Temporal composition of tannin and carbohydrate content in *Eucalyptus* leaves in South Africa

by

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ABSTRACT

In South Africa, the genus *Eucalyptus* plays an important role as a plantation tree and hence forms a major economic component in the forest sector. An insect pest of these *Eucalyptus* species, *Gonipterus scutellatus,* causes periodic defoliation in the plantations. Plants have extraordinary array of chemicals (secondary metabolites), which defend them from herbivores. This study reports on the seasonal variation of the tannin concentration and carbohydrate content of the leaves of nine *Eucalyptus* species. Soluble tannins were quantified using Hagerman test and the carbohydrate content with a refractometer. Generally high tannin concentrations were observed during spring and late summer with low concentrations during autumn and winter. During the months of February, April, July tannin concentration was usually found to be higher than the carbohydrate content. The higher concentration of tannin could be linked to the allocation of carbon for the production of tannin instead of carbohydrates for growth.

1. INTRODUCTION

The genus *Eucalyptus* has numerous species growing worldwide in a range of environmental conditions. In South Africa, *Eucalyptus* is of great economic importance and make up approximately 40% of forestry plantations. The wood is used for the production of timber and is the basis of pulp and paper (Anonymous, 1998).

Insects cause severe defoliation to the eucalypts, notably the Eucalypt snout beetle (*Gonipterus scutellatus,* Gyll.). The beetle was introduced to South Africa from Australia in 1916 and is now a common pest in many countries outside Australia. *Eucalyptus* leaves are generally tough and leathery and many insect species preferentially feed on newly flushed leaves which are much softer (Tooke, 1955).

Both the larvae and adults of the Eucalypt snout beetle feed on the eucalypt trees. The newly hatched larvae always emerge from the egg capsule ventrally by eating their way through the leaf so that the egg capsule appears intact (Tooke, 1955). Trees, which are not killed, are severely stressed and become more attractive and susceptible to the snout beetle (Tooke, 1955).

Plants have an extraordinary array of chemical and structural characteristics, which provide them partial protection from herbivores (Rosenthal and Janzen, 1979). Chemical defenses include secondary metabolites, and in this study the focus is tannins. Tannins are referred to as secondary metabolites because they are considered non-essential to plant metabolism (Sauvesty *et al.*, 1992).

Tannins can be defined as naturally occurring compounds of high molecular weight with the ability to form complexes with proteins. Tannins are a group of plant metabolites, which have the ability to precipitate protein. They act by complexing with leaf proteins, thereby reducing the suitability of plants for herbivores; they have a repellent, bitter taste (Hagerman, 1987).

The tannin content of various plants should be correlated with patterns of herbivory to establish the role of tannin as a defensive compound (Hagerman, 1987). Levels of tannins in plants can be influenced by factors such as climate, soil, water availability, age of a plant and maturity of the leaves. Tannins are phenolic compounds that are synthesized and accumulated when the plant is under stress. Phenols can be located in any part of the plant (Sauvesty *et al.*, 1992).

Tannin interacts with carbohydrates Woody plants can use carbohydrate to produce a variety of secondary compounds, which provide protection from insect attack. Tannins can form complexes with the carbohydrates and the herbivore's digestive enzymes when it consumes the plant, thereby disrupting the digestive process and reducing nutrients availability (Sauvesty *et al.*, 1992). Carbohydrate availability and carbon based secondary compounds are determined by the direct effect of light and carbon dioxide availability on photosynthetic rates (Koricheva *et al.*, 1998).

This study investigated the temporal relationship between levels of tannin and carbohydrate in the leaves of nine eucalypts species of, which had varying susceptibilities to the Eucalypt snout beetle (*Gonipterus scutellatus*).

2. LITERATURE REVIEW

2.1 IMPORTANCE OF *EUCALYPTUS* SPECIES

The eucalypts are of great economic importance both in Australia and in many other countries where it has been successfully introduced. They represent major timber resources but in many cases they also lend themselves to commercial exploitation for wood distillates, tannins, essential oils, nectar and pollen (Polynton, 1979). Wood from the *Eucalyptus* trees is used for the manufacture of paper, viscose and rayon, construction timber, mine props and for fuel. The leaves of some species are used for the production of essential or volatile oils in varying amounts (Polynton, 1979).

The major constituent of this oil is eucalyptol (cineol), which has diaphoretic, disinfectant, expectorant and antimalarial properties. As an antiseptic it is used in the treatment of infection of upper respiratory tract and in certain skin diseases. The gum is used to treat diarrhoea, cuts, haemorrhage and as an astringent in dentistry, (Sastri 1952; Penfold & Willis, 1961). Some plantings of these trees have proved useful for the prevention of soil erosion. Eucalypt trees are however, susceptible to environmental stress like water, fungal attack and insect damage (Polynton, 1979).

2.2 HERBIVORY

Herbivores can exert a major impact on plants. Insects have caused greater economic loss in agriculture and forestry than the combined effects of damage from droughts and freezing. They have caused greater tree mortality than does logging (Coley *et al.*, 1985). On average more than 10% of the plant production in natural *Eucalyptus* communities is consumed annually by herbivores (Coley *et al.*, 1985).

2.2.1 *Gonipterus scutellatus* (Eucalypt snout beetle)

The most important of the introduced insect pest of eucalypts in Southern Africa is the Eucalypt snout beetle (*Gonipterus scutellatus*), which reached South Africa from Australia in 1916 (Tooke, 1955). The colour of the body varies; newly emerged beetles are bright rusty red with an X-shaped mark over the elytra and a white scutellar mark often forming a median prothoracic stripe extending to the head. As the insect grows older it becomes dark brown. The ventral surface is usually light grey in colour (Tooke, 1955).

There is a distinct difference in the size of the sexes, the female being considerably larger than the male. The sex ratio is 3: 2, the female predominating. The adults are strong fliers and can attain the perfect local distribution unaided. In the past almost every homestead and railway station had at least a few eucalypt trees and the insects had great opportunities to spread long distances by flight in successive generations (Tooke, 1955).

Eucalypt transplants from nurseries may also have had egg capsules and larvae on their leaves or even pupae in the soil in which they are rooted, further enhancing the spread of the beetle in the past (Tooke, 1955).

Although *Gonipterus scutellatus* will mate readily in captivity, it is only on rare occasions that it will oviposit under such conditions, and then only on the youngest and tenderest leaves. The refusal to lay eggs on the older leaves is as noticeable in plantations as in the laboratory and would seem to point to the ability of holding back eggs when conditions are unsuitable for oviposition. About 1000 beetles produced 100-200 capsules eggs per day for several successive days, representing about 900-2000 eggs per day (Tooke, 1955).

Oviposition preferences of many insects are known to change depending on the availability of certain hosts. Alternate hosts may be used if a preffered species is absent, but largely or completely unused if it is present (Wiklund, 1981; Nylin and Janz, 1993; Bernays and Chapman, 1994).

The lifespan of the adult depends on temperature; the beetles can live from two to seven months (Richardson and Meakins, 1986) Both adults and larvae feed on the leaves and buds of their eucalypt hosts and can cause extreme defoliation of susceptible trees. Management of insect populations includes planting resistant eucalypt species and biological control using mymarid egg parasitoids (Richardson and Meakins, 1986)

The larvae cause the greatest damage; they devour the entire epidermis of the leaf, which soon becomes dry and brown. The first noticeable feature of infested trees is the brownish scorched appearance of the very young foliage due to the removal of the soft surface tissues. As the infestation increases, the young soft twigs are attacked and destroyed with the result that buds lower down are forced into growth (Tooke, 1955).

2.3. SECONDARY METABOLITES

Many plants appear to have broad-spectrum defenses against a large suite of enemies, including insects, herbivores, vertebrates and pathogens. These defenses can be physical or chemical in nature. Such chemicals are often called secondary plants compounds (Gullan & Cranston, 1994).

Plants have evolved an extraordinary array of secondary metabolites, which act, as anti herbivore defenses and which appear not to be waste products nor serve any other known function in the plant. They have been implicated as defensive agents in plant-plant, plant-pathogen and plant-herbivore interactions (Rhoades, 1979). Plants do not produce sufficient quantities of toxic compounds to provide complete protection of all tissues; instead the secondary metabolites occur in varying amounts in different parts of the plant (Rhoades, 1979).

Secondary compounds may act in one or two ways. At a behavioural level these chemicals may repel an insect or inhibit feeding or oviposition. At a physiological level, they may poison an insect or reduce the nutritional content of its food. The same chemicals that repel some insect species may attract others, for either oviposition or feeding. Such attracted insects are said to be adapted to the chemicals of their host plants, by tolerating, detoxifying or even sequestering them (Gullan & Cranston, 1994).

Secondary metabolites are so called because they are considered non-essential to plant metabolism (Harborne 1997). They have a broad spectrum of activities (deterrence of herbivores, allelopathic attraction of pollinators and organisms predating on herbivores) and when used by plants to deter antagonists it is adaptive for them to have such broad activity (Harborne 1997).

All plants have a characteristic phenolic fraction (Harborne 1997). The cell vacuole is generally assumed to be the main storage organ of phenols in the vegetative tissues of plants. Most phenols are found in combination with sugars or related compounds such as glycosides or esters (Haslam, 1974).

Secondary metabolites may include poisonous compounds whose concentration in the cell tends to be relatively low (e.g. cyanogenic glycosides, alkaloids). Some of these secondary metabolites accumulate to levels that are high enough to reduce the plant digestibility and palatability by herbivores (Haslam, 1989). Digestion reducing compounds are mostly of a phenolic nature, being predominantly tannins (Harborne, 1997).

Secondary compounds have been classified into two broad groups, the qualitative or toxic (are effective poisons in small quantities for example alkaloids), and the quantitative (act in proportion to their concentration, being more effective in greater amounts (for example tannins) (Gullan & Cranston, 1994).

Leaves of woody species contain higher concentrations of soluble phenols than leaves of herbaceous species (Poorter & Villa, 1997). Large long-lived trees are more apparent to an insect than small, scattered annual herbs. These apparent plants tend to have quantitative secondary compounds, produced at a high metabolic cost while unapparent plants often have qualitative or toxic secondary compounds, produced at little metabolic cost (Gullan & Cranston, 1994).

Fast growing trees adapted to resource rich habitats suffer higher rates of damage from herbivores and have both lower amounts and different types of defensive chemicals than slow growing species (Coley *et al.,* 1985). Defoliation has been shown to induce changes in the tree leaf quality (Werner, 1979, Haukioja *et al.*, 1981).

When defoliation events remove the foliage nutrients of trees growing in nutrient poor soils, it causes an increase in nutrient stress, which in turn results in a high production of carbon-based allelochemicals. The excess carbon that cannot be diverted to growth due to nutrient stress is diverted to the production of plant secondary metabolites (Tuomi *et al.,* 1984).

The diverse array of secondary metabolites in plants has been an important factor leading to dietary specialism and speciation in phytophagous insects (Rhoades, 1979). It is no accident that the most widespread defensive plant secondary metabolites (tannins and related phenolics) act mainly by inhibiting the digestive processes that provide protection against herbivores (Rhoades, 1979). Phenolics are the most widespread and major class of ecologically important secondary compounds. This group includes simple phenolic acids, flavonoids and polyphenols such as tannins (Rhoades, 1979).

2.4. TANNINS

2.4.1. Tannin

Tannins are polyphenolics of varying size and complexity, with molecular weights between 500 and 200000 Dalton (Haslam, 1974). Tannins can be defined as high molecular weight polyphenolic compounds with the ability to form complexes with natural polymers such as dietary protein, digestive enzymes and polysaccharides under suitable conditions of pH and concentration (McLeod, 1974).

Tannin extracts typically contain a high diversity of chemical species of varying chain length, linkage pattern and protein-complex mixture. Tannins are plant secondary compounds that have an anti-herbivory effect. In addition to a repellent, bitter taste, tannins form insoluble complexes with proteins and inhibit protein absorption. Tannin that is added to food lowers the body mass and food consumption, reduces protein availability and increases excreta nitrogen concentration (Robbins *et al.*, 1987). In general, herbivores avoid eating plants with high tannin concentration if alternative items are available (Robbins *et al.*, 1987).

Tannins are found in approximately 80% of woody and 15% of herbaceous dicotyledonous plant species and can occur at high levels in some forages, feeds and foods (Bryant *et al*, 1992). Tannins can be stored in high concentrations in vacuoles of plant cells, and intense deposits have been found in the epidermis of leaves and in leaf trichomes (MacKey *et al.*, 1978). Tannins are generally divided into two groups: the condensed tannin and the hydrolysable tannin. (Haslam, 1974).

2.4.1.1. Condensed tannin

Condensed tannins are polymetric, formed by acid catalysed condensation of two kinds of precursors termed proanthocyanidins. The monometric precursors are flavans in which either the 3 position alone, or position 3 and 4 of the heterocyclic ring are hydroxylated (Haslam, 1989).

Condensed tannin does not contain a sugar residue and are more resistant to breakage than the hydrolysable tannins. Condensed tannins are mainly responsible for defending plants against microbial and fungal attack (Zucker, 1983).

2.4.1.2 Hydrolysable tannin

Hydrolysable tannins contain a core of a polyhydric alcohol, usually glucose, which is esterified to a number of phenolic carboxylic acids. They are divided into two types, gallo-tannins and ellagi-tannins (Swain, 1979). Hydrolysable tannins decompose in water, acids, bases or a specific tannase to yield water-soluble products such as gallic acid. Hydrolysable tannin has a very restricted distribution, being found only in dicotyledons (Harborne, 1997).

Hydrolysable tannin serves to inactivate the digestive enzymes of the herbivore, especially insects. The combined effect of the tannins (condensed and hydrolysable) would result in reduction of fermentative activity and degradation of fibrous tissue (Zucker, 1983).

2.4.2. BIOSYNTHESIS OF TANNINS

Tannin biosynthesis involves all major physiological cycles. It can be affected by intrinsic and extrinsic factors, including, light, nutrition (availability of nitrogen, phosphorus and copper), growth rate or a hormone like ethylene that would affect a participating cycle and this would also result in a change in tannin production (Salisbury & Ross, 1978).

Both condensed and hydrolysable tannin are formed biosynthetically from products of shikimic acid pathway (Haslam, 1974). The amino acids from the shikimic acid pathway are important in the synthesis of proteins as well as a wide variety of phenolic compounds such as phytoalexins, lignin, coumarins and tannins. The shikimic acid pathway utilizes the products of photosynthesis. Therefore the processes, which regulate the production of photosynthesis products, will affect these products for tannin production and consequently may affect the rate at which tannins can be produced (Salisbury & Ross, 1978).

Tannin biosynthesis is indirectly dependent on the products of photosynthesis and therefore the understanding of the process regulating photosynthesis is essential to understand the variation in tannin production (Salisbury & Ross, 1978).

2.4.2.1. Nutrients

Plants growing on nutrient-rich soils, with a good supply of all minerals, should produce fewer tannins and phenolics than plants growing on nutrient-poor soils ((MacKey *et al.*,1978; Gartlan *et al.*, 1980; Tuomi *et al.*, 1984). These weakly defended, hence palatable plants are found where habitat quality and growth potential are high, and on nutrient rich sites associated with recurrent disturbance in northern ecosystems and light gaps in the forest canopy (Coley, 1983).

Heavily protected, low preference plant species are to be expected in climax vegetation, where relative shortage of nutrients and, or light limits plant growth (Coley, 1983). Nutrient stress favours evergreen plants that grow slowly with a slow rate of nutrient turn over, however, because evergreen leaves contain a higher proportion of the plant's nutrient capital than deciduous leaves, the plant is particularly sensitive to herbivory and hence evergreen leaves are protected by defensive compounds. These defenses should be best developed on infertile sites where plants can least afford to loose the nutrients contained in the leaves (Chapin, 1980).

On nutrient rich soils fallen leaves had low phenolic content and high nutrient content. Different rates of leaf turn over and nutrient turnover in different forest ecosystems (even when the same tree species is dominant) are due to the decomposing system, which is influenced by the phenolic and mineral contents of the leaves (Nicolai, 1988). Deciduous shrubs growing on nutrient rich sites have rapid growth, high leaf turnover and little investment in defense. Slow growing evergreen shrubs are favored on the nutrient poor sites; leaf turnover favors investment in defense of the leaves (MacLean & Jensen, 1985).

Plants with unconstrained supply of nutrients show significantly lower leaf starch and tannin concentrations as compared to plants grown in high light with moderate nutrient supply (Waring *et al*, 1985). Plants with balanced nutrition are expected to grow at moderately high units. The leaf rates are expected to have sufficient carbohydrates and soluble nitrogen resources to construct a variety of defensive compounds preceding or following attack by insects or pathogens (MacLean & Jensen, 1985).

Plants occurring on infertile soils do not have sufficient resources to support rapid growth. Such plants grow slowly even in most favourable environments and have low capacities to photosynthesize and absorb nutrients. The plant organs live longer, thus making them more available for attack by herbivores for longer times. Species adapted to growing on nutrient-poor soils will have slow growth rates and high concentrations of anti-herbivory defense chemicals (Coley *et al.*, 1985).

2.4.3 ROLE OF TANNIN

2.4.3.1 Plant tannins and herbivores

Tannins like any other chemical in the food, may be perceived by the insects through its peripheral chemoreceptors and may induce an overt behavioral response; either acceptance or rejection of the food. Alternatively or additionally tannins may produce some physiological effect after indigestion, this may be favourable to or adverse for the insect (Swain, 1979).

A reduced palatability and total intake of fodder in cattle can also be ascribed to tannin as it renders plant material characteristically astringent to the taste (Zucker, 1983). This can be explained by a loss of lubrication in the mouth, caused by a tannin precipitation with the saliva and glycoproteins of the mouth epithelium. When tannin occurs in high concentrations, a certain fraction of tannin (those that did not precipitate with the mucoprotein secretions of the digestive tract) can react with proteins of the outer cellular layer of the gut and thus inhibit the absorption of nutrients through the gut wall (McLeod, 1974).

Hydrolysable tannin can be degraded in the intestines to sugars and phenols and is then absorbed into the blood stream. If the detoxification mechanism of the herbivore is inadequate to handle these phenols, it can lead to gastritis, intestinal irritation, liver and kidney damage and eventually death (McLeod, 1974).

Some herbivores have adapted to feeding on tannin- rich diets and they can distinguish the quality of the tannin and prefer one plant species to another because of difference in the tannins present. This difference in feeding behaviour appears to be due entirely to chemical variation in the proanthocyanidins present (Clausen *et al.*, 1990).

Specialized insects are often attracted by defensive substances in or emitted by their food plants. The attractive response may not be due to any beneficial properties of the substance but to the fact that the plant emitting these substances, which are detoxifiable by the insects, is a known food source (Rhoades, 1979).

Baas (1989) describes carbon of the plant as the main driving force for the carbon and nutrient system, where phenolic biosynthesis (as a result of the excess carbon) is linked to plant stress as expressed in habitat quality (substrate fertility, availability of water and light) and defoliation patterns.

2.4.4. AVAILABLE METHODS FOR DETERMINING TANNIN

The analytical methods currently available for determining tannin have several disadvantages. The redox methods such as the Folin-Denis assay (Folin & Denis, 1951) are not specific for tannin, but detect phenolic compounds. The proanthocyanidin and vanillin assays (Bate-Smith, 1975; Price *et al.*, 1978) are known to be too selective.

The hydrolysable tannins, which are gallic acid derivatives (Haslam, 1979); do not react with acidic butanol or vanillin. Only the flavonoid-based condensed tannins (Haslam, 1979) can be detected with these reagents. Precipitation methods also have disadvantages. In one method a dye –labeled protein is used and the amount of protein precipitated by the tannin is determined spectrophotometrically (Asquith & Butler, 1985).

In another method, the tannin precipitated by excess protein is measured spectrophotometrically after reaction with ferric acid (Hagerman & Butler, 1978). Although these methods are straightforward, sample precipitation for these assays is complicated. Some solvents such as acetone interfere with the precipitation and must be removed from the extract before analysis (Hagerman, 1987).

Radial diffusion method for determining tannin in plant extracts, whereby tannin diffuses through a protein-containing gel and a visible disk-shaped precipitate develops as the tannin interacts with the protein overcomes all the problems. The method is simple, specific, sensitive and applicable to studies in which large numbers of samples are to be analyzed Application of the radial diffusion method in studies of herbivory should enable ecologists to establish whether tannins defend plants from insects and other herbivores (Hagerman, 1987).

2.5. CARBOHYDRATES

Carbohydrates are essential components of life as structural and energy storage components, as stabilization, recognition, signaling and communication agents (Coutinho & Henrissat, 1999).

They are the direct products of photosynthetic activity and constitute metabolites as well as structural building. They are amongst the various reserve compounds and they are quantitatively abundant (Bonicel *et al.*, 1987).

The starches and glycogens, long chain polymers of glucose, the structure of which differs from that of cellulose, are the media for energy storage in plants and animal. The amount of carbohydrate found in nature is larger than that of any other group of natural compounds. The most abundant organic substance on earth is cellulose, a polymer of glucose, which is the structural material of plants (Sharon, 1975).

An understanding of the various plant physiological processes frequently depends on accurate analyses of soluble and soluble sugars in different plants parts. The level of soluble sugars can be interpreted as a parameter of the plant's energy status. The soluble sugar pools in plants consist mainly of three individual sugars, glucose, fructose and sucrose (Mooney, 1972).

A study of the seasonal variations of carbohydrates provides an indication of the part they play in the tree's physiology and it also allows one to relate the various stages of phenological phases (Bonicel *et al.*, 1987). The changes in carbohydrate levels appear to depend on the species studied, the climatic and edaphic conditions of its plantation, amongst a variety of other influencing factors (Levitt, 1980).

Low temperatures are an important environmental factor limiting the productivity and geographical distribution of plants in larger areas of the world. Perennial plants such as woody species respond to the changes in environmental parameters associated with the seasonal cycles to become acclimated to winter temperatures. Acclimation in many plants is the process, which allows transition from a cold, tender state to the cold, hardy state. Several studies have shown a relationship between soluble carbohydrate accumulation and cold resistance (Bonice *et al.*, 1987 & Ficher and Höll, 1991).

The ability of carbohydrates to interact and protect membrane vesicles during freezing, thawing and drying has been reported and it is believed to be due to their capacity to interact with phospholipids in the manner which imitates the presence of water (Caffrey *et al.*, 1988). Pooled carbohydrate levels increase in response to drought and decrease in response to ozone exposure. Soluble sugars also increase in response to drought. Direct effects of light and CO_2 availability on photosynthetic rates are more important determinants of carbohydrate availability (Koricheva *et al.,* 1998).

Plants with an unconstrained supply of nutrients show partitioning of carbohydrates as compared to plants with moderate nutrient supply (Waring *et al.*, 1985). Therefore secondary metabolite production is inversely related to plant nutrients in species and in environmental conditions where growth is limited by nutrients (especially nitrogen and phosphorus) rather than by carbohydrate reserves (Mattson, 1980). Fast growing species have high concentrations of soluble sugars (Pooter & Villa, 1997).

3. MATERIALS AND METHODS

3.1. MATERIALS

Fully-expanded leaves of nine species of *Eucalyptus* (Myrtaceae) were used. According to previous studies, these species range from resistance to susceptible to the Eucalypt snout beetle (*Gonipterus scutellatus*). These species included *Eucalyptus fastigata, Eucalyptus benthamii, Eucalyptus cypellocarpa, Eucalyptus badjensis, Eucalyptus andrewsii/ campanulata, Eucalyptus dunnii, Eucalyptus macarthurii, Eucalyptus smithii* and *Eucalyptus nobilis.*

Leaves were collected from a tree breeding trial in Masonite plantations, which have twenty eucalypt species. The plantation is situated at Draycott, Central Drakensberg in the Kwazulu Natal Province (28° 59' S; 29° 37' E, Altitude 1580 m, soil type: Mispah and 10-35cm deep). The felled trees were allowed to coppice from August 2001 to July 2002. Different species plots were replicated three times and laid out in a randomized complete block design. Each plots consisted of 25 trees.

Ten to 12 leaves were collected from each eucalypt tree species every two months; starting in February, April and July 2002. During September 2002, juvenile, medium and old leaves were collected separately for comparison purposes. Mostly juvenile and medium leaves were evaluated in this study. The leaves were placed in brown bags, marked with the plot number and a tree number and frozen at -70° C overnight.

The samples were freeze-dried under reduced pressure in an Edward freeze drier (Mondulyo) for three days and grounded into a fine powder using a mortar and pestle. The powder was sifted with a tea strainer to ensure uniformity of particle size and to maximize contact area for efficient extraction, since a reduction in particle size favours solvent penetration into the material (Sauvesty *et al.*, 1992; Pooter and Bergkotte, 1992).

3.2. METHODS

3.2.1 Extraction (Appendix1)

The procedure for extracting plant material was adapted from (Sauvesty *et al.*, 1992; Pooter and Bergkotte, 1992, Regnier, 1994). Leaf powder, 0.05g was weighed into an Eppendorf tubes. Four replicates per sample were done. One ml of methanol/acetone/water (7:7:1, v: v: v) was added and vortexed in a MixiMatix 90W vortex (Jencons Scientific Ltd., England) for one minute. The Eppendorf tube was placed overnight at room temperature on a rotating shaker, 150rpm (Labcon 1077k). Suspensions were centrifuged at 10^4g for one minute in a microcentrifuge 7200g (Denver Instrumental Company, USA).

Supernatant was retained and one more extraction was performed using 1ml methanol/acetone/water (7:7:1, v: v: v) on the remaining material (retained as a pellet). The Eppendorf tube was vortexed, placed for 30 minutes on a shaker and then centrifuged. The second supernatant was added to the first supernatant. Two more extractions were performed using 1ml methanol/chloroform on the pellet. The tubes were vortexed, placed for 30minutes on a Labcon 1077k rotating shaker (150rpm) and centrifuged at 10^4g for 1 minute in a microcentrifuge.

Distilled water was added to each supernatant to separate the dark green non-polar chloroform precipitate from the clear polar alcohol and water-soluble supernatant (Pooter and Bergkotte, 1992). Tubes were centrifuged at 1000g for 1 minute to obtain a clear supernatant. If any chlorophyll was detected, more water was added and then centrifuged repeatedly.

All the supernatants were combined, partially evaporated in an Eppendorf Concentrator 5301 (Germany) at 45°C under reduced pressure. Water (0.5ml) was added until separation occurred and centrifuged for one minute at 1000rpm. The clean supernatant (water/methanol) containing the soluble tannins and the soluble carbohydrates were adjusted to 1ml with aqueous methanol (50%).

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3.2.2 MEASUREMENTS

3.2.2.1. TANNIN ASSAY (Radial Diffusion Assay, Hagerman, 1987)

Reagents:

1. Buffer:
   - 400ml distilled water
   - 1.425 ml concentrated acetic acid
   - 0.053g ascorbic acid
   - Adjust to pH 5.0 with 2M NaOH
   - Add water to 500ml
   - Autoclave the buffer solution for 1hr to prevent fungal and bacterial growth.

2. Agarose plates
   - 100ml acetate buffer
   - 1g agarose
   - Add 1g agarose to 100ml buffer and microwave for 2 min
   - Adjust temperature to 35-40^{0}C under running water or place in a water bath at 37^{0}C, place a thermometer inside the tube and stir continuously

- Add 0.1g Bovine Serum Albumin: [Fraction V powder, 96-99% albumin (Sigma A - 3350)] when mixture reaches about 37^0C,
- Mix and pour 10 ml per plate (8.5 cm diameter), quickly using a glass pipette, do not allow the liquid to bubble
- Let plates cool without condensation on the lid
- Seal with parafilm and store at 4^0C, up to four days

Assay:

- Punch four holes in a Petri dish filled with agarose solution (4.0 mm) with the rear-end of a glass syringe (Pasteur pipette) and remove the plug with forceps
- Dispense 80 µl on each hole, wait until liquid evaporates
- Close lid and seal with parafilm
- Incubate at 30^0C for 96 hours
- Measure the diameter of the precipitation ring formed (after 96h) twice, at right angles. Tannin molecules migrate through agarose gel, which contains Bovine Serum Albumin (BSA). The tannin-protein complex is formed in the gel, which appears as a ring. The diameter of the ring is a measure of protein precipitation/binding capacity of tannins.
- The square of the diameter is equal to amount of tannin
- Determine least significant difference (LSD) among different extracts
- Determine correlation co-efficient between methods (extraction with boiling water and methanol)
- Plot curves (standard curve and tannin concentrations in MeOH and water extracts)
- The diameters of the rings are measured, for each ring; two diameters at right angles to one another are measured to minimize errors due to nonuniform ring development.
- Tannin concentrations are calculated from the square of the average of the two diameters using an appropriate calibration curve (Fig. 1) set up with tannic acid as a standard ($R^2 = 0.9802, y = 25.888x$).

3.2.2.2. CARBOHYDRATES

The level of carbohydrates was determined using a hand refractometer, which is an easy to use instrument that measures soluble solids, mostly sugars (Westwood, 1978). Carbohydrates were expressed in % bricks.

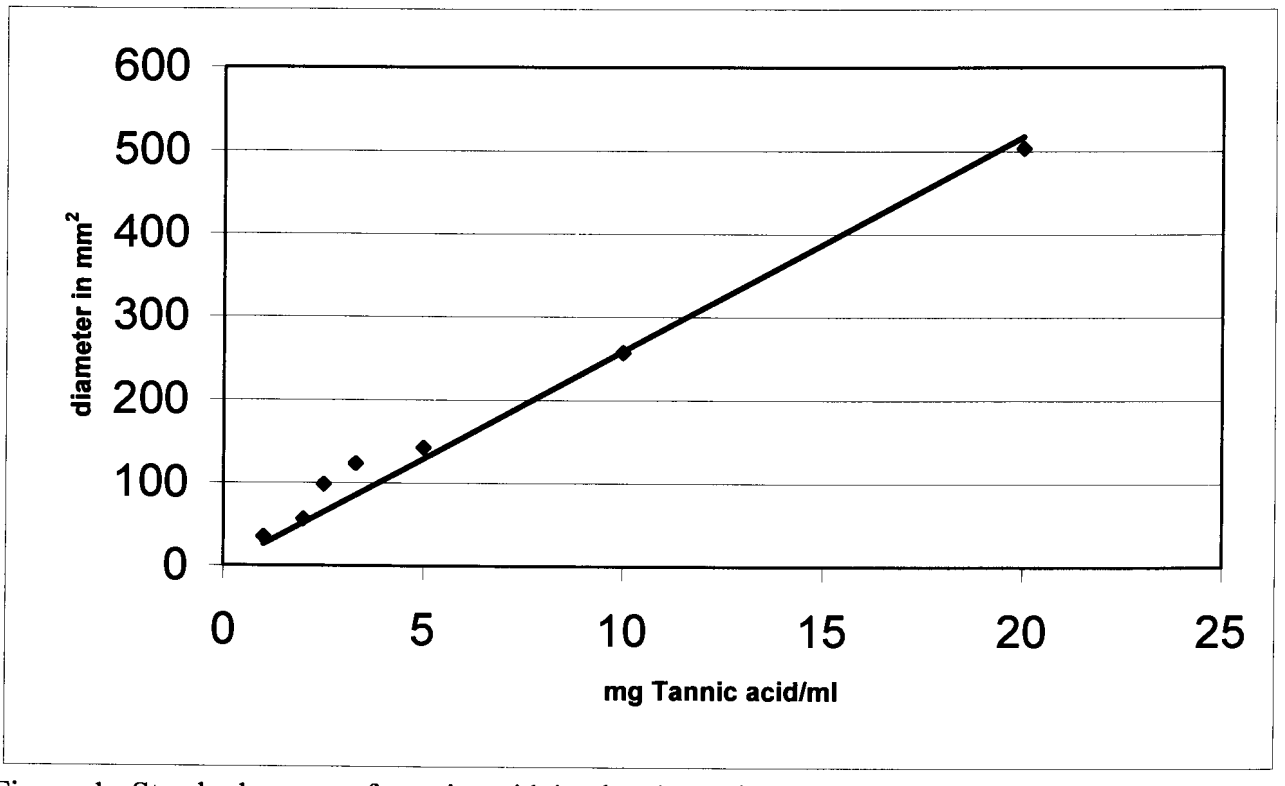

Figure 1: Standard curve of tannic acid in the determination of protein precipitating ability through the radial diffusion assay (Hagerman, 1987).

4. RESULTS

There were significant differences in the level of tannin between nine *Eucalyptus* species during February ($F_{1, 8} = 20.04$, $P < 0.0001$), with tannin concentrations ranging from 0.71(*E. dunnii*) to 75.23 *(E. nobilis*) mg/g dry weight (DW) (Table 1). There were no significant differences between *E. andrewsii/campanulata, E. marcathurii, E. smithii, E. fastigata* and E. *nobilis* which fell in the same group.

The carbohydrate content was significantly different between the species ($F_{1, 8} = 25.97$, $P < 0.0001$) during February (Table 1) and ranged from 0.67 – 2. 48 % bricks. There were two distinct groups with *E. andrewsii/campanulata, E. smithii, E. benthamii* and *E. badjensis* falling under the first group and *E. nobilis, E. cypellocarpa* and *E. fastigata* forming another group. Other cluster includes *E. dunnii* and *E. macarthurii* which were not significantly different from each other (Table 1). The tannin concentration (expressed as mg/g dry weight) was higher than the carbohydrate content (expressed as percentage bricks) during February in all nine species, except for *E. dunnii* (Table 1).

During April, there were significant differences in the level of tannin between species ($F_{1, 8} = 6.56$, $P < 0.0001$) with the concentration ranging from 3.28 to 61.17 mg/g DW (Table 2). Three groups were observed with *E. andrewsii/campanulata* and *E. benthamii* and falling under the first group, *E. badjensis* , *E. macarthuri, E. nobilis* and *E. dunnii* forming another group and *E. fastigata*, *E. smithii* and *E. cypellocarpa* forming the third group. These various groups overlap with each other. A significant difference for carbohydrate content was observed between the species in April ($F_{1, 8} = 33.06$, $P < 0.0001$). Seven species namely; *E. andrewsii/campanulata E. benthamii, E. macarthurii, E. badjensis, E. smithii, E. cypellocarpa* and *E. fastigata* had similar carbohydrate content level ranging from 0.72 to 1.16 % bricks, but which was significantly less than *E. nobilis* and *E. dunnii* ;the latter two species also being significantly different (Table 2).

Significant differences in the level of tannin between species during July ($F_{1, 8} = 10.92$, $P < 0.0001$) with the concentration ranging from 0.76 to 60.75 mg/g DW were observed (Table 3). *Eucalyptus macarthurii, E. dunnii, E. smithii* and *E. fastigata* were not significantly different to one another but were significantly different to *E. benthamii, E. badjensis* and *E. nobilis* which fell in the same group. *Eucalyptus cypellocarpa* was significantly different from the two groups. There was a pattern in April and July, whereby *E. dunnii, E. smithii* and *E. macarthurii* had similar tannin concentration. Significant differences for carbohydrate content was observed between the species in July ($F_{1, 8} = 26.12$, $P < 0.0001$) with a range from 1.02 to 2.35 % bricks (Table 3).with *E. smithii, E. badjensis, E. andrewsii/campanulata* and *E. nobilis* forming a group.

Eucalyptus benthamii was significantly different from *E. smithii* but not significantly different from *E. badjensis, E. andrewsii/campanulata* and *E. nobilis*. *E. dunnii* and *E. cypellocarpa* formed a group which was significantly different from *E. macarthurii,* and *E. fastigata,* the latter having the same carbohydrate content (Table 3).

During September similar tannin content in the following species *E. dunnii, E. benthamii, E. andrewsii/campanulata, E. badjensis, E. nobilis* and *E. smithii* was observed in juvenile and medium leaves (Table 4). For older leaves the same pattern was observed except for *E. smithii* and *E. dunnii* which differed from the cluster. Other broad cluster in juvenile, medium and old leaves was *E. macarthurii, E. fastigata* and *E. cypellocarpa.*

A distinct group was observed whereby *E. dunnii, E. andrewsii/campanulata, E macarthurii* and *E. smithii* had similar carbohydrate content in the juvenile leaves. Two groups were observed in medium leaves with *E. dunnii* and *E. cypellocarpa* forming a group and *E macarthurii* and E. *fastigata* forming another. These groupings were different from other species, *E. badjensis, E. nobilis, E. andrewsii/campanulata, E. benthamii* and *E. smithii*, which were significantly different form each other (Table 4). *E. benthamii, E. nobilis* and *E. cypellocarpa* fell in the same group in older leaves with *E. dunnii* and *E. macarthurii* not significantly different from one another but different from the other species (Table 4).

There were significant differences in the level of tannin between the species over time (February, April and July ($F_{1,8} = 18.20$, $P < 0.0001$)). There was a decrease in the tannin concentration for *E. benthamii, E. nobilis, E. smithii* and *E. fastigata* observed during February, April and July followed by an increase in September. *Eucalyptus andrewsii/campanulata, E. macarthurii, E. nobilis* and *E. fastigata* had high tannin concentration in February and September. (Table 5).

Significant differences in the carbohydrate content between the species over time (February, April and July ($F_{1,8} = 49.93$, $P < 0.0001$)) were observed.

The carbohydrate content for *E. benthamii E. andrewsii/campanulata, E. badjensis* and *E. smithii* was low during February and high during September, with the other eucalypt species, *E. dunnii, E. macarthurii, E. nobilis, E. fastigata* and *E. cypellocarpa* having high amounts in February and low amounts in September (Table 5).

5. DISCUSSIONS

In all *Eucalyptus* species during the course of the year, a variation of the amount of tannin was generally observed with low amounts of tannins during April and July and high amounts during September. The lower amount of tannin in the leaves coincides with the hibernation of *Gonipterus scutellatus* during the dry winter months (April to July) and with the first generation emerging in September and the second generation in February (Tooke, 1955). A trend was observed for the level of tannins which supports observations that *E. benthamii E. andrewsii/campanulata, E. badjensis, E. dunnii, E. nobilis* are susceptible and that *E. smithii E macarthurii, E. fastigata* and *E.* cypellocarpa are more resistant to herbivore attack (P. Govender, personal communication).

Tannins are linked to herbivory. *Eucalyptus* species with higher concentrations of tannin appear to benefit by being less infested and damaged by *Gonipterus scutellatus*. Herbivores tend to avoid eating plants with high tannin concentrations (Coley, 1986). It has been reported that mature leaves contain higher concentrations of tannins than immature leaves so as to protect the tree from being damaged (Cornelissen & Fernandez, 2001).

The differences in the tannin concentration of the various tree species may be due to a number of factors, which include, defence, water availability and genetic composition (Tuomi *et al.*, 1984, Coley, 1986). Secondary metabolites act as anti-herbivory defences and the reduction in herbivore infestation is a function of increased defence (Rhoades, 1979; Coley, 1986). We can therefore reason that the increase in tannin concentration in September was due to the infestation by the *Gonipterus scutellatus.*

Summer rainfall and dry winter months are experienced in the trial site where the samples of leaves were collected.

In situations where defence is not considered as the main reason for tannin production, the low concentrations of tannin in young leaves can be explained by the lack of excess carbon for secondary compound production, as most carbon in the young leaves are channeled towards primary growth (Baas, 1989).

According to Koricheva *et a*l., 1998 the allocation of carbon in preference to tannin is determined by resource availability. There was no trend in the carbohydrate level and this could be due to a number of factors including growth, light and nutrient availability.

The carbon-nutrient hypothesis by Hamilton *et al.*, 2000 assumes that carbon and nitrogen are allocated to the production of secondary metabolites after the requirements of growth have been met. Carbon based secondary compounds will increase in instances where carbohydrate accumulate in excess of growth demands. Direct effects of light and carbon dioxide availability on photosynthesis rates are more important determinants of carbohydrate availability and carbon based secondary compounds compared with the indirect effects through changes in nutrients availability (Bricks, 1990; Koricheva *et a*l., 1998).

The relationship between tannin concentration and carbohydrates indicates that the carbon nutrient status of the plant as determined by resource availability, directly controls allocation to plant secondary metabolites .This allocation in turn affects palatability of the leaves and its resistance to herbivores (Hamilton *et al.*, 2000).

6. CONCLUSIONS

An increase in the tannin concentration during September and February and a decrease during April and July supported reports that the first and second generation of *Gonipterus scutellatus* occurs during spring and late summer and that these beetles hibernate during

autumn and winter. There appears to be an inverse relationship between tannin and carbohydrate contents and this confirmed that the allocation of secondary metabolites is controlled by carbon nutrient status of the plant, which affects palatability and resistance to herbivores. This indicated that there is a relation between tannin, carbohydrates which would impact on defoliation susceptibility of various *Eucalyptus* species.

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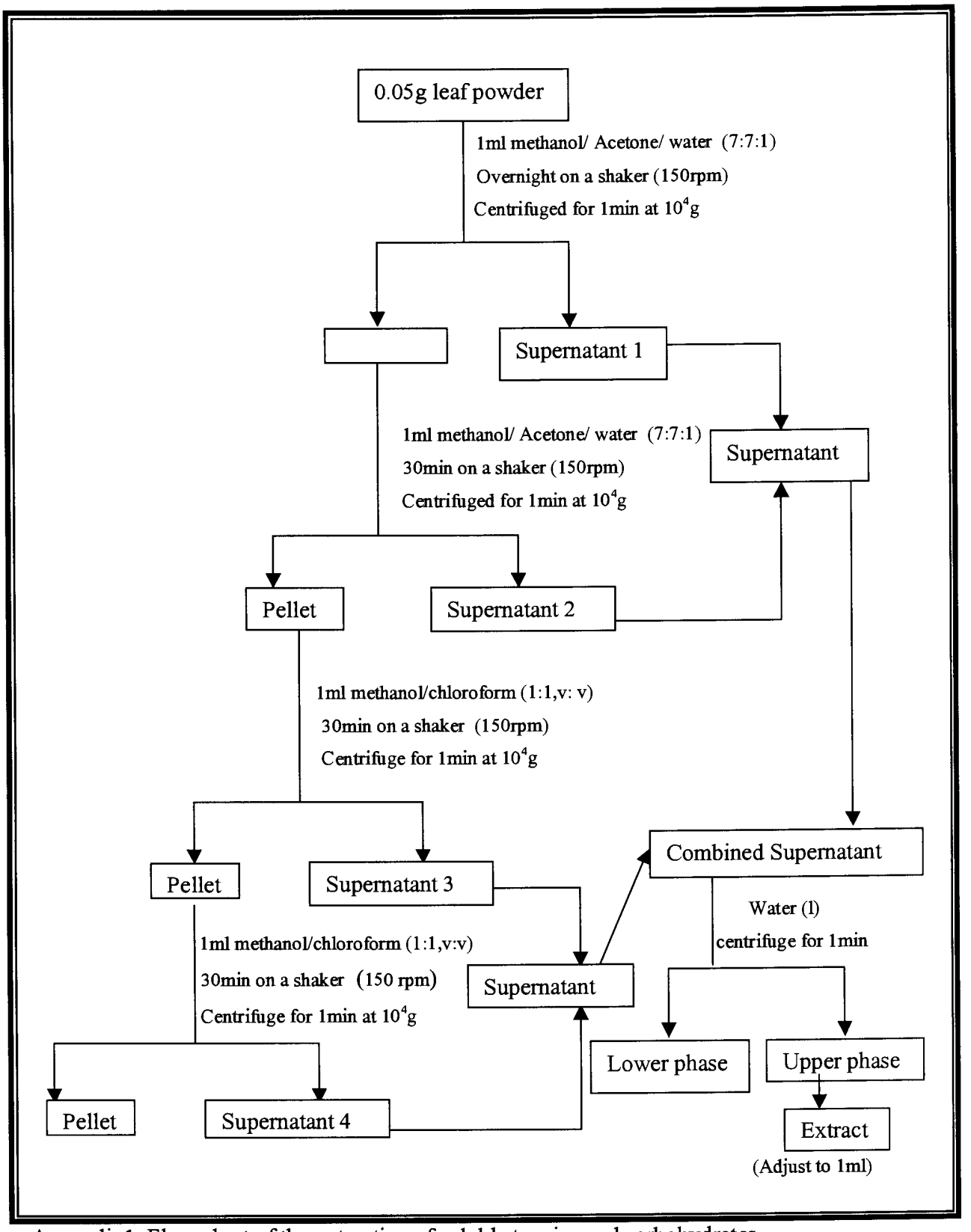

Appendix1: Flow chart of the extraction of soluble tannins and carbohydrates.

Table 1: Comparison of the Least Squares Means of tannin and carbohydrate contents between the species during February

Eucalyptus species	Tannin (mg/g DW)*	Carbohydrate (% bricks)*
E. dunnii	0.71 a	1.67 b
E. benthamii	12.36 b	0.89 a
E. badjensis	23.79 bc	0.94 a
E. cypellocarpa	37.30 cd	2.48 c
E. andrewsii/campanulata	59.26 de	0.67 a
E. macarthurii	61.44 de	1.60 b
E. smithii	64.93 e	0.86 a
E. fastigata	69.11 e	2.23 c
E. nobilis	75.23 e	2.30 c

Comparison within the columns only; tannin and carbohydrate values followed by the same letter are not significantly different ($p<0.05$) from one another.

Table 2: Comparison of the Least Squares Means of tannin and carbohydrate contents between the species during April

Eucalyptus species	Tannin (mg/g DW)*	Carbohydrate (% bricks)*
E. benthamii	3.28 a	1.00 a
E. andrewsii/campanulata	6.95 ab	0.72 a
E. nobilis	18.01 bc	1.21 b
E. dunnii	20.96 cd	1.91 c
E. macarthurii	27.71 cde	0.92 ab
E. badjensis	28.17 cde	1.16 ab
E. smithii	39.70 def	0.90 ab
E. cypellocarpa	42.17 ef	1.06 ab
E. fastigata	61.17 f	0.98 ab

*Comparison within the columns only; tannin and carbohydrate values followed by the same letter are not significantly different ($p<0.05$) from one another.

Table 3: Comparison of the Least Squares Means of tannin and carbohydrate contents between the species during July.

Eucalyptus species	Tannin (mg/g DW) *	Carbohydrate (% bricks)*
E. benthamii	0.76 a	1.43 bc
E. andrewsii/campanulata	12.23 bc	1.14 ab
E. nobilis	1.06 a	1.20 ab
E. dunnii	31.04 d	2.34 e
E. macarthurii	29.30 d	1.64 cd
E. badjensis	7.42 ab	1.09 ab
E. smithii	29.02 d	1.02 a
E. cypellocarpa	60.75 e	2.35 e
E. fastigata	26.70 cd	1.89 d

*Comparison within the columns only; tannin and carbohydrate values followed by the same letter are not significantly different ($p<0.05$) from one another.

Table 4: Comparison of the Least Squares Means of the level of tannin and carbohydrate contents between the species during September.

Eucalyptus species	Tannin (mg/g DW)			Carbohydrate (% bricks)		
	Juvenile	Medium	Old	Juvenile	Medium	Old
E. dunnii	17.79 a	30.07ab	30.45 b	0.86 a	0.93 a	4.9 f
E. benthamii	22.19 a	28.64a	8.11 a	2.04 d	2.43 f	4.33 e
E. andrewsii/campanulata	22.77 a	44.13abc	23.96ab	0.94 a	2.06 e	2.31 b
E. badjensis	26.52 a	26.16a	27.25ab	2.14 d	3.03 g	2.83 c
E. nobilis	27.43 a	31.63abc	28.73ab	1.4 b	1.1 b	4.44 e
E. smithii	30.84 ab	32.33abc	53.30 bc	0.96 a	1.87 d	1.87 a
E. macarthurii	60.89 bc	65.22 cd	77.07 c	0.76 a	1.7 c	4.74 f
E. fastigata	82.56 c	87.26 d	93.27 c	1.21 b	1.58 c	3.51 d
E. cypellocarpa	93.95 c	59.26 bcd	92.08 c	1.67 c	0.9 a	4.17 e

*Comparison within the columns only; tannin and carbohydrate values followed by the same letter are not significantly different ($p<0.05$) from one another.

RELATION CARBOHYDRATE RESISTANCE OF AGAINST

University of Pretoria

BETWEEN CONTENT 9 *EUCALYPTUS GONIPTERUS*

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TANNIN AND AND THE SPECIES *SCUTELLATUS*

INTRODUCTION

In S.A. Eucalypts are grown commercially and makes up more than 50% of all newly afforested areas. They represent major timber resources and are basis of pulp and paper industry. Insects cause severe damage to eucalypts (Fig. 1a) notably Eucalyptus snout beetle (*Gonipterus scutellatus*) (Fig. 1b).

Tannin are thought to defend the plants from herbivores. They act by complexing with leaf proteins thereby reducing the suitability of plants for herbivores. Tannin also interact with carbohydrate. High level of tannin is associated with high level of carbohydrate.

AIM

To study the relation between levels of tannin carbohydrate found in the leaves and the damage by the Eucalyptus snout beetle (*Gonipterus scutellatus*).

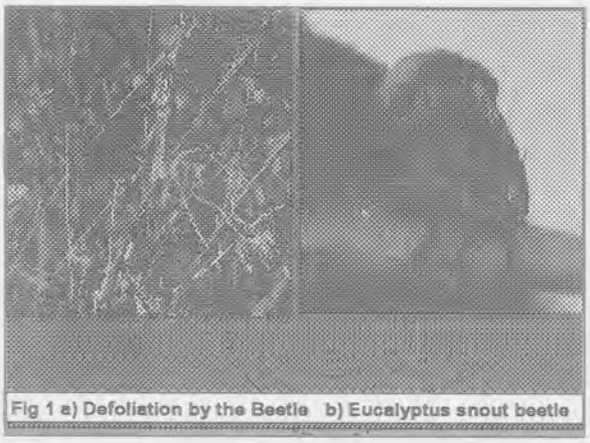

Fig 1 a) Defoliation by the Beetle b) Eucalyptus snout beetle

MATERIALS AND METHODS

- Leaves of 9 *Eucalyptus* species were collected from the Eucalyptus plantations in Natal, frozen,freeze-dried and grinded into fine powder.
- Extraction method (Poorter and Bergkotte,1992;Reguier,1994)
- 2 solutions were used(1ml methanol/Acetone/water (7:7:1) and 1ml Chloroform/methanol (1:1))
- The top part of the solution containing the tannin and carbohydrate is retained and the bottom green solution thrown away.
- Tannin assay-done using (Hagerman, 1987);Buffer and Agarose plates
- The concentration of carbohydrate was determined using a hand refractometer (Westwood, 1978)

RESULTS

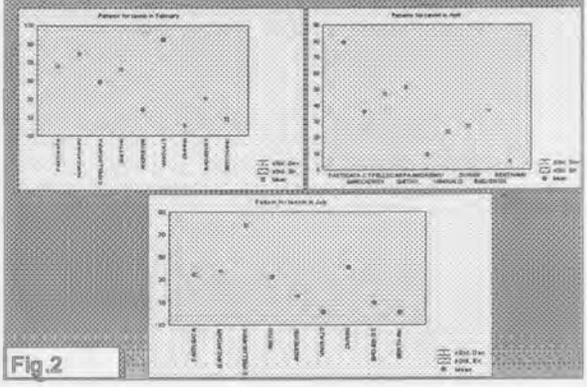

Fig.2

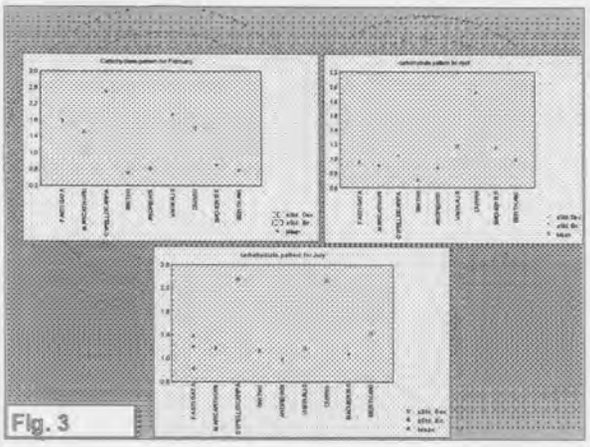

Fig. 3

DISCUSSION

Variation between species in the leaf concentration of tannins was due to resistance and susceptibility of the trees to insect attack.Plants with high levels of tannins are known to have lower damage levels of herbivory (Cates, 1986).

High levels of carbohydrates were found in February and low levels in April and July.Previous studies have shown that carbohydrates increases in the second half of the dry season (January-March) and decrease during the second half of the rainy season and the beginning of the long season (July – November) (Laur,T.,2004).

CONCLUSION

- Eucalyptus species with high levels of tannins are more resistant to defoliation than species with low levels of tannin.
- No link between carbohydrates and resistance.
- This experiments was done for 3 months and should be repeated for another year, linking the data to environmental factors.
- More extractions need to be done to find out what concentration is toxic to the beetles.

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