Product Society.* Forest Product Society, Madison.

SCORE, A.J. and PALFREYMAN, J.W. 1994. Biological control of the dry rot fungus *Serpula lacrymans* by *Trichoderma* species: The effects of complex and synthetic media on interaction and hyphal extension rates. *International Biodeterioration & Biodegradation,* **33:** 115-128. Elsevier Science Ltd. Great Britain.

SEIFERT, K. A., W.E. HAMILTAON, C. BREUIL, and M. BEST. 1987. Evaluation of *Bacillus subtilis* C168 as a potential biological control of sapstain and mould on unseasoned lumber. *Canadian Journal of Microbiology,* **33:** 1102-1107.

SEIFERT, K., A, BREUIL, C, ROSSIGNOL, L., BEST, M. and SADDLER, J.H. 1988. Screening microorganisms with the potential for biological control of sapstain on unseasoned lumber. *Material und Organismen,* **23:** 81-95.

SEIFERT, K.A. 1993. Sapstain of commercial lumbers by species of *Ophiostoma* and *Ceratocystis*. In: Wingfield, M.J., K.A. Seifert, and J.J. Webber, (Eds.), *Ceratocystis* and *Ophiostoma*: Texonomy, Ecology and Pathogenicity. *American Phytopathology Society*, Minneapolis, pp 141-151.

SEXTON, C.M., MARISTANY, A.G., BRUNNER, C.C. and MORRELL, J.J. 1993. *Using image analysis to rate wood stain trials.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/93/10034.

SHARMA, S.N. and KUKRETI, D.P. 1981. Seasonal behavior of rubberwood, an under-utilised non-conventional timber resource. *Journal of Timber Development Association of India*, **27(2):**19-29.

SHARPE, P.R. and DICKINSON, D.J. 1992. *Blue stain in service on wood surface coatings; Part 1–The nutritional requirements of Aureobasidium pullulans.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/1556-92.

SHEWMAKER, C.K., BOYER, C.D., WIESENBORN, D.P., THOMPSON, D.B., BOERSIG, M.R., OAKES, J.V. and STALKER, D.M. 1994. Expression of *Escherichia coli* glycogen synthase in the tubers of transgenic potatoes (*Solanum tuberosum*) results in a highly branched starch. *Plant Physiology*, **104:** 1159-1166.

SHIELDS, J.K. and ATWELL, E.A. 1963. Effect of a mold, *Trichoderma viridae*, on decay of birch by four storage-rot fungi. *Forest Products Journal,* **13(7):** 262-265.

SIAU, J.F. 1971. *Flow in Wood.* Syracuse University Press, New York.

SIAU, J.F. 1984. *Transport process in wood.* Springer-Verlag, New York.

SILVA, A.A. and MORRELL, J.J. 1998. Inhibition of wood-staining Ophiostoma picea bu Bacillus subtilis on Pinus ponderosa sapwood. *Material und Organismen,* **32(4):** 41-52.

SIMATUPANG, M.H. 1986. Wood cement boards, In: M.B. Bever (Ed.), *Encyclopedia of Material Science and Engineering.* Massachusetts Institute of Technology, Massachusetts.

SMITH, J.E. and BERRY, D.R. 1975. *The Filamentous Fungi. Volume 1- Industrial mycology.* Edward Arnold (Publishers) Ltd. London.

SMITH, R.S. 1960. The permeability of wood. *Proceedings of the 5th World Forestry Congress*, pp. 1546-1548.

SRINIVISAN, U., BRUCE, A. and STAINES, H.J. 1992. *Effect of media composition on the antagonistic properties of Trichoderma sp against wood decay fungi.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/92-1538.

STAINER, R. Y., INGRAHAM, J.L., WHEELIS, M.L. and PAINTER, P.R. 1986. *The Microbial World. 5th edn.* Prentice-Hall, New York.

TABARIH, P.K. and SEEHAN, G. 1984. Effect of *Botryodiplodia theobromae* attack on Abachi wood (*Triplochiton scleroxylon* K. Schum). *Holzforschung,* **19**: 9-21.

TAPPI. 1978. *TAPPI Testing Procedures.* Technical Association of the Pulp and Paper Industry, USA.

TERZIEV, N. 1995. Migration of low-molecular sugars and nitrogen in *Pinus sylverstris* L during kiln and air drying. *Holzforschung,* **49(6):** 565-574.

TERZIEV, N. and BOUTELJE, J. 1998. Effects of felling time and kiln drying on colour and susceptibility of wood to mould and fungal stain during an above ground field test. *Wood and Fibre Science,* **30(4):** 360-367.

THOMAS, A.V. and LANDON, F.H. 1953. The timber of para *rubber (Hevea brasiliensis*). *The Malayan Forester,* **16**: 217-219.

TROYA, M. T. and NAVARRETE E.A. 1989. Blue stain fungi (*Ceratocystis* spp.) found in Spain on pine wood. International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/1410.

TSUNODA, K. and NISHIMOTO, K. 1988. *Japanese standardized method for evaluating effectiveness of anti-sapstain and anti-mold chemicals.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/88-2299.

TSUNODA, K., TAKAHASHI, M. and NISHIMOTO, K. 1983. Studies on low toxicity anti-sapstain chemical. (I) Chemical control to prevent sapstain and mould on rubberwood. *Wood Research Technical Notes,* **17**: 122-131.

VERRAL, A.F. 1939. Relative importance and seasonal prevalence of wood staining fungi in the Southern states. *Phytopathology,* **29**: 1021-1051.

WALKER, J.F. 1993. Timber preservation. In: J.C.F. Walker, B.G. Butterfield, T.A.G. Langrish, J.M. Harris and J.M. Uprichard (Eds.), *Primary wood processing: Principles and practice.* Chapman & Hall, London. Pp. 285-320.

WALKLEY, A. 1947. A critical examination of a rapid method for determining organic carbon in soils: Effect of variations in digestion conditions and of inorganic soil constituents. *Soil Science,* **63**: 251-263.

WALKLEY, A. and BLACK, I.A. 1934. An examination of the Degtjareff method for determining soil organic matter and proposed modification of the chromic acid titration method. *Soil Science,* **37:** 29-38.

WALLER, R.A. and DUNCAN, D.B. 1969. A Bayes rule for the symmetric multiple comparison problem. *Journal American Statistics Association,* **64:** 1484-1499 and *Corrigenda,* 1972, **67:** 235-255.

WAZNY, J., RUDNIEWSKI, P. KRAJEWSKI, K.J. and WAZNY, T. 1989. The reflectance method for testing the effectiveness of fungicide against surface mould growth on materials; I. Wood. *Wood Scicience Technology,* **23:** 179-189.

WEBBER, J.F. 1990. *Guidelines for water stoarage of timber.* Forestry Commission Research Information Notes: 175. Farnham, England.

WHITE-McDOUGALL, W.J., BLANCHETTE, R.A. and FARREL, R.L. 1998. Biological control of blue stain fungi on *Populus tremuloides* using selected *Ophiostoma isolates. Holzforschung*, **52(3):** 234-240.

WIENDLING, R. 1934. Studies on a lethal principle effective in the parasitic action of *T. lignorum* on *Rhizoctinia solani* and other soil fungi. *Phytopathology,* **24:** 1153-1179.

WONG A.H.H. and A.P. SINGH. 1997. Micrographic evidence for degradation of normal fibre walls in rubberwood (*Hevea brasiliensis*) by the common blue-stain fungus *Botryodiplodia theobromae.* Paper read at First ASEAN Microscopy Conference. Malaysia.(unpublished)

WONG A.H.H., L.T. HONG and J. SHAPIEI. 1995. Sap-stain in timber: Evaluation of sapstain preservatives. *Timber Technology Bulletin No 4.* Forest Research Institute Malaysia, Kuala Lumpur.

WONG, A.H.H. 1988. Natural decay resistance of kempas (*Koompassia malaccensis*) with included phloem against rot fungi. *Journal of Tropical Forest Science,* **1(2):** 162-169.

WONG, A.H.H. 1993. Susceptibility to soft rot in untreated and copper-chrome-arsenic treated Malaysian hardwood. Doctor of Philosophy thesis. Oxford University. Oxford.

WONG, A.H.H. and KOH, M.P. 1991. *Decay resistance of densified ammonia-plasticized stems of oil palm (Elaeis guineensis).* International Research Group on Wood Preservation, Stockholm, Sweden. Document No. IRG/WP/3673.

WONG, A.H.H. and SABRI, A. 2000. *Variation In infection rates of blue-stain, mould and white rot tropical fungi on mixed light Malaysian woods.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/00-10334.

WONG, A.H.H. and WILKES, J. 1988. Association of tissue characteristics with susceptibility to decay in the sapwood of *Pinus radiata. Holzforschung,* **42:** 399-402.

WONG, A.H.H. and WOODS, T.L 1997. *Performance of Tuff-Brite C™ and other formulations against blue-stain, mould and brown-stain in freshly sawn rubberwood (Hevea brasiliensis) in humid tropics of Peninsular Malaysia.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/97-30163.

WONG, A.H.H., ABDUL RAZAK, M.A. and LING, W.C. 1999a. Sapstain control in Malaysia- a research perspective. Paper presented at 5th Conference on Forestry and Forest Products Research (CFFPR-99), Series "Challenges to wood preservation industries in the next millennium", 30 pp.

WONG, A.H.H., EDEN, D., CHITTENDEN C., HEDLEY, M. and WAKELING, R. 1999b. *Comparison of the FRIM and* ***Forest Research*** *laboratory methods for screening of anti-sapstain formulations.* International Research Group on Wood Preservation, Stockholm, Sweden. Document No: IRG/WP/99-20170.

WONG, A.H.H., WOODS, T.L., HONG, L.T. and LEACH, T.J.1997. A Malaysian performance evaluation of selected environmentally acceptable preservatives against sapstain in green rubberwood. Paper presented at International Union of Forestry Research Organizations (IUFRO) All division 5 Conference, 7-12 July, 1977, Pullman, Washington, USA, Working Unit No. S5.03.09 (Wood Durability) 10pp. (unpublished).

WONG, W.C. 1980. Density and pH values of exotic and indigenous tree species grown in Peninsular Malaysia. *The Malaysian Forester* **42(2):** 219 - 231

WRIGHT, B. 1989. Gene-spliced insecticide uncorked in Australia. *New Scientist*, 4-March: 23.

YAHYA, M.P. 1998. The availability of rubberwood resources in Peninsular Malaysia up to year 2000 and beyond. *Proceedings of The Colloquium on Rubberwood Resources and Technologies.* Forest Research Institute Malaysia, Kuala Lumpur, pp. 6-11

YAP, Y.J. 1998. Status of the rubberwood processing and its utilization by the Malaysian furniture industry. *Proceedings of The Colloquium on Rubberwood Resources And Technologies.* Forest Research Institute Malaysia, Kuala Lumpur. Pp. 28-29.

ZABEL, R.A. 1954. Variations in preservative tolerance of wood destroying fungi. *Forest Product research Society,* **4:** 166-169.

ZABEL, R.A. and MORRELL, J.J. 1992. *Wood Microbiology: Decay and Its Prevention*. Orlando: Academic Press. 476 pp.

ZHENG, Y., J.N.R. RUDDICK, and C. BREUIL. 1994. Factors affecting the growth of *Ophiostotna piceae* on lodgepole pine heartwood. *Material und Organismen,* **29:** 105-117.

Association of contents of nitrogen and sugars in rubbererwood (*Hevea brasiliensis*) clones with susceptibility to sapstain by *Botryodiplodia theobromae, Aureobasidium pullulans* and *Aspergillus niger*. International Research Group on Wood Preservation Document No: IRG/WP/99-10307.

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THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Section 1 Biology

Association of contents of nitrogen and sugars in rubberwood (*Hevea brasiliensis*) clones with susceptibility to sapstain by *Botryodiplodia theobromae, Aureobasidium pullulans* and *Aspergillus niger*

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Association of contents of nitrogen and sugars in rubberwood (*Hevea brasiliensis*) clones with susceptibility to sapstain by *Botryodiplodia theobromae, Aureobasidium pullulans* and *Aspergillus niger*

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ABSTRACT

The purpose of this study was to determine if nitrogen and sugar contents in rubberwood from three selected varieties (clones) rubber trees would affect the rate of colonisation by *Botryodiplodia theobromae* (a tropical sapstain fungus), *Aureobasidium pullulans* (a temperate sapstain fungus) and *Aspergillus niger* (a common mould fungus). Sapstain growth was rated daily until at least 50% mycelial coverage was achieved for 40x20x5mm samples from three rubberwood clones (GT1, PB217 and RRIM600) inoculated with the test fungi and incubated in a humidified petri dish assembly under aseptic conditions. All samples had more than 50% coverage of mycelium after 9 days The results indicate that the nitrogen content of clone RRIM600 (1.061mg/g) was significantly higher when compared to that of clone GT1 (0.73mg/g) and clone PB217 (0.78mg/g). After drying (45°C), clone PB217 contained significantly higher amounts of fructose (5.55mg/g), glucose (2.30mg/g) and total sugar (13.15mg/g), as compared to clone GT1 and RRIM600. Clone GT1 had the lowest fructose (0.23mg/g), glucose (0.14mg/g), sucrose (2.20mg/g) and total free sugar (2.56mg/g). RRIM 600 however had the highest amount of sucrose. *B. theobromae* spread significantly faster on RRIM600 (4.3 days when >50% mycelial coverage is achieved) than PB217 (5.6 days) or GT1 (5.8 days) which was associated with the overall higher nutrient availability in clone RRIM600. Light sanding (down to 1-2mm depth) of sapstain infected samples revealed that deep sapstain typical of *B. theobromae* remained in the wood, contrasting with the absence of stain by *A. pullulans* and *A. niger* (both are typical superficial stainers) in the sanded material.

KEY WORDS: clone, nitrogen, sugar, stain fungi, *Botryodiplodia theobromae, Aureobasidium pullulans, Aspergillus niger,* discoloration, blue-stain, sapstain, rubberwood, *Hevea brasisliensis*, Malaysia,

1. INTRODUCTION

1.1 Rubber tree clones

Among the clones that have been planted in the Rubber Research Institute of Malaysia (RRIM) and the Golden Hope Plantations, a total of 64 genotypes have been identified as potential timber producers; however only twenty-five are suitable as planting materials for both timber and latex production (Ramli *et al.,* 1995). While some growth characteristics such as bole volume, vigour, trunk appearance, resistance to wind damage and major leaf diseases of these clones have been established and evaluated, their susceptibility to sapstain fungi and specific methods for sapstain control have not been studied.

1.2 Nitrogen content and sugar contents in wood.

The relative quantities of nitrogen and sugar vary in different types of wood. Rubberwood consists of 77.8% holocellulose, 39.7% α-cellulose, and 17.8% klason lignin (Guha and Lee, 1972). Azizol and Rahim (1989) determined the content of free sugars in freshly cut as well as in seasoned rubberwood logs. Freshly cut logs contained 1.05 to 2.29% of free sugars and 7.53 to 10.17% of starch. After 20 weeks of seasoning in the open, the sugar content and the starch content decreased to 0.23 and 1.08%, respectively.

Woody plant tissues also contain very small quantity of nitrogen in the range of 0.3 – 1.0 mg/g. As the nitrogen content of wood is low it is generally considered a major limiting factor controlling decay by both fungi (Cowling 1970; Cowling & Merill, 1966) and insects (Bletchly, 1966). Lilly *et al.* (1991) suggested that under nutrient limitation, fungi may not necessarily sporulate, but may divert all energy resources to continued hyphal extension in order to increase the chance of reaching a fresh nutrient supply. Autolytic breakdown of proteins in older mycelia can be the major source of translocatable nitrogen (Fenn and Kirk, 1981). Abraham and Breuil (1993) observed that proteolytic activity was detected after 24 hours of growth and increased throughout growth.

1.3 Sapstain Fungi

In Malaysia the tropical fungus *Botrydiplodia theobromae* is known to cause blue stain in rubberwood and light coloured (e.g. Jelutong and Ramin) sapwood timbers (Hong 1976; Hong & Wong, 1994). Recently, however this common tropical blue-stain fungus has been reported to significantly degrade the wood cell wall in the sapwood of certain timber species (Encinas & Daniel, 1995; Wong & Singh, 1997), as well as the gelatinous fibres of 'tension wood cells' (Encinas & Daniel, 1997). The degradation of cell wall and the gelatinous fibres apparently reduces certain wood strength properties (Arenas *et al.,* 1967; Encinas and Daniel, 1996).

Aureobasidium pullulans occurs commonly in the soil, wood and on plant surfaces. This fungus forms an extra-cellular carbohydrate (pullulan), which facilitates tenacious adherence to the surface. It thrives over broad temperature and pH ranges. It can utilise many simple carbon compounds and displays resistance to some toxicants (Zabel and Morrell, 1992). *Aureobasidium pullulans* is a well-known causal agent of sapstain in lumber (Käärik, 1974).

To minimise degradation and occurrence of sapstain, it is normal practice to apply anti-sapstain formulations by spraying over the length of logs and by end coating with bituminous based emulsion, incorporating preservatives. Similarly a number of wood preservatives comprising insecticides and fungicides or mixtures of both are now widely used for the treatment of sawn rubberwood against fungal attack and insect infestation (Hong *et al.,* 1994).

At present the most commonly used anti-sapstain formulation is a mixture of sodium pentachlorophenate (NaPCP) and borax. It is used both in the control of sapstain in green rubberwood logs and sawn timber.

However as NaPCP preservative is toxic to human beings, there is an increasing health and environmental concern among users. In India for example, the use of NaPCP is now totally banned (Florence and Sharma, 1997). Laboratory evaluation is required to test the efficacy of potential anti-sapstain material. In addition information on sapstain susceptibility of various rubber tree clones is required to optimise the level of anti-sapstain treatment needed for different clones.

Moulds are particularly common on hardwoods when the wood is very wet (Zabel and Morrell, 1992) which feed mainly on sugar and simple carbohydrates present in parenchyma cells of the sapwood (Hunt and Garret, 1967). Surface moulds which infest rubberwood cause superficial staining on unseasoned or partially seasoned wood or logs. Several fungi such as *Aspergillus niger*, *Fusarium* spp. *Sphaeronema* spp, *Trichorderma* spp., and *Curvularia* spp. Cause superficial staining (Kaarik, 1974). In contrast to mould fungi, which have colourless hyphae, the fungi causing sapstain have pigmented (coloured) hyphae, secrete soluble pigments and cause the formation of coloured deposits in wood ray cells. This results in the development of deep-seated stains producing a most undesirable blemish under natural finishes (Butcher, 1974).

The objectives of the current study were:

i) To examine the nitrogen and sugar contents in rubberwood from three selected clones of rubberwood (GT1, RRIM600 and PB217).
ii) To examine whether there is an association between the nitrogen and sugars contents of rubberwood clones and their colonisation by *Botryodiplodia theobromae* (a tropical sapstain fungus), *Aureobasidium pullulans* (a temperate sapstain fungus) and *Aspergillus niger* (a common mould fungus).

2. MATERIALS AND METHODS

The level of nitrogen and sugars were examined in

i. Green samples
   Green disc samples were obtained to investigate radial (outer sapwood and inner sapwood) and height (top and bottom) variation of sugars and nitrogen.

ii. Green/dried sawn wood samples (45°C)
   Samples were obtained after drying to examine the redistribution of nitrogen and sugars as would happen during the drying of timber.

2.1 Sample Preparations

Two clones (PB217 and RRIM 600) of rubber trees were obtained from Bukit Pilah Estate in Negeri Sembilan and the third variety (GT1) came from Tangkah Estate in Johor

From each clone, five trees were selected at random. During felling, discs were taken at 20% height, 50% height and 80% height (to the first branch). The discs were sealed in plastic bags to prevent loss of moisture. Immediately after arrival in the laboratory, the outer sapwood, inner sapwood and heartwood sections (approximate dimension; 20mm x 20mm x 50mm) were cut and separated from each individual disc and labelled.

The wood specimens were then dried in the oven overnight at 45°C. The oven-dried samples were then milled (Willey mill), screened (40 mesh), then placed in separate airtight containers and stored in the freezer (-20°C) until required for nitrogen (King et al., 1981) and sugar analysis.

2.2 Determination of nitrogen

The nitrogen contents of the three rubberwood clones were determined by the micro-Kjedahl method (Kjeldahl, 1883) using Kjeldatherm digestion system KT-40 (C. Gerhardt, Bonn, Germany). The

digestion system consisted of an aluminium block with 40 borings for thermostatically controlled heating at 400°C. On conclusion of the digestion, the sample solution recovered from the unit was analysed for nitrogen contents with the help of Vapodest-5 (C.Gerhardt, Bonn, Germany) distillation and titration unit.

Approximately 0.5 g of wood meal (oven dried to constant weight at 100°C) was accurately weighed and placed into a 100ml digestion tube and 10 ml of 98% concentrated sulphuric acid was added, followed by addition of 2.5g catalyst (K_2SO_4:SeO_2;1000:1). The samples were then allowed to digest under reflux for 3 to 4 hours. When digestion reached the end point (as indicated by the development of a clear liquid), the reaction mixture was then cooled to room temperature. Ammonium sulphate standards at 500 and 1000 ppm were run with each set of wood samples. The digest solutions were then analysed for nitrogen using a Vapodest-5 distillation and automatic titration unit, programmed to calculate the results based on the formula:

%N (w/w) = 1.4007 x titre / weight of wood analysed
= 1.4007 x (xx.xx ml used – xx.xx ml blank value) / weight of wood

2.3 Determination of sugar contents

Wood samples were extracted in Erlenmeyer flasks (250ml). 3g of oven dried wood samples were extracted in 50ml deionised water. The flask and content were allowed to equilibrate for five minutes and the flask was then placed on an orbital shaker at 200 r.p.m. After sixty minutes, the contents were first filtered through filter paper (Whatman's No.1) and then through a membrane filter (pore size, 0.45 μm) to remove any residual wood.

The clear solution (60 μl) of each sample was injected into a Shimadzu LC-10AT high performance liquid chromatograph equipped with a 5μm, 4.6mm (I.D.) x 250mm spherical Apex carbohydrate column.

Free sugar contents were calculated by comparison to standards. The standards injected were 0.4 mg/ml each of xylose, arabinose, fructose, glucose and sucrose. Two replicates were undertaken for each sample extract.

2.4 Sapstain Exposure Tests

2.4.1 Wood Samples

The three clones tested were PB217, GT1 and RRIM 600. Small sample specimen (5x20x40mm) were sterilised with gamma radiation of about 25 kGray.

2.4.2 Inoculum

Two sapstain and a mould fungi were used in this study, viz. *Aureobadidium pullulans* and *Lasiodiplodia (Botryodiplodia) theobromae* (both sapstain) and *Aspergillus niger* (mould).

2.4.3 Testing against sapstaining fungi and moulds

The sample surface was then treated with spore inoculum of sapstain or mould fungi. Treated samples of each clone were positioned on an inert plastic mesh placed in a petri dish over moistened filter paper to maintain humid conditions and reduce drying out of the wood. The petri dish was then incubated at 25°C. Observations and assessments were made daily for 4 weeks. The performance of the clone was measured as the extent of fungal colonisation based on rating as shown below:

0 = No growth is apparent under the microscope
1 = Fungal growth does not cover more than 5% of the test surface.
2 = The growth does not cover more than 20% of the test surface.
3 = The growth does not cover more than 35% of the test surface.

4 = The growth does not cover more than 50% of the test surface.
5 = The growth cover more than 50% of the test surface.

The data on a) nitrogen content; b) sugar contents and, c)rate of fungal growth based on the above score were analysed using the analysis of variance (Snedecor and Cochran, 1976) and the Waller-Duncan (1969) multiple range tests, standard deviation, minimum and maximum values were also obtained (SAS, 1979).

3. RESULTS AND DISCUSSION

3.1 Nitrogen Content

3.1.1 Nitrogen content in green (disc) samples

i) Analysis of variance

Analysis of variance for nitrogen content (mg/g) for the three test clones is shown in Table 1. A highly significant correlation was observed between clone/height and nitrogen content. The effect of cross-section was not significant. The grand mean nitrogen content of all samples was 0.855 mg/g.

Table 1. Mean squares from the analysis of variance of mg/g nitrogen for green samples.

Source	*Df*	*Nitrogen*
Clone	2	0.388 **
Height	1	1.390 **
Section	1	1.227 NS
Clone x height	2	0.246 **
Height x section	2	0.125 *
Clone x section	2	0.298 **
Error	26	0.023

NS, *, ** - Mean square is non-significant, significant at 0.05 and significant at 0.01 levels of probability, respectively.

ii) Mean nitrogen content

Variability in nitrogen content was observed in relation to height (Table 2). The top portion (80% height) had significantly lower mean nitrogen content (0.821 mg/g) when compared to (20% height) portions (1.052mg/g). Abraham and Breuil (1993) also reported a decrease in nitrogen content with increase in height, consistent with the finding of this study.

Table 2. Clone, height and section means for nitrogen content in green samples.

	Nitrogen (mg/g)
Clone[1]	
GT1	0.727 a
PB217	0.777 a
RRIM 600	1.061 b
Height[2]	
Top (80% height)	0.650 a
Bottom (20% height)	1.052 b
Section[2]	
Outer sapwood	0.821 a
Inner sapwood	0.890 a

Means having the same letter down the column show non-significant differences according to the Waller-Duncan multiple range tests. [1]Means are averages of 12 replications. [2]Means are averages of 18 replications.

The mean nitrogen content of clone RRIM 600 (1.061 mg/g) was significantly higher when compared to that of clone GT 1 (0.727 mg/g) and that of PB217 (0.777mg/g). These values were much higher than the nitrogen content of lodgepole pine (0.49 mg/g) as reported by Abraham and Breuil (1993).

3.1.2 Nitrogen content in dried (sawn timber) samples

i) Analysis of variance

Highly significant effect was observed for surface section and significant effect was observed for clone and interaction of clone and surface section (Table 3).

Table 3. Mean squares from the analysis of variance of mg/g nitrogen for air-dried samples.

Source	*Df*	*Nitrogen*
Clone	2	0.078 *
Surface section	1	0.800 **
Section x clone	1	0.140 *
Error	24	0.027

*, ** - Mean square is significant at 0.05 and 0.01 levels of probability, respectively.

ii) Mean nitrogen content

(Table 4). The highest difference in content of nitrogen (mg/g) for the core to that of the surface is in the clone RRIM600 (86.50%). King et al. (1981) explained the difference as due to migration of nitrogen during drying of wood. The extent of this migration depends on the amount of soluble material present.

Table 4. Clone, height and section means for nitrogen content (mg/g) in air-dried samples.

	Core (mg/g)	*Surface (mg/g)*	*% difference*
Clone[1]			
GT1	0.538	0.657	22.12
PB217	0.488	0.838	71.72
RRIM 600	0.578	1.078	86.50

3.2 SUGAR CONTENT

3.2.1 Sugar content in green (disc) samples

i) Analysis of Variance

Clone effect was highly significant for fructose and glucose but was not significant for sucrose and total free sugars (Table 5). Height shows highly significant effects for fructose, glucose and total free sugar, and significant effects for sucrose. Section had no effect on fructose and glucose but was highly significant for sucrose and significant for total free sugars.

Table 5. Mean squares from the analysis of variance of mg/g fructose, glucose, sucrose and total sugar in green samples.

Source	*Df*	*Fructose*	*Glucose*	*Sucrose*	*Total*
Clone	2	6.015 **	6.453 **	8.142 NS	13.406 NS
Height	2	3.629 **	2.077 **	25.892 *	59.385 **
Section	2	0.292 NS	1.006 NS	5.708 **	4.539 *
Error	74	0.325	0.407	6.186	8.889
Total	80	0.549	0.599	7.456	11.051

NS, *, ** - Mean square is non-significant, significant at 0.05 and highly significant at 0.01 levels of probability, respectively.

ii) Mean sugar contents of disc samples

Three sugars, which were detected in all the rubberwood extracts, eluted in the following order: fructose, glucose and sucrose (Table 6). Sucrose was the predominant sugar found accounting for 74% of the total sugars in PB217, 85% in PB217 and 92% in GT1. The means for sugar ranked in ascending order were sucrose, fructose and glucose.

Table 6. Clone, height and section means[1] for mg/g fructose, glucose, sucrose and total sugars for green samples.

	Fructose	*Glucose*	*Sucrose*	*Total*
Clone[2]				
GT 1	0.446 a	0.275 a	8.477 a	9.195 a
RRIM 600	0.813 b	0.557 a	7.471 a	8.842 a
PB 217	1.380 c	1.227 b	7.593 a	10.200 a
Height[2]				
20% H	0.455 a	0.372 a	7.036 a	7.863 a
50% H	1.092 b	0.792 b	8.935 b	10.819 b
80% H	1.088 b	0.896 b	7.571 ab	9.555 b
Section[2]				
Outer sapwood	0.987 a	0.721 a	7.089 a	8.797 a
Inner sapwood	0.780 a	0.550 a	7.290 a	8.620 a
Heartwood	0.869 a	0.788 a	9.163 b	10.820 b

[1] Means are averages of 27 samples. [2]Means having the same letter down the column show non-significant differences according to the Waller-Duncan multiple range test.

There were no significant differences for sucrose and total sugar content among clones. However, a high degree of variability among clones was observed for fructose and glucose contents. The clone PB217 contained significantly higher amounts of fructose (1.380mg/g) and glucose (1.227mg/g) as compared to those of clones GT1 and RRIM600. Clone GT1 had the lowest fructose (0.446mg/g) and glucose (0.275mg/g) contents.

3.2.2 Sawn timber (dried) samples

i) Analysis of variance

Three sugars, which were detected in the surface and core samples of all the rubberwood extracts, eluted in the following order: fructose, glucose and sucrose. Statistical analysis of the data (Table 7) showed that clone effect was highly significant for fructose, glucose and sucrose and significant for total free sugars. Position showed highly significant effect for fructose, glucose and sucrose, and significant for total free sugars: clone effect was highly significant for fructose, glucose and sucrose and significant for total free sugars. Position showed highly significant effect for fructose, glucose and sucrose, and significant for total free sugars.

Table 7. Mean squares from the analysis of variance of mg/g fructose, glucose, sucrose and total sugar for air-dried samples.

Source	*Df*	*Fructose*	*Glucose*	*Sucrose*	*Total*[1]
Clone	2	72.625 **	10.191**	48.482 **	225.999**
Position	1	11.116 **	3.537 **	155.632**	312.951**
ClonexPosition	2	3.592 *	0.430 NS	17.317**	25.767*
Error	18	0.958	0.231	2.158	7.257

*, ** and NS - Mean square is significant at 0.05, highly significant at 0.01 levels of probability and not significantly different, respectively.

ii) Mean sugar content from sawn timber(dried) sawn timber

After drying, sucrose was still the predominant sugar found, accounting for 74% of the total sugars in clone RRIM600, 49% in clone PB217; and 31% in clone GT1 (Table 8).

Table 8. Clone and position from surface means (mg/g oven dry weight) for fructose, glucose, sucrose and total sugar for air-dried samples.

	Fructose	*Glucose*	*Sucrose*	*Total*
Clone[1]				
GT 1	0.233 a	0.135 a	2.196 a	2.563 a
RRIM 600	0.889 a	0.679 b	7.090 c	8.649 b
PB 217	5.745 b	2.304 c	5.105 b	13.154 c
Surface position[2]				
Core	1.605 a	0.655 a	2.250 a	4.511 a
Surface	2.966 b	1.423 b	7.343 b	11.733 b

Means having the same letter down the column show non-significant differences according to the Waller-Duncan multiple range tests. [1]Means are averages of 24 samples from each clone.[2]Means are averages of 12 samples for each surface position.

A higher degree of variability was observed for fructose, glucose, sucrose and total free sugar content after drying. After drying, clone PB217 contained significantly higher amounts of fructose (5.745mg/g), glucose (2.304mg/g) and total sugar (13.154mg/g) as compared to those of clones GT1 and RRIM600. Clone GT1 had the lowest fructose (0.233mg/g) and glucose (0.135mg/g), sucrose (2.196mg/g) and total free sugar (2.563mg/g) contents. RRIM600 however had the highest amount of sucrose (7.090mg/g).

For all the three clones, means for fructose (2.966mg/g), glucose (1.423mg/g), sucrose (4.511mg/g) and total free sugar (11.733mg/g) contents for the surface samples after controlled (below 50°C) drying were significantly higher than those of the core.

3.3 Sapstain Exposure Tests

Analysis of variance

There was a highly significant difference in the number of days to develop sapstain between clones and between fungi (Table 9). However there were no significance differences between untreated samples and samples treated with 2% Sinesto-B (threashold value of 8%).

Table 9. Mean squares from the analysis of variance of number of days to develop 50% coverage of mycelium (Score 5) for air-dried clone samples inoculated with *B. theobromae, A, pullulans* and *A. niger.*

Source	*Df*	*MS*
Treatment	1	4.408 NS
Clone	2	26.175 **
Fungi	3	24.475 **
Treat*Clone	2	5.308 NS
Treat*Fungi	3	3.031 NS
Clone*Fungi	6	10.442 **
Tretment*Clone*Fungi	6	4.397 NS
Error	96	2.00

NS, *, ** - Mean square is not significant, significant at 0.05 and highly significant at 0.01 levels of probability, respectively.

3.3.1 Effect of Clones on development of fungi

i) **Aspergillus niger**

Table 10 and Figure 1 show that for *A. niger,* growth is slowest in clone GT1 (6.9 days), with no significant different between clone PB217 (4.4 days) and RRIM 600 (3.3 days). This could be explained from the total free sugar of core portion as tabulated in Table 10. Total sugar in clone GT1 (5.459 mg/g) is significantly lower than PB217 (9.409mg/g) and RRIM600 (8.674 mg/g).

Table 10. Clone means[1] for days to develop more than 50% coverage of mycelium (score 5) on air-dried rubberwood samples with respect to sugar and nitrogen contents of test samples.

Clone [1]	Score 5 **(days)**	Sugar (mg/g) **(mg/g)**	Nitrogen (mg/g) **(mg/g)**
GT 1	5.800 b	5.459a	0.538a
RRIM 600	4.300 a	8.674b	0.578b
PB 217	5.575 b	9.409c	0.488a

[1]Means were average of 40 samples from each clone. Means having the same letter down the column show non-significant differences according to the Waller-Duncan multiple range test (0.05 level of probability).

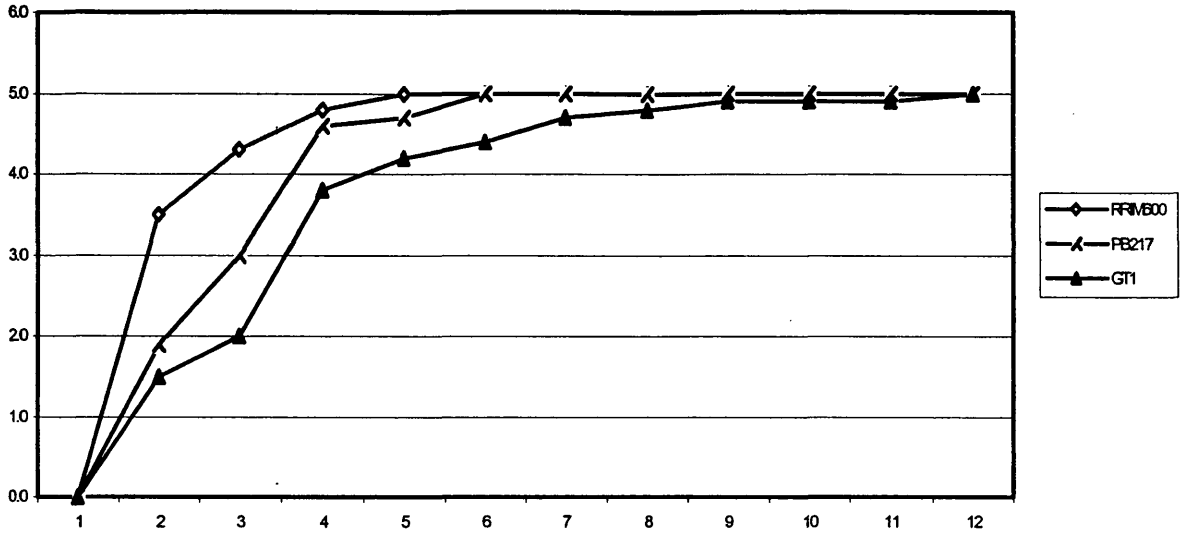

Figure 1. Plot of scores versus time for A. niger spore inoculum

It was also noted that mycelium appeared relatively earlier for *A. niger* and mix spore inoculum on both PB217 and RRIM 600 wood samples. Both PB217 and RRIM600 have relatively higher amount of total sugars. All samples of PB217 and RRIM 600 obtained a score of five within 6 days while clone GT1 took more than 10 days.

ii) Botryodiplodia theobromae

B. theobromae growth was relatively faster on RRIM 600 (Figure 2) which contained significantly higher amount of total sugar. For the first 3 days there was no difference in growth of mycelium for clone GT1 (low in total sugar but high in nitrogen) and PB217 (low in nitrogen but high in total sugar). However more samples of GT1 achieved higher scores after 3 days. This probably shows that Nitrogen is the limiting factor for development of *B. theobromae*. All samples achieved scores of 5 (more than 50% coverage of mycelium) after 9 days.

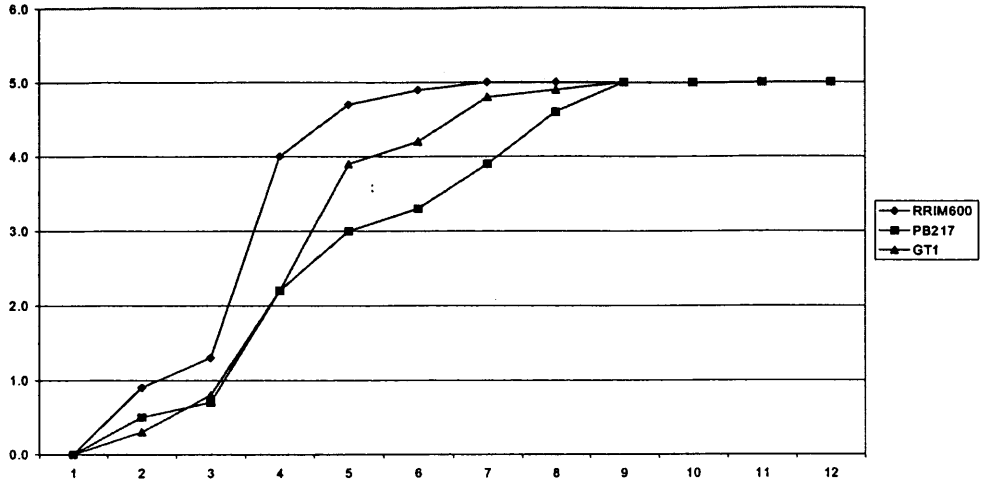

Figure 2. Plot of scores versus time for B. theobromae spore inoculum

iii) Aureobadisium pullulans

A. pullulans was relatively slow to develop on PB217, however there was no significant difference between GT1 and RRIM 600 (Figure 3).

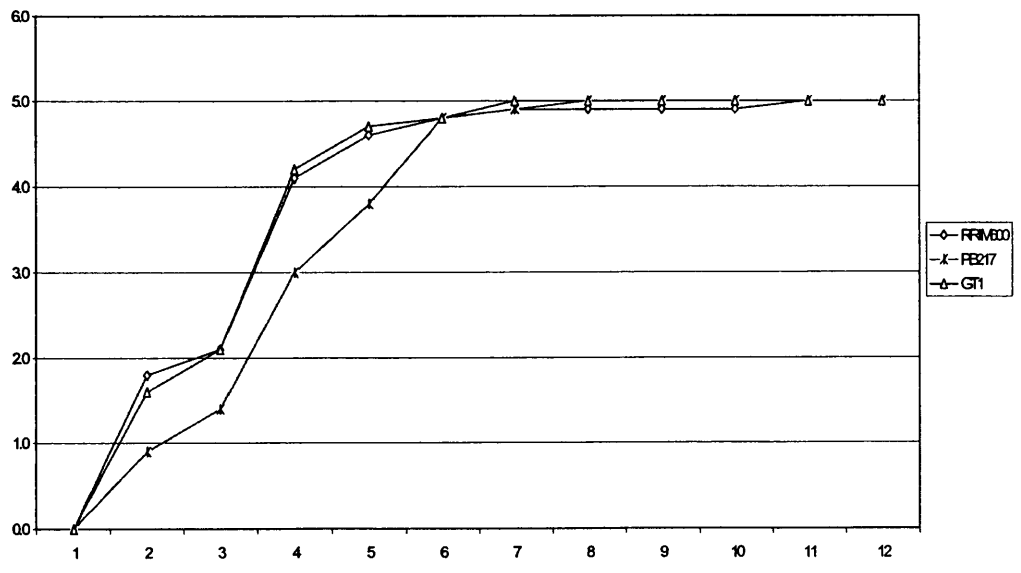

Figure 3. Plot of scores versus time for A. pullulans spore inoculum

4. CONCLUSION

The results indicate that the nitrogen content of clone RRIM600 (1.061mg/g) was significantly higher when compared to that of clone GT1 (0.73mg/g) and clone PB217 (0.78mg/g). After oven drying (45°C), clone PB217 contained significantly higher amounts of fructose (5.55mg/g), glucose (2.30mg/g) and total sugar (13.15mg/g), as compared to clone GT1 and RRIM600. Clone GT1 had the lowest fructose (0.23mg/g), glucose (0.14mg/g), sucrose (2.20mg/g) and total free sugar (2.56mg/g). RRIM 600 however had the highest amount of sucrose. The variation in sugar and nitrogen contents in the various rubberwood clones would probably influence its susceptibility to sapstain fungi. This is clearly shown by the rate of development of sapstain on the various rubberwood clones. It was shown that *B. theobromae* spread significantly faster on RRIM600 (4.3 days when >50% mycelial coverage is achieved) than PB217 (5.6 days) or GT1 (5.8 days) which is associated with the overall higher nutrient availability in clone RRIM600.

Light sanding (down to 1-2mm depth) of sapstain infected samples revealed that deep sapstain typical of *B. theobromae* remained in the wood, contrasting with the absence of stain by *A. pullulans* and *A. niger* (both are typical superficial stainers) in the sanded material. Visual assessment of the surface stain was not able to differentiate differences between clones. Therefore a quantitative assessment of sapstain assessment need to be developed. Currently research is in progress to examine two quantitative methods of colour assessment viz-a-vis the traditional method of visual assessment. The best of the two methods of assessment will then be used to investigate in detail the superficial and penetrative effect of sapstain fungi on the selected clones of rubberwood. With the available color image assessment tool several biological, biochemical sapstain control systems would also be evaluated.

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REFERENCES

Abraham L.D. and C Breuil. (1993). *Organic nitrogen in wood: Growth substrates for a sapstain fungus.* International Research Group on Wood Preservation Document No: IRG/WP/93-10019. Stockholm, Sweden.

Arenas. C.V. Mendoza, E.M. & Banstin, C.C. (1967). Studies on the effect of the staining fungus Diplodia theobromae (Pat.) Nowell on the thoughness and bending strength properties of Dklitan (Pouteria duclitan (Blanco) Baehni). Wood Pres. Rep., Univ. Laguna, Philippines.

Azizol, A.K and Rahim S. (1989). Carbohydrates in rubberwood *Hevea brasiliensis* Muell. *Holzforschung.* **43**(3): 173 – 178.

Bletchly, J.D. (1966). Aspects of the habits and nuitritium of the *Anobidae* with special reference to *Anobium punctatum. Material und Organismen.* 371-381.

Butcher, J.A. (1974). *A practical guides to fungal damage of timber and wood products.* New Zealand Forest Service, Wellington. Information Series No. 65: 8-10.

Cowling, F.B. and W. Merrill. (1966). Nitrogen in wood and its role in wood deterioration. *Canadian Journal of Botany* **44**:1533-1544.

Cowling, F.B. (1970). Nitrogen in forest trees and its role in wood deterioration. *Abstract of Uppsala Dissertations in Science, 164.*

Encinas, O. & Daniel, G. 1995. Wood cell wall degradation by the blue stain fungus *Botryodiplodia theobromae* Pat. *Material und Organismen* 29:255-272.

Encinas, O. & Daniel, G. 1996. Decay capacity of the different strains of the blue stain fungus *Botryodiplodia theobromae* Pat. on various wood species. *Material und Organismen* 30:237-259.

Encinas, O. & Daniel, G. 1997. Degradation of the gelatinous layer in aspen and rubberwood by the blue stain fungus *Lasidiplodia theobromae. IAWA Journal* 18:107-115.

Fenn, P. and T.K. Kirk. (1981). Relationship of nitrogen to onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium. Arch Microbial.* **130**; 59-65.

Florence, E.J.M. and Sharma J.K. (1990). *Botryodiplodia theobromae* associated with blue staining in commercially important timbers of Kerala and its possible biological control. Material und Organismen 25(3): 193-199.

Florence, E.J.M. and Sharma J.K. (1997). Control of sapstain and mould on rubberwood using sodium azide – Laboratory evaluation. *Journal of Tropical Forest Products* **2**(2): 187- 192. Forest Research Institute Malaysia, Kepong.

Gan, L.T., C.Y. Ho and O.K. Chew. (1985). Rubberwood sawn timber production and recovery studies. Pp 97 – 122 in *Proceedings of the Second Rubberwood Seminar.* Nov. 19 – 20. 1985. Forest Research Institute Malaysia, Kepong.

Guha, S.R.D. and T.W. Lee. (1972). Structure of hemicelluloses isolated from rubberwood. *Journal of the Indian Academy of Wood Science.* **3**(2): 49-52.

Hong, L.T. (1976). A blue stain organism of jelutong (Dyera costulata Hk.f.) Malaysian Forester 39:177-188.

Hong, L.T. & Wong A.H.H. (1994). Biodeterioration and preservationn of rubberwood (Hevea brasiliensis). International Research Group on Wood Preservation Document No: IRG/WP/94-10084. Stockholm, Sweden.

Hong, L.T., A. H.H. Wong and Y.F. Ho. (1994). Durability of rubberwood, in *Rubberwood Processing and Utilisation.* (L. T. Hong, ed). Malaysian Forest Records No 39. Pp 46-49. Forest Research Institute Malaysia, Kepong.

Hunt G.M. and G.A. Garrett, (1967). Wood Preservation 3rd Ed. Mc Graw-Hill, New York.

Kaarik, A.A. (1974). *Sapwood-staining Fungi.* International Research Group on Wood Preservation Document No: IRG/WP/125. Stockholm, Sweden.

King, B., T. A. Oxley and K.D. Long. 1981. Soluble nitrogen in wood and its redistribution on drying. *Material und Organismen* 16:241-254.

Kjedahl, J. (1883). A new method for determining nitrogen in organic materials. *J. Anal. Chem.* **22**:366-369.

Lilly, W.W., Wallweber, G.J. and Higgins, S.M. (1991). Proteolysis and amino acid recycling during nitrogen deprivation in *Schizophyllum commune. Curr. Microbiol.* **23**:27-32.

Ramli O., N.L. Arshad, S.H. Ong, H. Othman, M. Benong, M.G. Wan Chik, A.Z. Mohd Zain, A.G. Zawawi and A.G. Mohd Noor. (1995). Potential Hevea genotypes for timber production. *Proceedings Rubber Growers' Conference 1995*. Rubber Research Institute Malaysia, Kuala Lumpur.

SAS. (1985). *User's Guide*. SAS Institute, Inc., Cary, NC.

Snedecor, G.W. and W.G. Cochran. (1976). *Statistical Methods*. 6th Ed. The Iowa State Univ. Press, Ames.

Waller, R.A. and D.B. Duncan. (1969). A Bayes rule for the symmetric multiple comparison problem. *J. Am. Stat. Assoc.* **64**:1484-1499 and *Corrigenda* 1972, **67**:235-255.

Wong A.H.H. and A.P. Singh. (1997). Micrographic evidence for degradation of ht normal fibre walls in rubberwood (*Hevea brasiliensis*) by the common blue-stain fungus Botryodiplodia theobromae. Paper read at First ASEAN Microscopy Conference. Malaysia.(unpublished)

Zabel R.A. and J.J. Morrell. (1992). *Wood microbiology: Decay and its prevention.* Academic Press, Orlando, 476pp.

APPENDIX B

Comparison of three methods of quantitative evaluation of sapstain in rubberwood. International Research Group on Wood Preservation Document No: IRG/WP/00-20188.

Document No: IRG/WP 00-20188

IRG/WP

The International Research Group on Wood Preservation

Working Group 2 **Test Methodology and Assessment**

COMPARISON OF THREE METHODS OF QUANTITATIVE EVALUATION OF SAPSTAIN IN RUBBERWOOD

by

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ABSTRACT

Assessing the extent of fungal stain on wood during laboratory trials is a subjective process and is subjected to considerable variation between individual evaluators and specimen. The purpose of this paper was to explore the potential of three quantitative methods of wood surface measurement of rubberwood specimens degraded by sapstain and mould fungui *Botrydiplodia theobromae* (BT), *Aureobasidium pullulans* (AP) *and Aspergillus niger* (AN) and to identify the best possible method for quantitative assessment of sapstain on wood surface. The three methods evaluated were Spectrophotometry (Spectroflash 500), colorimetry (Minolta Croma Meter CR200) and Densitometry. Test samples, 40x20x5mm from three rubberwood clones (GT1, PB217 and RRIM600) were inoculated with the test fungi and incubated in a humidified petri dish assembly under aseptic conditions. After four weeks, the stained test samples were air dried and sanded (approximately 0.5mm) and evaluated by the three quantitative methods and visually. The quantitative ratings generated by the colorimeter were the most closely correlated ($R^2 = 0.836$) with the subjective visual evaluation.

KEY WORDS: clone – quantitative - blue-stain – sapstain – rubberwood – clone – spectrophotometry – densitometry - colorimetry

INTRODUCTION

Laboratory trials using small wood specimens are widely used in the screening of biocides for preventing fungal stain. Several methods have been used for testing and assessing stain, but the procedures remain largely subjective (Sexton *et al.* 1993). Many reports assign numbers to categories based on percent fungal coverage, or other criteria (Laks *et al.* 1991; Tsunoda and Nishimoto 1988; Hong 1989; Wong *et al.* 1997 & Wong *et al.* 1999) such as the use of percentage of wood discolored as the measure of biocide effectiveness (Morrell and Sexton 1992). Although these ratings are no doubt practicable, however the reports rarely describe, in detail, the scales employed, the number of personnel making the evaluations or whether the samples are coded to conceal the treatment groups. These parameters may bias ratings thereby influence the results (Sexton *et al.* 1993). Quantitative assessments of systematically assigned spot on the wood samples provide accurate estimate on degree of discoloration and would minimise operator subjectivity and provide more consistent assessments.

Objective ratings have been reported in several studies. Bjurman 1989 used ergosterol measurements to evaluate the degree of fungal mould and stain. Ergosterol levels were shown to be correlated with the fungal biomass but not necessarily relate to the degree of discoloration (Sexton *et al.* 1993). Wazny *et al* 1989 and Grant 1973 used the changes in reflectance of the wood to directly measure discoloration (Grant, 1973 & Wazny et. al., 1989). Reflectance has been used successfully to detect defects, including blue stain, in Douglas-fir veneer (Maristany *et. al.*1992). Sexton *et al.* 1993 explored on the use of image analysis to assess the extent of fungal discoloration and found that the results obtained were comparable to those produced by human evaluators.

The objective of this study is to examine three quantitative methods vis-a-vis subjective visual rating. The first two methods, spectrophotometry (D65-daylight exposure and A-tungsten light exposure) and colorimetry are based on CIELAB color space. Both instruments are designed to measure light in a way equivalent to how the human eye perceives light (Minolta 1994). The third method estimated the changes in reflectance from the wood samples using a scanning densitometer.

MATERIALS AND METHODS

Wood Samples

Three *H. brasiliensis* clones tested were PB217, GT1 and RRIM 600 grown in Peninsular Malaysia. Two varieties (PB217 and RRIM 600) of rubber trees were obtained from Bukit Pilah Estate, Negeri Sembilan and the third variety (GT1) came from Tangkah Estate, Johor. The trees were obtained in November 1997 from locations of similar soil type, terrain and climate. From each variety, five trees were selected at random. The billets (below the first branch) were sawn to 25x20x200 mm and dried at 50^{0}C within 24 hours. Sample specimens (5x20x40mm) were then prepared and sterilised with gamma radiation of 25 kGray.

Fungi

Inoculum from two sapstain fungi and a mould fungus were used in this study. The sapstain fungi were *Aureobasidium pullulans* (UiTM isolate) and *Botryodiplodia (Lasiodiplodia) theobromae* (FRIM isolate). The mould fungus was *Aspergillus niger* (UiTM isolate). Fungi were grown on

2% malt agar plates at 25°C in the dark for approximately 14 days. Mycelial fragment/spore suspensions of sapstain fungi and mould were prepared separately by mixing the fungal colony plus agar with 400 ml of water in a Waring blender. It was then filtered with sterile muslin cloth.

Experimental protocol

Seven combinations (Table 2) of fungal inocula were use as the treatment regimes. Control samples were treated with 2%NaPCP and rubberwood samples of natural color. Five replicate samples of each clone were treated for 5 seconds by dipping in each inoculum/mixture. They were then placed in pairs on to glass petri dish. The paired blocks (with similar fungal treatment) rested on netlon mesh placed over moistened filter paper at the base of the dish. The petri dish assembly was incubated at 25°C for 4 weeks.

Stain assessment

After 4 weeks the wood samples from the sapstain exposure tests were dried to below 20% moisture content in an oven at 60°C for 48 hours to prevent any further fungal growth. After drying, the samples were sanded to remove approximately 0.5 mm of the surface layer. The relative darkness were then determined by a) assessing percent reflectance of the surface using spectrophotometry (D65-daylight exposure or A-tungsten light exposure); colorimetry and scanning densitometry and b) visual assessment.

Use of the Spectrophotometer (Spectroflesh 500)

Spectroflash 500 is a fast, accurate dual beam spectrophotometer design to measure the percent reflectance or transmittance of sample in visible wavelength region. The condition s for sample measurement are controlled by a computer.

Calibration of the Spectrophotometer involved measurement of the zero reflecting light trap (or blocking the lens for transmission), and using a white calibration tile (maximum reflectance). These measurements establish the lower and upper reflectance limits. Measurements were made using a 6mm viewing area. Two readings were obtained for each sample as follows: the sample was placed against the reflectance measurement aperture and the sample test description keyed in to the computer. Light reflected from the sample was collected and measured after careful positioning of the sample, by checking exactly what the spectrophotometer will be measuring. This checking was achieved by viewing the sample through the viewing periscope provided on the top of the spectrophotometer. The reflectance values were recorded on a scale of 0–100. The darkness means reported in the paper being represented by percent of light absorbed = (100 - percent lightness).

Use of the Colorimeter (Minolta Croma Meter CR200)

Minolta Croma Meter CR200 is a compact tristimulus color analyser for measuring reflective color of surfaces. The measuring head has an 8mm-diameter measuring area and uses diffuse illumination and a 0° viewing angle.

Unlike spectrophotometer the tristimulus colorimeter take measurements under only standard illuminant C or Standard Illuminant D65, both of which represent daylight and which have very similar spectral power distribution (Minolta 1994). Calibration of the colorimeter involved setting the readings to a standard (Y; x; y) readings using the calibration plate (white) provided. For

standard illuminant C the (Y; x; y) were set to (94.2; 0.3131; 0.3201) and for standard illuminant D65 the (Y; x; y) were set to (94.2; 0.3156; 0.3329).

Three readings were obtained for each sample as follows: the "observer" of the portable colorimeter was placed on the sample, once the reflectance measurement were taken the reflectance values were automatically calculated based on CIE standard daylight illuminant on a scale of 0–100.

Use of the Densitometer

Calibration of the densitometer involves establishing a zero value in the reflectance value of unstained wood and a maximum value, i.e. the reflectance of the darkest sample. All other samples were evaluated against this scale. Two readings were obtained for each sample as follows: the amount of light reflected from the sample surface was evaluated by scanning crosswise. The average value obtained represents the average percent reflectance. Darkness was represented by percent of light absorbed = (100 - percent reflectance).

Visual Assessment

The samples were also evaluated for darkness by visual observation and matching with a guided darkness scale of 0 to 100. All the data were analysed using the analysis of variance (Snedecor & Cochran 1976), the Waller-Duncan (1969) multiple range tests at 0.05 level of probability with the help of SPSS 9.0. Standard deviation, minimum and maximum values were also obtained.

RESULTS AND DISCUSSION

Assessment Methods

The concept of white light is important to color. It may be defined as radiant energy with a wavelength distribution that evokes a neutral, or hueless, sensation in the observer with normal color vision. For colorimetry it is especially important to be able to identify any particular 'white' reference source. For this purpose the concept of color temperature is useful. It is the absolute, or Kelvin (K), temperature of a perfect radiator, called a black body, whose chromaticity (color without regard to brightness) is the same as that of the body in question. At a temperature of 800°K to 900°K a black body is red; as the temperature becomes higher, the color becomes yellow at about 3000°K, white (neutral) at about 5000°K. Yet light sources whose color temperature range from the 3000°K TO 6500°K of so-called average daylight are all considered in the range of white light when used as sources of illumination. General-purpose incandescent (tungsten) lamps used in the home are usually about 2820-3000°K. In 1966 relative spectral energy distribution were adopted as an international standard for average daylight at a correlated color temperature of 6500°K. Two Commission Internationale de l'Eclairage (CIE) standards, namely 'CIE illuminant A' (an incandescent lamp operated at 2854°K) and 'CIE illuminant D6500' (representing average daylight) were used (Anon 1992).

Unlike the subjective expressions commonly used by people to describe color verbally, spectrophotometer and colorimeter expresses color numerically according to International Standards. By expressing color this way it make it possible for anyone to understand what color

is being expressed. The two most widely known methods of expressing color are the Yxy color space (devised in 1931) and the L*a*b (also referred to as CIELAB) color space devised in 1976 to provide a more uniform color difference in relation to visual differences.

The CIELAB color space is presently one of the most popular color space for measuring color and is widely used in virtually all fields (Minolta 1994). In this color space L indicates lightness and a* and b* indicates color directions. Since this study attempts to quantify darkness, only variation in L was examined.

Analysis of variance for percent reflectance for the various method of sapstain assessment was as shown in Table 1. The table shows that the quantitative assessment made by the colorimeter had the lowest error mean square (13.87), indicating least variation between sample measurements. The highest was shown to be assessment made using the densitometer (499.9). However the difference in the error mean square from assessment made by the spectrophotometer with D65 and A-tungsten illuminant did not differ more than 5%. Among the methods of quantitative assessment spectrophtometry and colorimetry were more precise when compared to those made either visually or by using densitometry.

Table 1. Mean squares from the analysis of variance of percent reflectance (D65 and A light source) and percent darkness (visual) from sanded sapstain exposed samples.

Source	*Df*	*S'photometer D65 – Daylight*	*S'photometer A- Tungsten*	*Colorimeter*	*Densitometer*	*Visual*
Clone	2	109.597 **	109.595 **	80.764**	18562.8**	252.40*
Treatment	8	2022.544 **	2248.964 **	1530.981**	13361.6**	2838.67**
Clone x treatment	16	40.292 **	45.921 **	41.540**	4231.8**	53.04NS
Error	123	17.29	18.23	13.870	499.9	55.08

NS, *, ** - Mean square is non-significant, significant at 0.05 and highly significant at 0.01 levels of probability, respectively.

The visual assessment scores were regressed against the scores of the quantitative methods and correlations as measured by the R^2 were as shown in Table 2. It can be shown that the highest R^2 value of 0.837 was that of for colorimetry. This indicates that the colorimetry scores for measuring darkness contribute about 80% of those of visual assessment. The lowest R^2 value 0.313 was that for densitometer. The poor correlation of the densitometer with visual assessment was due to the measurement being affected by the small variation in thickness of the wood samples. As calibration is relative to the height of the scanner above the wood surface any variation in thickness is reflected in the score obtained.

Table 2. R^2 values and slope of regression line with visual assessment as dependent variable

	Spectrophotometer (D65 –daylight)	*Spectrophotometer (A –tungsten)*	*Colorimeter*	*Densitometer*
R^2	**0.645**	**0.644**	**0.837**	**0.313**
Slope (B)	0.953	0.903	1.052	0.678
Constant	28.44	31.70	15.610	41.983

The effect of variety and different treatments of the 3 clones assessed by the four methods of assessments were as shown in Table 3. The ranking of the clones was the same for the four methods. The darkest being RRIM 600 and the lightest being PB217. However subtle differences in significance as attributed by the quantitative methods and visual assessment can be seen in Table 3.

Table 3. Overall clone means darkness for rubberwood sapstain exposed samples.

Clone[1]	*Spectrophotometer (D65 – Daylight)*	*Spectrophotometer (A- Tungsten)*	*Colorimeter*	*Densitometer*	*Visual*
RRIM600	41.85 a	40.43 a	38.23a	67.50 a	62.47 a
GT1	39.67 ab	38.36 ab	36.02b	66.50 a	47.48 b
PB217	38.49 b	36.99 b	35.82b	62.80 b	45.10 b

[1]Means having the same letter down the column show non-significant differences according to the Waller-Duncan multiple range tests at 0.5 level of probability. Means are averages of 50 replications.

Effect of sapstain exposure treatments

Table 4 shows the overall mean for each sapstain treatment as obtained by the four methods of assessment. All methods were in agreement that rubberwood samples exposed to *B. theobromae* were darkly stained even after slight sanding. Samples assessed by spectophotometry and colorimetry showed significant differences between the unstained sample and samples treated with 2% NaPCP + BT, however these differences were not distinguishable by visual assessment. However results obtained by using the densitometer were not in agreement, especially for BT+AN which was rated the lightest (29.51) and AN (63.33) which was rated darker than the other two method of assessments. No further analysis will be undertaken on the densitometry system.

Table 4. Overall treatments means darkness for rubberwood sapstain exposed samples.

Treatments[1]	*Spectrophotometer (D65 – Daylight)*	*Spectrophotometer (A- Tungsten)*	*Colorimeter*	*Densitomete*	*Visual*
B. theobromae (BT)	56.50 a	56.20 a	55.16a	68.04a	81.33 a
BT + AN	44.60 b	43.26 b	45.47b	29.51c	71.67 b
BT + AP	42.67 bc	41.76 b	42.14c	41.91b	71.00 bc
AN + AP + BT	39.13 cd	37.46 c	36.51d	48.13b	68.33 bc
A. niger (AN)	38.24 d	36.20 c	36.16d	63.33a	67.33 cd
AN + AP	36.45 d	34.75 c	34.26de	45.81b	63.00 e
A. pullulans (AP)	35.88 d	33.88 c	32.61e	46.05b	64.00 de
2% Na PCP + BT	28.57 e	26.58 d	27.21f	NA	44.00 f
Control (untreated)	21.50 f	19.64 e	20.70g	NA	44.00 f

[1]Means having the same letter down the column show non-significant differences according to the Waller-Duncan multiple range tests at 0.5 level of probability. Means are averages of 15 replications.

Differences between treatments by clone.

Table 5 shows the darkness values for each of the rubberwood clones after the various treatments assessed using the spectrophotometer (Cmeter), Colorimeter (Cmeter) and visual assessment. To simplify discussion the treatments were discussed in 3 separate groups. In group I (unstained and NaPCP + BT), there were no significant differences. For group II (AP, BT+AN+AP, AN+AP and AN), all the quantitative methods except densitometry werein agreement with visual assessment and demonstrated that there were no significant difference in all the treatments. In group III (BT+AN, BT+AP and BT), all the methods of assessments were in agreement that there were no significant difference for clone GT1. For clone PB217 all the three methods were in agreement that BT exposed samples were the darkest. For clone RRIM600 the two quantitative methods were in agreement that samples exposed to BT were the darkest but the visual methods did not show any significant difference. Therefore in the case of RRIM600 the results show that the two quantitative methods were more sensitive and able to distinguish subtle differences in darkness of sapstain exposed samples.

Table 5. Treatment means darkness for rubberwood sapstain exposed samples by clones for evaluation by spectropotometer (SMETER), colorimeter (CMETER) and visual assessment.

TREATMENT	CLONE GT1			CLONE PB217			CLONE RRIM600		
	SMETER	CMETER	VISUAL	SMETER	CMETER	VISUAL	SMETER	CMETER	VISUAL
Group I									
Naatural color	**20.70a**	**20.00a**	**43.0a**	**21.18a**	**22.04a**	**48.0a**	**22.63a**	**20.07a**	**41.0a**
NaPCP + BT	**28.76ab**	**27.20b**	**43.0a**	**27.93a**	**27.34b**	**48.0a**	**29.00ab**	**27.09b**	**41.0a**
Group II									
AP	34.30b	34.71c	57.0ab	33.87b	32.17c	63.0b	39.48b	30.93b	72.0bc
BT + AN + AP	35.37bc	35.00c	66.0bc	39.23bc	35.09c	70.0b	42.81b	39.43c	69.0b
AN + AP	37.34bc	36.34c	60.0b	34.53b	34.53c	63.0b	37.49b	37.60c	66.0b
AN	40.91bc	29.94b	65.0b	36.45bc	34.51c	69.0b	37.37b	38.33c	68.0b
Group III									
BT + AN	**46.63c**	**46.52d**	**70.0bc**	42.52c	42.82d	**72.0bc**	44.65b	47.07d	**73.0bc**
BT + AP	**46.94c**	**45.15d**	**70.0bc**	38.96bc	39.13d	69.0b	42.12b	42.13c	**74.0bc**
BT	**52.88c**	**49.27d**	**77.0c**	**55.10d**	**54.75e**	**81.5c**	**61.50c**	**61.45e**	**85.5c**

[1]Means having the same letter down the column show non-significant differences according to the Waller-Duncan multiple range tests at 0.05 level of probability. Means are averages of 15 replications.

The advantage of these quantitative methods is that the value obtained does not require a rating scale (at present there is no universally accepted scale). Both spectrophotometry and colorimetry were able to overcome any errors caused by subjective human judgement, however the study indicates that the colorimetry scores correspond better to visual assessment. Being hand-held the colorimeter is more practical and much easier to use. The other advantage of the colorimeter is that it costs only 30% of the spectrophotometer.

CONCLUSION

The quantitative assessment 0.5mm below the surface of sapstain exposed rubberwood, were able to assess, in detail, the susceptibility of rubberwood clones to sapstain fungi. There is a statistically justifiable case for using the colorimeter to investigate the sapstaining activity and the cosmetic degrade caused by dominant sapstain fungi such as *B. theobromae*. The results obtained by this method could explain more than 80% of the variation made by visual assessment and the method is free of human bias. The detail analysis of the results were in agreement with visual assessment and in several instances more sensitive.

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REFERENCES

ANON. 1992. *Spectrophotometer operations.* Datacolor International. 51 Neil Road #03-10, Singapore.

BJURMAN, J. 1989. Influence of storage on mould susceptibility of wood at RH values lower than 100%. International Research Group on Wood Preservation Document No: IRG/WP/1413. Stockholm, Sweden

HONG, L.T. 1976. A blue stain organism of jelutong (Dyera costulata Hk.f.) *Malaysian Forester* 39:177-188.

HONG, L.T. 1989. Perlindungan kayu getah dengan bahan awet. *FRIM Technical report* No. *12.* 8pp.

GRANT, C. 1973. The use of a reflectance method for estimating surface mould growth on chipboard. *Int. Biodetn. Bull.* 8 (4)139:140.

LAKS, P.E., PICKENS, J.B., WOODS, T.L. BELL, J.P. & NOLF, J.M. Performance of chlorothalonil and chlorthalonil/biocide combinations in anti-sapstain tests. *Forest Prod. J.* 41(5):23-30.

MARISTANY, A.G., LEBOW, P.K., BRUNNER, C.C., BUTLER, A.D. & FUNCK, J.W. 1992. Classifying wood-surface features using dichromatic reflection. *SPIE (International Society for Optical Engineering) Proceedings* Vol. 1936. Bellingham, WA. USA.

MINOLTA. 1994. *Precise Color Communication – color control from feeling to instrumentation.* Minolta Co. Ltd. Osaka, Japan.

MORRELL, J.J. & SEXTON, C.M. 1992. Effect of nutrient regimes, temperature, pH and wood sterilisation method on performance of selected bioprotectants against wood staining

fungi. International Research Group on Wood Preservation Document No: IRG/WP/229. Stockholm, Sweden.

SEXTON, C.M., MARISTANY, A.G., BRUNNER C. C. & MORELL J.J. 1993. Using image analysis to rate wood stain trials. International Research Group on Wood Preservation Document No: IRG/WP/93-10034. Stockholm, Sweden.

SNEDECOR, G.W. & COCHRAN, W.G. 1976. *Statistical Methods*. 6th Ed. The Iowa State Univ. Press, Ames.

TSUNODA, K. & NISHIMOTO, K. 1988. Japanese standardized method for evaluating effectiveness of anti-sapstain and anti-mold chemicals. International Research Group on Wood Preservation Document No: IRG/WP/88-2299. Stockholm, Sweden.

WALLER, R.A. & DUNCAN, D.B. 1969. A Bayes rule for the symmetric multiple comparison problem. *J. Am. Stat. Assoc.* **64**:1484-1499 and *Corrigenda* 1972, **67**:235-255.

WAZNY, J., RUDNIEWSKI, P. KRAJEWSKI, K.J. & WAZNY, T. 1989. The reflectance method for testing the effectiveness of fungicide against surface mould growth on materials; I. Wood. *Wood Sci. Technol.* 23:179-189.

WONG, A.H.H. & WOODS, T.L 1997. Performance of Tuff-Brite C™ and other formulations against blue-stain, mould and brown-stain in freshly sawn rubberwood (*Hevea brasiliensis*) in humid tropics of Peninsular Malaysia. International Research Group on Wood Preservation Document No: IRG/WP/97-30163. Stockholm, Sweden.

WONG, A.H.H., ABDUL RAZAK, M.A. & LING, W.C. 1999. Sap-stain control in Malaysia: A research perspective. "Challenges to wood preservation industries in the next millennium". *Fifth Conference on Forestry and Forest Products Research (CFFPR)* 1999 Series. Kuala Lumpur, Malaysia.

APPENDIX C

Spectrophotometric analysis of sapstain caused by *Botryodiplodia theobromae, Aureobasidium pullulans* and *Aspergillus niger* on three rubberwood clones. Journal of Tropical Forest Products 6(2): 152-164.

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SPECTROPHOTOMETRIC ANALYSIS OF SAPSTAIN CAUSED BY *BOTRYODIPLODIA THEOBROMAE, AUREOBASIDIUM PULLULANS* AND *ASPERGILLUS NIGER* ON THREE RUBBERWOOD CLONES

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ASHARI, A. J., WONG, A. H. H. & PALFREYMAN, J. W. 2000. **Spectrophotometric analysis of sapstain caused by *Botryodiplodia theobromae, Aureobasidium pullulans* and *Aspergillus niger* on three rubberwood clones.** Rubberwood blocks from three clones (GT1, PB217 and RRIM600) were inoculated with the test fungi and incubated for four weeks, after which they were air dried and sanded (about 0.5 mm), and then quantitatively assessed for sapstain by spectrophotometry. Blue stain from *B. theobromae* was still present and pronounced after sanding, and was darker (absorbance: 56.5%) compared with wood infected by *A. niger* (38.2%) or *A. pullulans* (35.9%). Clone RRIM600 had higher darkness (41.9%) compared with clone PB217 (38.5%). The mean per cent darkness of clone GT1 (39.7%) was not different from clones RRIM600 and PB217. Single or mixed inocula *of A. pullulans* and *A. niger* effectively controlled *B. theobromae* infection in wood of clones RRIM600 and PB217, thus suggesting a potential for biocontrol of sapstain in rubberwood.

Key words: Rubberwood – *Hevea brasiliensis* – clone – sapstain – quantitative assessment – visual rating – spectrophotometry

ASHARI, A. J., WONG, A. H. H. & PALFREYMAN, J. W. 2000. **Analisis spektrofotometri ke atas cacat warna gubal disebabkan oleh kulat *Botryodiplodia theobromae, Aureobasidium pullulans* dan *Aspergillus niger* pada tiga klon kayu getah.** Sampel ujian ketiga-tiga klon kayu getah (GT1, PB217 dan RRIM600) diinokulat dengan kulat ujian. Selepas empat minggu, sampel-sampel tersebut dikeringkan dalam udara dan dihaluskan (anggaran 0.5 mm). Cacat warna gubal di permukaan sampel ditentukan secara kuantitatif menggunakan spektrofotometri. Cacat warna biru disebabkan *B. theobromae* masih lagi ketara walaupun permukaan kayu telah dihaluskan. Kayu yang dijangkiti *B. theobromae* adalah lebih gelap (serapan: 56.5%) berbanding dengan kayu yang dijangkiti *A. niger* (38.2%) atau *A. pullulans* (35.9%). Klon RRIM600 (41.9%)

mempunyai nilai serapan yang lebih tinggi daripada klon PB217 (38.5%). Nilai serapan klon GT1 (39.7%) tidak berbeza daripada klon RRIM600 dan klon PB217. Inokulasi *A. pullulans* dan *A. niger* secara berasingan atau bersama-sama dapat mengawal *B. theobromae* dalam kayu klon RRIM600 dan klon PB217. Ini bermakna inokulasi sedemikian berpotensi sebagai kawalan cacat warna gubal dalam kayu getah.

Introduction

Variation in wood quality between latex-producing clone varieties of rubberwood (*Hevea brasiliensis*) has become an area of research interest in the plantation forestry in Peninsular Malaysia. Recently the Malaysian Rubber Board has recommended 64 clones as potential timber producers but only 25 were found suitable as planting materials for timber and latex production (Ramli *et al.* 1996). While growth characteristics (Ramli *et al.* 1996) such as bole volume, vigour, trunk appearance, resistance to wind damage and major leaf diseases of these clones have been examined, the clonal variation in sapstain susceptibility of rubberwood has not been studied. Schmidtling and Amburgey (1982) reported the clonal variation in the decay susceptibility of loblolly pine.

The common tropical fungus *Botryodiplodia theobromae* (syn. *Lasiodiplodia theobromae*) causes deep blue stain in rubberwood and light-coloured sapwood timbers such as jelutong and ramin (Hong 1976, Hong & Wong 1994, Wong *et al.* 1999a) in Malaysia. *Aureobasidium pullulans* is a superficial stainer and occurs commonly in temperate soils, wood and on plant surfaces. This fungus is one of the most frequently isolated sapstain fungus in temperate regions (Sharpe & Dickinson 1992). *Aspergillus niger*, a primary mould, with non-pigmented hyphae represents tropical moulds that compete for nutrients and space in the rubberwood, possibly reducing staining by other fungi. This paper investigated the sapstain susceptibility of three rubberwood clones to these fungi.

Materials and methods

Wood samples

Wood samples from two *H. brasiliensis* clones (PB217 and RRIM600) were obtained from Bukit Pilah Estate, Negeri Sembilan while the third clone (GT1) from Tangkah Estate, Johor in Peninsular Malaysia. These estates are being managed by Golden Hope Plantations. The trees were felled in November 1997. They were sampled from locations of similar soil type, terrain and climate. Five trees were selected at random from each clone. The stem height to first branch ranged from 4 to 6 m. Disc samples for nitrogen and sugar content determinations were taken from the top portion (80% height) and at breast-height (BH). The remaining part of the stem (between BH and 80% height) was cut into billets. The billets were sawn to 2.5 × 2.0 × 200.0 cm size and dried at 50 °C within 24 h. Wood blocks measuring 5 × 20 × 40 mm were prepared, double sealed in plastic bags and sterilised by gamma radiation at a dose of 25 kGray.

Determination of nitrogen

The nitrogen content of the three rubberwood clones was determined by first digesting two wood meal samples (0.5 g) by the micro-Kjeldahl method (Kjeldahl 1883) using the Kjeldatherm™ digestion system KT-40, as described by Ashari *et al.* (1999). The carbon-nitrogen ratio was calculated based on carbon being arbitrarily assigned as 40% of the dry wood mass (Levi & Cowling 1969).

Determination of sugar content

Approximately 3 g of oven-dried wood meal were added into 100 ml deionised water in a 250 ml Erlenmeyer flask and placed on an orbital shaker (200 r.p.m.) for 60 min at 30 °C. The solution was filtered through a membrane filter (pore size 0.45 μm). Aliquots (60 μl) of the water extract were injected into a Shimadzu™ (model: LC-10AT) high performance liquid chromatograph equipped with a 5 μm, 4.6 mm (ID) × 250 mm spherical Apex carbohydrate column. After injection and passing through the column at a rate of 1.5 ml min^{-1}, the sample was mixed with a steady stream of nebulising nitrogen gas (2 standard litre per min), forming an aerosol. This was immediately followed by solvent vaporisation in the drift tube (recommended temperature, 79 °C) to produce a cloud of solute droplets, which entered a light scattering detector. The sample particles scattered the laser light but the evaporated mobile phase (acitonitrile:water 80/20) did not. The scattered light was detected by a silicone photodiode located at a 90° angle from the laser. The photodiode produced a signal which was sent to the analog output for collection via a Shimadzu™ chromatopac C-R6A data processor. Free sugar contents were calculated based on sugar standards (i.e. 0.4 mg l^{-1} of xylose, arabinose, fructose, glucose and sucrose). Duplicate readings were obtained for each sample extract.

Test fungi

Cultures of *B. theobromae* (BT) isolated from blue-stained rubberwood were obtained from the Forest Research Institute Malaysia, Kepong while those of *A. pullulans* (AP) and *A. niger* (AN) were obtained from the Universiti Teknologi MARA, Shah Alam. The fungi were grown on 2% malt agar plates at 25 °C in the dark for approximately 14 days.

Laboratory sapstain test

Seven combinations (see Table 5) of fungal inocula were used as the treatment culture regimes comprising single inoculum, dual and tri-culture mixed inocula. Control samples consisted of samples treated with 2% sodium pentachlorophenate (NaPCP) which were then inoculated with BT or samples which were uninoculated. Mycelial fragment/spore suspension of fungi were prepared separately by blending the fungal colony agar mixture with 400 ml of sterilised distilled water in a blender

and filtered through sterilised cotton gauze. Five replicate samples of each clone were dipped for 5 s in a given treatment regime, then placed in glass Petri dishes. The blocks of wood (with similar treatment regime) were rested on nylon mesh placed over moistened filter paper in Petri dishes. The dishes were incubated at 25 °C for up to four weeks. The surface mycelial coverage of fungi was daily and visually assessed based on a rating scale (Wong *et al.* 1999b): 0 = sound, 1 = up to 5% coverage (median: 3%), 2 = up to 20% coverage (median: 13%), 3 = up to 35% coverage (median: 23%), 4 = up to 50% coverage (median: 43%) and 5 = more than 50% coverage (median: 75.5%) until a rating of 5 was reached. The mean fungal coverage was calculated from the replicated median values for that treatment combination.

Quantitative stain assessment (spectrophotometry)

After four weeks, the wood samples from the sapstain exposure tests were dried to below 10% moisture content in an oven at 60 °C for 48 h to arrest further fungal growth. The samples were manually sanded to remove approximately 0.5 mm of the surface layer. The per cent reflectance of the surface was determined using a dual beam Spectroflash500™ spectrophotometer. The spectrophotometer was calibrated by measuring the zero reflecting light trap (or blocking the lens for transmission), and a white calibration tile (maximum reflectance) to establish the lower and upper reflectance limits. Measurements were made using a 6 mm diameter viewing area. The mean darkness obtained was represented by the per cent of light absorbed (inversed expression of per cent reflectance). Also after sanding, the samples were again rated based on the scale by Wong *et al.* (1999b) for comparison.

Statistics

All data were subjected to ANOVA incorporating Tukey's pair wise comparison and the Waller-Duncan multiple range tests.

Results

Nitrogen content and C:N ratio

The nitrogen content of clone RRIM600 was significantly higher than the other two clones (Table 1). Variation in nitrogen content was observed for stem height where the top portion (80% height) had significantly lower mean nitrogen content than the breast height. However, there was no significant difference between the outer and inner sapwood. The clone with the lowest C:N ratio was RRIM600.

Sugar content

There were no significant differences for both sucrose and total sugar content among the clones (Table 2). However, clone PB217 contained significantly higher amounts of fructose and glucose than the other two clones. Clone GT1 had the lowest fructose and glucose contents. There were significantly lesser amounts of fructose, glucose, sucrose and total sugar at breast height than above this height. The inner sapwood was also found to contain significantly more sucrose and total sugar than the other sections of sapwood.

Table 1. Nitrogen content and estimated carbon:nitrogen ratio in rubberwood of different clones, stem heights and radial positions

	No.	Nitrogen (mg g^{-1})	C:N
Clone			
GT1	12	0.727 a	550:1
PB217	12	0.777 a	514:1
RRIM600	12	1.061 b	377:1
Height			
Top (80% height)	18	0.650 a	615:1
Breast height	18	1.052 b	380:1
Section			
Outer sapwood	18	0.821 a	487:1
Inner sapwood	18	0.890 a	449:1

Means in the same column followed by the same letter are not significantly different ($p > 0.05$).

Table 2. Sugar contents in rubberwood of different clones, heights and radial positions

	No.	Free sugar contents in wood (mg g^{-1})			
		Fructose	Glucose	Sucrose	Total sugar
Clone					
GT1	27	0.446 a	0.275 a	8.477 a	9.195 a
RRIM600	27	0.813 b	0.557 a	7.471 a	8.842 a
PB217	27	1.380 c	1.227 b	7.593 a	10.200 a
Height					
Breast height	27	0.455 a	0.372 a	7.036 a	7.863 a
50% height	27	1.092 b	0.792 b	8.935 b	10.819 b
80% height	27	1.088 b	0.896 b	7.571 ab	9.555 b
Section					
Outer sapwood	27	0.987 a	0.721 a	7.089 a	8.797 a
Middle sapwood	27	0.780 a	0.550 a	7.290 a	8.620 a
Inner sapwood	27	0.869 a	0.788 a	9.163 b	10.820 b

Means in the some column followed by the same letter are not significantly different ($p > 0.05$).

Visual assessment of fungal growth before sanding

Table 3 shows the mean number of days taken to develop sapstain ratings 2, 3 or 5 on air-dried unsanded surfaces of rubberwood block samples. For rating 2 (up to 20% coverage), the three clones were all significantly different. Clone RRIM600 was the fastest to achieve rating 2 (2.2 days) and PB217 the slowest (3.2 days). However, clone RRIM600 was significantly the fastest to achieve rating 3 (2.7 days) and 5 (4.3 days) compared to clone GT1 and PB217.

Table 3. Number of days required for wood of different rubberwood clones to develop different sapstain ratings

Clone	Rating 2	Rating 3	Rating 5
GT1	2.74 b	3.56 b	5.80 b
RRIM600	2.22 a	2.70 a	4.30 a
PB217	3.15 c	3.75 b	5.57 b

Mean of 45 samples (9 treatments x 5 replicates) from each clone. Means in the same column followed by the same letter are not significantly different ($p > 0.05$).

Quantitative stain assessment (darkness rating) after sanding

Clonal effects

Clone RRIM600 had significantly higher overall mean darkness than clone PB217 (Table 4). However, the mean darkness of clone GT1 was not significantly different from that of clones RRIM600 and PB217.

Table 4. Overall mean per cent darkness (spectrophotometry) and fungal coverage for infected rubberwood samples between clones pooled across the fungal treatment regimes

Clone	Per cent darkness	Per cent mean fungal coverage
RRIM600	41.85 a	25.57 a
GT1	39.67 ab	22.61 b
PB217	38.49 b	18.44 b

Mean of 45 samples (9 treatments x 5 replicates) from each clone. Means in the same column followed by the same letter are not significantly different ($p > 0.05$).

Fungal treatment regime effects

Rubberwood samples exposed only to BT were the darkest and the control (non-inoculated rubberwood) samples were the lightest (Table 5). Wood samples inoculated with the fungal combinations BT + AN + AP showed lower stain than other fungal combinations mixed with BT. However, they showed significantly higher stain than those treated with a combination of 2% NaPCP + BT. Also, BT + AN + AP combinations were rated similar to those samples receiving single inoculum of AN or AP or the AN + AP mixture.

Table 5 also shows the overall mean surface fungal coverage (%) over the sanded samples for each fungal treatment regime pooled across all clones. Rubberwood samples exposed only to BT sustained the highest mean per cent coverage. Those inoculated with AP were not significantly different from those treated with 2% NaPCP + BT and the non-inoculated control blocks.

Table 5. Overall mean darkness (spectrophotometry) and fungal coverage for variously stained rubberwood samples between fungal treatments regimes pooled across clones

Treatment	Per cent darkness	Per cent mean fungal coverage
B. theobromae (BT)	56.50 a	75.50 a
BT + AN	44.60 b	39.33 b
BT + AP	42.67 bc	36.67 b
BT + AN + AP	39.13 cd	10.67 d
A. niger (AN)	38.24 d	11.00 d
AN + AP	36.45 d	22.67 c
A. pullulans (AP)	35.88 d	3.00 e
2% NaPCP + BT	28.57 e	1.00 e
Control (non-inoculated)	21.50 f	0.00 e

Mean of 15 samples (3 clones x 5 replicates) per treatment. Means in the same column followed by the same letter are not significantly different ($p > 0.05$).

Table 6 shows that RRIM600 treated with BT had significantly higher mean per cent darkness (61.5%) compared to PB217 (55.1%) and GT1 (52.8%). It is important to note that all fungal treatments omitting BT had significantly lower darkness values [i.e. AN (38.2%), AN + AP (36.5%) and AP (35.9%)] than those containing BT, except for the samples treated with 2% NaPCP + BT (28.6%). Treatment with 2% NaPCP prevented BT growth and staining of the wood. In two out of seven treatments, RRIM600 was significantly darker than GT1. In two out of seven treatments, clones RRIM600 and GT1 were of similar darkness. PB217 was the lighest in the majority of treatments while RRIM600 was consistently darker than PB217. PB217 and RRIM600 therefore appear to the be least and most susceptible to sapstain respectively.

Table 6. Tukey's pairwise comparison of mean per cent darkness (spectrophotometry) of variously stained treatment regimes compared with *B. theobromae* infected samples among different clones

Treatment	GT1	% Diff	PB217	% Diff	RRIM600	% Diff
AN (*A. niger*)	40.91	-22.5 **	36.45	-33.8 **	37.37	-39.2 **
AP (*A. pullulans*)	34.30	-35.0 **	33.87	-38.5 **	39.48	-35.8 **
BT (*B. theobromae*)	52.80	–	55.10	–	61.50	–
AN + AP	37.34	-29.3 **	34.53	-37.3 **	37.49	-39.0 **
AN + BT	46.63	-11.7 ns	42.52	-22.8 **	44.65	-27.4 **
AP + BT	46.94	-11.1 ns	38.96	-29.3 **	42.12	-31.5 **
AN + AP + BT	35.37	-33.0 **	39.22	-28.8 **	42.81	-30.4 **
2% NaPCP + BT	28.76	-45.5 **	27.93	-49.3 **	29.00	-52.8 **
Control (non-inoculated)	20.70	-60.8 **	21.18	-59.9 **	22.63	-57.1 **

ns = Not significantly different (stained as dark as samples exposed to *B. theobromae*),
** = Significantly less stained

Visual stain rating after sanding

Clonal effects

Clone RRIM600 had significantly higher overall mean per cent fungal coverage than clones PB217 and GT1 (Table 4). However there was no significant difference between GT1 and PB217.

Fungal treatment regime effects

Table 5 shows that samples exposed only to BT obtain the highest per cent coverage. Samples inoculated with AP were not significantly different from those treated with 2% NaPCP + BT and the control samples.

Comparison of clonal means (compare with BT)

For clones PB217 and RRIM600, the mean darkness of all stain treatment regimes was significantly lower than that of samples infected with BT alone (Table 6). However, the darkness ratings for GT1 samples exposed to AP + BT and AN + BT were both comparable statistically with BT-infected samples.

Comparison of clonal means (compare with NaPCP + BT)

For all the three clones, the mean darkness of control samples was not significantly different from the 2% NaPCP + BT treatment (Table 7). For PB217 and GT1, the darkness of samples exposed to AP and AN + AP was also not significantly different from the 2% NaPCP + BT samples. Similarly, RRIM600 samples exposed to AN and AN + AP were not significantly different from samples

treated with 2% NaPCP + BT. For GT1, the darkness of samples exposed to AN + AP + BT was not significantly different from samples treated with 2% NaPCP + BT.

Table 7. Tukey's pairwise comparison of mean per cent darkness (spectrophotometry) of variously stained treatment regimes with that of 2% NaPCP + BT treated samples among different clones

Treatment	GT1	% Diff	PB217	% Diff	RRIM600	% Diff
AN (*A. niger*)	40.91	42.1 **	36.45	30.5 **	37.37	28.9 ns
AP (*A. pullulans*)	34.30	19.2 ns	33.87	21.3 ns	39.48	36.1 **
BT (*B. theobromae*)	52.80	91.4 **	55.10	97.3 **	61.50	112.1 **
AN + AP	37.34	29.7 ns	34.53	23.6 ns	37.49	29.3 ns
AN + BT	46.63	62.0 **	42.52	52.2 **	44.65	54.0 **
AP + BT	46.94	63.1 **	38.96	39.5 **	42.12	45.2 **
AN + AP + BT	35.37	22.9 ns	39.22	40.4 **	42.81	47.6 **
2% NaPCP + BT	28.76	–	27.93	–	29.00	–
Control (non-inoculated)	20.70	-28.1 ns	21.18	-24.2 ns	22.63	-22.0 ns

ns = Not significantly different, ** = significantly darker.

Discussion

Woody plant tissues usually contain very small quantities of nitrogen, in the range of 0.03 to 0.10% (0.3–1.0 mg g^{-1}). The C:N ratio of most woody plant species is between 350–500:1 although in some species this ratio may be higher (Cowling & Merrill 1966). The results for nitrogen content and C:N ratio in rubberwood (Table 1) fall within the levels reported by Cowling and Merrill (1966). Nitrogen in wood is usually attributed to proteins and considered to be part of dried remnants of cytoplasm in dead wood cells. Nitrogen content is highest in the cambial zone and in the pith and lowest just outside the pith region (Cowling 1970). Variability in nitrogen content in rubberwood was also observed both between clones and between stem height, although not radially in the stem (Table 1). Variation in nitrogen levels between stem heights was similarly reported elsewhere (Abraham & Breuil 1993). It was also found that clone RRIM600, among all three clones, was most susceptible to BT. Since clone RRIM600 took significantly less time to develop stain ratings of 2 (2.2 days), 3 (2.7 days) or 5 (4.3 days) compared to the other two clones (Table 3), this may be associated with clonal variations in nitrogen levels.

It was reported that at C:N ratios of 4:1 to 400:1 the amount of mycelial growth was not limited by nitrogen but by the total carbon available, whereas at higher ratios growth was limited by the amount of nitrogen available (Levi & Cowling 1966). Although the C:N ratio of clone RRIM600 was within the range where total carbon would apparently be the limiting factor for fungal growth, the relatively higher nitrogen levels in RRIM600, would imply that both carbon and nitrogen have a role in rapid sapstain development in this clone.

In the field, BT typically penetrates the ends of rubberwood logs within a week after felling, causing severe infection particularly during the rainy season, and even resulting in internal sapstain in rubberwood within a few weeks storage (Wong & Woods 1997). Single organism treatments (present study) clearly showed that BT stained deeply into the sample exceeding 0.5 mm surface layer for all clones. On the other hand, the relative staining effect due to both AP and AN was significantly lighter (superficial). Two major sapstain control options have been adopted to date, i.e. (i) the use of anti-sapstain chemical and (ii) the hitherto experimental trial with potential biocontrol agents (e.g. antagonistic mould fungi) (Morrell & Sexton 1992, Kreber & Morrell 1993, Hong & Wong 1994, Wong *et al.* 1999a). The mixed fungal inocula incorporating BT in rubberwood clones (present study) provide invaluable insights into the potential biocontrol effects of combinations of AP and AN on controlling sapstain caused by BT. Unlike other clones, the mean darkness of GT1 samples exposed to AN + AP + BT was not significantly different from samples treated with 2% NaPCP + BT (Table 7), suggesting an effective biocontrol of BT. However, both AP + BT and AN + BT (Table 6) combinations inoculated on GT1 failed to control BT penetration. This suggests that there are other unknown factors influencing the biocontrol of BT growth on rubberwood.

The effectiveness of biological control fungi is known to be related to the nutrient content of growth media (Srinivasan *et al.* 1992a, 1992b, Score & Palfreyman 1994). The results presented here further suggest that the effect of biocontrol can vary among rubberwood clones containing different nutrient levels. Biological control organisms were also reported to limit but not completely inhibit the physiological activity of the target stain organisms (Florence & Sharma 1990, Highley & Croan 1991, Kreber & Morrell 1993). Several other reports have also described potential organisms for biological protection against stain fungi but field performance of these organisms has been inconsistent (Benko 1989, Florence & Sharma 1990). These observations may again be related to the nutrient variability in the wood or the sensitivity of the bioprotectants to environmental factors. Most of these studies were reported from observation and evaluation of the surface of wood samples exposed to sapstain fungi. However, no studies have so far been undertaken to quantitatively examine the degree of subsurface sapstain control (as reported in this study), a situation that reflects the condition of the wood after planing and moulding in the mills.

Objective ratings of sapstain have been reported elsewhere as an attempt to reduce operator bias in stain evaluation. For example, use of ergosterol-based evaluations of fungal mould and stain (Bjurman 1989) revealed good correlations with the fungal biomass, although not necessarily related to the degree of discoloration (Sexton *et al.* 1993). Wazny *et al.* (1989) and Grant (1973) used the changes in reflectance of the wood to directly measure discoloration, somewhat similar to the present spectrophotometric analysis. Reflectance has been used successfully to detect defects, including blue stain, in Douglas-fir veneer (Maristany *et al.* 1992). Sexton *et al.* (1993) attempted the use of image analysis to assess the extent of fungal discoloration and found that the results obtained were comparable

to those produced by human evaluators. The prominent, sub-surface or deep internal sapstain infection caused by BT is commonly observed and qualitatively rated on rubberwood (Wong & Woods 1997). However, the novel quantitative assessment, as reported here, apparently for the first time on BT-infected rubberwood clones, can also provide invaluable insights into the outcome of biological control and anti-sapstain preservative tests.

Conclusion

The least and most susceptible rubberwood clones to sapstain were respectively clones PB217 and RRIM600. There was indication of possible biological control effect of a mixed inocula of *A. pullulans* and *A. niger* on *B. theobromae* growth especially for clones PB217 and RRIM600. This study using NaPCP was undertaken prior to its ban effective 1 January 2000. Therefore, this study does not imply an endorsement for continued use of NaPCP in the market.

Much more needs to be understood about the variation in wood quality among rubberwood clones, as well as the relative antagonism or synergism among fungi which may influence relative stain susceptibility of wood when researching into a non-toxic (biological control) approach for sapstain control in rubberwood and other sapwood species.

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References

ABRAHAM, L. D. & BREUIL, C. 1993. *Organic Nitrogen in Wood: Growth Substrates for a Sapstain Fungus.* Document No. IRG/WP/93-10019. International Research Group on Wood Preservation, Stockholm. 15 pp.

ASHARI, A. J., PALFREYMAN, J. W. & WONG, A. H. H. 1999. *Association of Contents of Nitrogen and Sugars in Rubberwood (Hevea brasiliensis) Clones With Susceptibility to Sapstain by Botryodiplodia theobromae, Aureobasidium pullulans and Aspergillus niger.* Document No. IRG/WP 99-10307. International Research Group on Wood Preservation, Stockholm. 15 pp.

BENKO, R. 1989. *Biological Control of Blue Stain on Wood With Pseudomonas cepacia 6253. Laboratory and Field Test.* Document No. IRG/WP/1380. International Research Group on Wood Preservation, Stockholm. 6 pp.

BJURMAN, J. 1989. *Influence of Storage on Mould Susceptibility of Wood at RH Values Lower Than 100%.* Document No. IRG/WP/1413. International Research Group on Wood Preservation, Stockholm. 8 pp.

COWLING, E. B. 1970. Nitrogen in forest trees and its role in wood deterioration. *Abstract of Uppsala Dissertations in Science* No. 164. 19 pp.

COWLING, E. B. & MERRILL, W. 1966. Nitrogen in wood and its role in wood deterioration. *Canadian Journal of Botany* 44: 1533–1544.

FLORENCE, E. J. M. & SHARMA, J. K. 1990. *Botrydiplodia theobromae* associated with blue staining in commercially important timbers of Kerala and its possible biological control. *Material und Organismen* 25: 193–199.

GRANT, C. 1973. The use of a reflectance method for estimating surface mould growth on chipboard. *International Biodeterioration Bulletin* 8: 139–140.

HIGHLEY, T. & CROAN, C. S. 1991. *Laboratory Studies on Control of Sapstain and Mould on Unseasoned Wood by Bacteria.* Document No: IRG/WP/1493. International Research Group on Wood Preservation, Stockholm. 7 pp.

HONG, L. T. 1976. A blue stain organism of jelutong (*Dyera costulata* Hk.f.). *Malaysian Forester* 39: 177–188.

HONG, L. T. & WONG, A. H. H. 1994. *Biodeterioration and Preservation of Rubberwood (Hevea brasiliensis).* Document No. IRG/WP/94-10084. International Research Group on Wood Preservation, Stockholm. 12 pp.

KJELDAHL, J. 1883. A new method for determining nitrogen in organic materials. *Journal Analytical Chemistry* 22: 366–369.

KREBER, B. & MORRELL, J. J. 1993. Ability of selected bacterial and fungal protectants to limit fungal stain in Penderosa pine sapwood. *Wood and Fiber Science* 25(1): 23–34.

LEVI, M. P. & COWLING, E. B. 1969. Role of nitrogen in wood deterioration. VII. Physiological adaptation of wood-destroying and other fungi to substrate deficient in nitrogen. *Phytopathology* 59: 460–468.

MORRELL, J. J. & SEXTON, C. M. 1992. *Effect of Nutrient Regimes, Temperature, pH and Wood Sterilisation Method on Performance of Selected Bioprotectant Against Wood Staining Fungi.* Document No. IRG/WP/1551-92. International Research Group on Wood Preservation, Stockholm. 11 pp.

MARISTANY, A. G., LEBOW, P. K., BRUNNER, C. C., BUTLER, A. D. & FUNCK, J. W. 1992. Classifying wood-surface features using dichromatic reflection. Pp. 56–64 in de Shazer, J. A. & Meyer, G. E. (Eds.) *Optics in Agriculture and Forestry. Proceedings of the International Society for Optical Engineering* Vol. 1836. Bellingham, Washington.

RAMLI, O., ARSHAD, N. L., ONG, S. H., OTHMAN, H., BENONG, M., WAN CHIK, M. G., MOHD ZAIN, A. Z., ZAWAWI, A. G. & MOHD. NOOR, A. G. 1996. Potential *Hevea* genotypes for timber production. Pp. 340–359 in Abdul Aziz, S. A. K. (Ed.) *Proceedings of Rubber Growers' Conference.* 17–19 July 1995. Rubber Research Institute Malaysia, Kuala Lumpur.

SCHMIDTLING, R. C. & AMBURGEY, T. L. 1982. Genetic variation in decay susceptibility and its relationship to growth and specific gravity in loblolly pine. *Holzforschung* 36: 159–161.

SCORE, A. J. & PALFREYMAN, J. W. 1994. Biological control of the dry rot fungus *Serpula lacrymans* by *Trichoderma* species: the effects of complex and synthetic media on interaction and hyphal extension rates. *International Biodeterioration & Biodegradation* 33: 115–128.

SEXTON, C. M., MARISTARY, A. G., BRUNNER, C. C. & MORRELL, J. J. 1993. *Using Image Analysis to Rate Wood Stain Trials.* Document No. IRG/WP/93/10034. International Research Group on Wood Preservation, Stockholm. 7 pp.

SHARPE, P. R. & DICKINSON, D. J. 1992. *Blue Stain in Service on Wood Surface Coatings. Part 1—The Nutritional Requirements of Aureobasidium pullulans.* Document No. IRG/WP/1556-92. International Research Group on Wood Preservation, Stockholm. 25 pp.

SRINIVASAN, U., BRUCE, A. & STAINES, H. J. 1992a. *Effect of Media Composition on the Antagonistic Properties of Trichoderma spp. Against Wood Decay Fungi.* Document No. IRG/WP/1538-92. International Research Group on Wood Preservation, Stockholm. 25 pp.

SRINIVASAN, U., STAINES, H. J. & BRUCE, A. 1992b. Influence of media type on antagonistic modes of *Trichoderma* spp. against wood decay basidiomycetes. *Material und Organismen* 27: 301–321.

WAZNY, J., RUDNIEWSKI, P., KRAJEWSKI, K. J. & WAZNY, T. 1989. The reflectance method for testing the effectiveness of fungicide against surface mould growth on materials: I. Wood. *Wood Science & Technology* 23: 179–189.

WONG, A. H. H. & WOODS, T. L. 1997. ***Performance of Tuff-Brite C™ and Other Formulations Against Blue Stain, Mould and Brown Stain in Freshly Sawn Rubberwood (Hevea brasiliensis) in Humid Tropics of Peninsular Malaysia.*** Document No. IRG/WP/97-30163. International Research Group on Wood Preservation, Stockholm. 20 pp.

WONG, A. H. H., ABDUL RAZAK, M. A. & LING, W. C. 1999a. Sapstain control in Malaysia—a research perspective. Paper presented at the Fifth Conference on Forestry and Forest Products Research. Series: Challenges to Wood Preservation Industries in the Next Millennium. 18 May 1999. Forest Research Institute Malaysia. 30 pp.

WONG, A. H. H., EDEN, D., CHITTENDEN, C., HEDLEY, M. & WAKELING, R. 1999b. Comparison of the FRIM and Forest Research laboratory methods for screening of anti-sapstain formulations. Document No. IRG/WP/99-20170. International Research Group on Wood Preservation, Stockholm. 12 pp.