TEMPORAL COMPOSITION OF TOTAL SOLUBLE PHENOLIC 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 major economic component in the forest and mining sector. Unfortunately, this genus has problems due to damage by pests. The *Eucalyptus* snout beetle, *Gonipterus scutellatus*, feeds and defoliates the leaves of eucalypts. Plants produce secondary metabolites, which protects them against defoliation by insects and herbivores. This study focuses on the variations of total soluble phenolic content of nine *Eucalyptus* species between the species and within the species throughout the year. Total soluble phenols were quantified with the Folin-Ciocalteau reagent. There was a general increase in the concentration of total soluble phenols throughout the year. The content of total soluble phenols were generally higher compared to carbohydrates. This experiment needs to be done on a continuous basis so as to formulate a screening method for eucalypt species that are resistant to *G. scutellatus*.

Chapter 1

General introduction

Agriculture and forestry play an important role in developing countries like South Africa. Forestry in South Africa makes a large contribution to the economy (Forestry South Africa, 2001/2002). For the year 2001/2002 the forestry industry contributed R2.7 billion, with an annual turnover of R2.3 billion, and the contribution of the forest products industry to gross value of manufacturing was R11.8 billion (Forestry South Africa, 2001/2002). *Eucalyptus* species comprise about 38.0% of the 1,33, 944 ha. of plantation area in South Africa (Forestry South Africa, 2001/2002).

Eucalyptus species, hybrids and clones are planted commercially in South Africa for the production of pulp and paper and various other forest products (Govender, 2002). Although they grow better than in their native homerange, they do have constraints when planted as exotic species (Richardson and Meakins, 1986). Some of their constraints include being severely attacked by fungi, viruses, pathogens and insect pests. One of these insect pests is the *Eucalyptus* snout beetle, *Gonipterus scutellatus* (Gyll.). It has been observed that this pest attacks or favours a few *Eucalyptus* species whilst some species have been recorded as resistant (Tooke, 1953; Richardson and Meakins, 1986).

Tooke (1953) investigated the degree of resistance of some eucalypt species by correlating the proportion and types of essential oils with the susceptibility of eucalypts in South Africa. Although results were not consistent, it was demonstrated that eucalypt species containing low levels of cineol were generally resistant while those with high cineol levels were susceptible. Tooke (1953) also investigated the defoliation of the *Eucalyptus* species with respect to taxonomic groupings and his results showed that whilst other genera contained species that are resistant, the majority of eucalypt species had a variable degree of susceptibility.

When Richardson and Meakins (1986) in their experiments on the inter- and intraspecific variation of eucalypts to the susceptibility of the *Eucalyptus* snout beetle, found variations between provenances of the same species. Especially in the case of *E. viminalis* Labill. some provenances

appeared to be resistant whilst others were susceptible, which led to the suggestion that secondary plant metabolites might have an effect on the palatability of eucalypt leaves. Therefore to understand the possible susceptibility of various *Eucalyptus* species to the eucalypt snout beetle, an investigation of the chemical composition of the leaves of eucalypts was necessary.

Objectives

The objective of this study was to investigate the temporal change in the amount of total soluble phenols of nine *Eucalyptus* species and to record and compare the amount of total soluble phenols within each species throughout the year. This was investigated with a view to formulate a method for screening *Eucalyptus* species against *G. scutellatus* using phenolic concentration in leaves.

Chapter 2

2.1 *Eucalyptus*: as a plantation tree species

2.1.1. *Eucalyptus* in South Africa

The genus *Eucalyptus* L'Herit (Family: Myrtaceae) was first recorded to the scientific world by a British botanist, Joseph Banks, although a French botanist, L'Heritier, effectively described it (Hillis, 1966). This family is one of the most speciose of the aromatic trees, comprising more than 500 species (Hillis, 1966; Siddiqui *et al.*, 2000). *Eucalyptus* is endemic to Australia, Tasmania and surrounding islands (Papua New Guinea and the Philippines) (Tooke, 1953). The widespread success of eucalypts (outside their native range) as exotic trees is largely due to their ability to grow well under a wide range of environmental conditions, for example they grow at altitudes ranging from sea level to above 1800m above sea level (Polynton, 1979). Their performance as exotic trees is often superior compared to their indigenous home range.

This species is cultivated in warm and subtropical climates for their industrial (making paper and furniture) and medicinal value (Siddiqui *et al.*, 2000). Essential oils from leaves of some species are used for their economic and medicinal value (Siddiqui *et al.*, 2000). The major constituent of this oil is cineol (eucalyptol), which has a wide range of medicinal uses including being a diaphoretic disinfectant, expectorant, it has antimalaria properties, it is used for the treatment of infection of the upper respiratory tract and certain skin diseases, against diarrhoea and as a stringent in dentistry, haemorrhage and cuts (Siddiqui *et al.*, 2000).

The success of *Eucalyptus* is based on its ability to adapt to a wide range of climatic conditions. Eucalypts can be found in regions ranging from semi-arid with an annual precipitation of 10mm. to humid areas with an annual precipitation of over 100-200mm. These areas can be divided into summer and winter rainfall areas. Also eucalypts can be found in altitudes ranging from sea level to 2000m above sea level and temperatures from 0°C to above 30°C. *Eucalyptus* can be found in soils with varying amounts of nutrients but in most cases they are found on low nutrient soils of various acidity (Hillis, 1966).

Eucalypts were introduced into South Africa around the 1800's as ornamentals (Tooke, 1953) but today they are an important plantation tree species in the commercial forest industry (Govender, 2002). *Eucalyptus* trees are the most important source of hardwood in the world (Louw, 2001) with a wide range of uses, which include paper pulp, fuel wood, poles, charcoal, plywood, fiberboard and veneer (Department of Water Affairs and Forestry, 2000). This species has also proved to be useful for the prevention of soil erosion (Polynton, 1979). *Eucalyptus* species have been used for successful commercial afforestation in many countries, which includes Brazil (largest area of *Eucalyptus* plantations), South Africa, China, India, Portugal, Spain, and Ethiopia. In South Africa there are several eucalypt species planted commercially; *E. grandis being* dominant (Louw, 2001).

2.1.1. Health constraints to *Eucalyptus* silviculture

There are many pathogens and pests that damage *Eucalyptus* species. In South Africa, insects, mammals, nematodes and fungi have been implicated to the damage caused to this genus. These can either be establishment pests or post establishment pests. Some of the most common insects that damage this genus include the termite, *Macrotermes natalensis* Haviland (Isoptera: Termitidae) (Atkinson, 1999), the eucalypt tortoise beetle, *Trachymela tincticolis* (Coleoptera: Chrysomelidae) (Tribe and Cillie, 1997), the *Eucalyptus* snout beetle, *Gonipterus scutellatus* Gyllenhal (Coleoptera: Curculionidae) (Tooke, 1953) and *Phoracantha semipuncta* and *Phoracantha recurva* (Tooke, 1928). There are also pathogens that attack and severely destroy *Eucalyptus* trees. These include the *Phytopthora cinnamoni, Cylindrocladium, Pythium species, Botrytis species and Pantoea ananatis* (Govender, 2002).

2.2. *Gonipterus scutellatus*: as an insect pest

The adult insect is rusty red to brown in colour but this varies according to the age of the beetle, for example, a newly emerged beetle from the pupal case is rusty-red in colour and has an X-marking over the elytra (Tooke,

1953). The X-shaped mark fades over time and the colour changes to a dark brown colour (Tooke, 1953). The size of the adult beetle ranges from 8-9 mm in length with a short snout. The ventral surface is usually light grey in colour. There is a distinct difference between the sexes, with the female being larger than the male. The larva is sluggish and yellowish in colour and becomes green as it matures, with a faint dark median dorsal line and a heavy dark lateral stripe (Tooke, 1953). It usually measures about 11mm in length and 4mm in width. As the larva is excreting, the pellets become dry and form a filament, which is dragged by the larva as it moves (Tooke, 1953). Both the larvae and adult lifestage damage the leaves of *Eucalyptus* trees. Larvae after emerging from the capsule feed on the epidermis and mesophyll of the tender shoots but gradually move to the edges of the leaf as they mature. If the larva does not destroy the leaf, then the leaf tends to dry up. The adult beetle feeds and destroys the older leaves of the eucalypts (Tooke, 1953).

2.2.1. Life cycle of *G. scutellatus*

Gonipterus scutellatus Gyll. (Coleoptera: Curculionidae) has 2-3 generations per year (Tooke, 1953). The life cycle of the eucalypt snout beetle has regional differences with winter rainfall areas being different to summer rainfall areas and differences at high and low altitudes (Tooke, 1953). Egg laying starts two to three weeks after mating. Females deposit about 20 to 30 egg capsules during their lifetime with each capsule containing an average of about nine eggs (Hanks *et al.*, 2000). Oviposition only takes place on young tender foliage. Larvae take about two weeks to emerge depending on temperature. The larva emerges from the capsule through a hole on the ventral side of the capsule and immediately starts feeding on the epidermis of the leaf (Tooke, 1953). There are four larval instars and each takes about seven to ten days to develop depending on temperature. As the larva becomes mature it drops to the ground, enters the soil and forms a pupal cell. The pupal period lasts for 30-40 days before an adult emerges (Tooke, 1953). Generations of the eucalypt snout beetle vary according to temperature and available moisture. Due to these factors there are three regions (where the life cycle vary) namely the summer rainfall area below 1200m altitude, summer rainfall area above 1200m and winter rainfall area.

In summer rainfall areas below 1220m and coastal areas there are two generations per year. Oviposition commences on a large scale in September, increases in October and reaches a maximum in November. It takes four to six weeks for the larva to reach maturity whilst the pupal stage lasts for three to five weeks (Tooke, 1953). Adults from eggs laid during this period start emerging in December. Eggs laid by these beetles commence in January and reaches a maximum in February and decrease in March. From April through May pupal cells have already formed. Generations tend to overlap in these regions and egg capsules are always present (Tooke, 1953).

In summer rainfall areas with altitudes above 1200m, oviposition does not commence before the beginning of October and ceases in December. The eggs take fourteen to sixteen days to hatch. The larvae take four to five weeks to reach maturity and there are two generations in a year. By January the second brood start laying eggs and reaches its maximum in February and March and ceases completely in April. During March to April larvae are fully-grown. Overlapping of generations is not pronounced as in other regions (Tooke, 1953).

In winter rainfall areas oviposition commences in mid July and continues throughout the year since young tender foliage is always present. There is no definite hibernation period although adults may disappear during cold days and reappear during warm days to lay eggs. Therefore the beetle continues to breed even in winter (Tooke, 1953).

2.2.2. Distribution of *G. scutellatus*

Gonipterus scutellatus Gyll. (Coleoptera: Curculionidae) was first discovered in South Africa in 1912 in Newlands, Cape Town (Tooke, 1953). *Eucalyptus* in South Africa did not have any insect pests at that stage. This insect rapidly spread across eucalypt plantations in South Africa; in 1922 it was found in King Williams Town (in the Eastern Cape); in 1924 it was observed in Pietermaritzburg (in KwaZulu-Natal) and by 1925 it was present in plantations of the Witwatersrand (Gauteng Province) (Tooke, 1953). Currently the pest can be observed throughout plantation forests in South Africa in the winter rainfall areas (Western Cape), in the coastal regions and in the summer rainfall areas above and below 1200m in altitude.

The quick movement of the beetle is because they are strong fliers and can therefore move long distances within a short period of time (Tooke, 1953). It has a widespread distribution, which includes New Zealand (Tooke, 1953), Tasmania (Tooke, 1953), Central and South Africa (Tooke, 1953), Madagascar, Argentina, Brazil, Uruguay, Spain (Cordero *et al.*, 1999), Zimbabwe (Mossop, 1955), Mauritius (Williams *et al.*, 1955) and California (Hanks *et al.*, 2000).

2.2.3 Role of climate in the life history *G. scutellatus*

Temperature and rainfall play an important role in the life history of the eucalypt snout beetle (Tooke, 1953). It was shown that it is the onset of low temperatures that trigger hibernation and the lower the temperature, the more pronounced the hibernation. Breeding during the winter months is frequent in those areas with high temperatures. During low temperatures the incubation and development period of the larvae and pupae is lengthened (Tooke, 1953). Females oviposit when there is new flush and young tender foliage. The presence of this flush depends on the availability of rain. In the winter rainfall areas, oviposition does not cease, as there is new flush available throughout the year (Tooke, 1953)

2.3 Control of *G. scutellatus* on *Eucalyptus*

2.3.1 Chemical control

For the control of *Gonipterus scutellatus* experimental chemical trials were performed where fenvalerate or cypermethrin were administered. These were applied at 40g active ingredient per hectare (g a.i./ha) (Atkinson, 1999). Spraying was usually done in early summer and sometimes during late autumn if necessary. The synthetic pyrethroid cypermethrin, applied at the above dosages was extremely effective. Also these chemicals were observed to have low toxicity against birds and mammals but can be toxic to fish if directly applied on open water (Atkinson, 1999), but these treatments are not yet registered for use.

2.3.2 Biological control

Mossop (1929) argued that chemical control of the eucalypt snout beetle was impractical because of the frequency of applications needed. The use of chemicals for ten to fifteen years will cost more for the plantation industry and increase the cost of production. Hence biological control was investigated as an alternative control measure (Mossop, 1929).

The eucalypt snout beetle has a natural enemy, which is native to Australia (an arc from Adelaide around to Sydney, and Penola) (Tooke, 1953). Tooke (1953) introduced this mymarid egg-parasite to South Africa in 1926. *Anaphes nitens* Girault (formerly *Patasson (=Anaphoidae) nitens* Girault) (Hymenoptera: Mymaridae) has effectively controlled the snout beetle in many countries where it was introduced including South Africa (Mossop, 1929; Tooke, 1953), Spain (Cordero *et al.*, 1999), Zimbabwe (Mossop, 1955), Mauritius (Williams *et al.*, 1955) and California (Hanks *et al.*, 2000).

The parasite oviposits inside the eggs (endoparasitic) of the snout beetle. When the eggs of *A. nitens* hatch, the larvae feed on the embryos and egg content of the beetle. Pupation of the egg parasite takes place inside the egg capsule of the beetle. The egg to adult development of the parasite takes two to four weeks (Hanks *et al.*, 2000), depending on temperature. The adult wasps emerge through a hole that they chew in the host's capsule. Sometimes adult parasites emerge from the exit hole of beetle larvae. The pest has become a relatively minor forestry pest in South Africa compared to the 1930's, since the establishment of biological control. In South Africa, in the summer rainfall regions above 1200m, biological control frequently fails in early spring (Tooke, 1953).

2.3.3 Cultural control

There were several cultivational control measures that were tested in South Africa. The method of ploughing (with irrigation and fertilisation at the same time), which exposed overwintering pupae in the ground was tested (Tooke, 1953; Richardson and Meakins, 1986). The exposure of the pupal cells to cold and warm temperatures caused desiccation and breaking. This method proved to be successful although it was never practised on a large scale to have any significant effect on the eucalypt snout beetle populations

(Tooke, 1953). Another method, which was tested, was coppice reduction and pruning (Richardson and Meakins, 1986). This would reduce the food availably to the eucalypt snout beetle. Another method that was tested with little success was felling of infected stands and burning of infected stands (Richardson and Meakins, 1986).

2.4. Phenolic compounds

Phenolic compounds embrace a wide range of substances, which possess an aromatic ring bearing a hydroxyl substituent and other functional derivatives (Thomson, 1964), such as an associated sugar. These compounds are widespread in the plant kingdom (pteridophytes, gymnosperms and angiosperms) in leaves, fruits, bark, roots or wood. Sometimes they form up to 50% of the dry weight (Mila *et al.*, 1996) of the plant material. They are referred to as secondary compounds or secondary metabolites because they are considered non-essential to plant metabolism (Sauvesty *et al.*, 1992).

These phenolic compounds are synthesised and accumulated when the plant is under stress, which suggests that they could be used as physiological indicators of environmental stress. Phenolics can be located in any part of the plant (Sauvesty *et al.*, 1992), for example, in the plant cell wall where they can have a structural role and decrease digestibility (Lam *et al.*, 1990), on leaves as wax (Faina *et al.*, 1999), needles and cones of pine or as essential oils of the leaves of tobacco (Harborne and Simmonds, 1964). Phenolics can also occur as free compounds or phenolic glycosides in vacuoles and cell wall components. According to Sosulski *et al.* (1982) phenolic acids can contribute unpalatable flavours and can contribute to grey, brown or green colour of food. Among the natural phenolic compounds (of which hundreds are known) flavonoids and phenolics form the largest group of phenolic compounds while phenolic quinines; xanthones and other groups form the smaller group (Thomson, 1964).

Phenolic compounds have a wide range of functions. Within living plant tissue, phenolic compounds as well as terpenoids, can act as toxins, for example, consumption of *Acacia berlandieri* Benth. by domestic livestock during periods of drought results in a locomotor ataxia, as well as having

adverse negative effects on food intake and male fertility (Clement *et al.*, 1997). Four phenolic amines (*N* methyl-phenethylamine, tyramine, *N*-methyltyramine, and hordenine) had previously been extracted from *A. berlandieri*. *N*-methyl-phenethylamine has been shown to negatively impact fertility in female Angora goats (Clement *et al.*, 1997). They can also act as deterrents to pathogens and herbivores as well as plant litter decomposing animals (Louw, 2001). Phenolics have a defensive role to play in plants against animals, pathogens and insects (Castellanos and Espinosa-Garcia, 1997). Some phenolics affect root symbionts and alter site quality through interference with decomposition, mineralization and humification (Kainulainen and Holopainen, 2002).

There is also growing interest in phenolic compounds from the food industry because they have properties in promoting and sustaining human health (Louw, 2001). They can be anti-oxidants, antibacterial, antivirals, and antimutagenic, antiallergic and anticarcinogenic compounds. For example, polyphenols found in tea and red wine are said to be protective against a wide spectrum of cancers, arterioscrosis, heart diseases and blood problems (Louw, 2001).

Secondary compounds have been classified into two broad groups based on their inferred biochemical actions. The first group is qualitative and toxic with chemicals effective in small quantities, for example, alkaloids. The other group is quantitative and more effective in larger amounts, for example, tannins (Gullinan and Cranston, 1994). Secondary plant compounds function in two ways: at a behavioural level they repel insects, inhibit feeding and oviposition by insects. At a physiological level they may poison an insect and reduce the nutritional content of its food and discourage feeding (Gullinan and Cranston, 1994).

Secondary compounds are generally present in plants as mixtures that can be highly diverse. These mixtures protect plants against insect and pathogen attack by conferring resistant traits against these plant consumers (Castellanos and Espinosa-Garcia, 1997). Some hypotheses have been proposed about diversification of the secondary metabolic mixtures and their effects on plant consumers. Plants possess a variety of compounds, which act as defensive mechanisms against herbivores (Coley, 1983). The first

hypothesis demonstrates that highly diverse secondary metabolite mixtures can reduce herbivory more effectively than low diversity mixtures or single compounds. Conversely in some cases, the concentration of a single secondary metabolite within a mixture can be the trait that can account for the defensiveness of the plant against herbivores (Castellanos and Espinosa-Garcia, 1997). These secondary compounds function in two ways:

(1) each compound in the mixture affects a particular herbivore target and is therefore effective against a group of herbivores.

(2) compounds are functioning together as a mixture and are effective against a particular group of plant consumers, where the compounds function additively (Castellanos and Espinosa-Garcia, 1997).

Secondary metabolites can either be involved in qualitative or quantitative defences. Qualitative defences were found in plants, which were hidden and difficult for herbivores to find, like young shoots, and successional plants because of their unpredictable distribution. These plants produced less costly defences, which are effective against the majority of herbivores. Quantitative defences were found in plants with a high risk of being discovered, for example, old mature leaves and late successional plants. These plants were expected to invest largely in broadly effective defences (Coley, 1983). Therefore the allocation of secondary plant metabolites in plants, as defences has to do with risk of being found by predators.

2.4.1. Phenolic compounds against herbivory and defoliation

There are about 200 thousand species of plants and one million species of phytophagous insects on earth, which interact closely and are dependent on each other. These plants supply food for the insects and in return the plants are pollinated (Hanover, 1980). The interaction of plants and insects assist in the investigation of the co-evolution of plant and animals mediated by plant secondary compounds (Li and Lui, 2001). Browsing can eliminate and alter the relative chemical composition and the growth of an individual plant species within an environment. Secondary compounds inhibit foraging mammalian herbivores by affecting the intake, digestion, metabolism and reproduction of the animal. High concentrations of noxious compounds, like phenolics and resins and low concentrations of positive nutritional factors

like nutrients may protect plants against herbivores (Haukioja, *et al.,* 1985). It is assumed therefore that these compounds play a defensive role against herbivory, defoliation and phytophagy, although they may have other metabolic functions or simply be metabolic wastes (Gullinan and Cranston, 1994). This could also be demonstrated by the removal of the terminal shoot in plants during spring and autumn. In the first growing season this may not significantly damage the plant. However, higher levels of defoliation, such as the removal of the crown, significantly reduced the survival of the plant (Wilkinson and Nielson (1995). For a tree to be defoliated three things must occur: firstly the insect must be attracted to land on the foliage, secondly the foliage must be acceptable for the insect to feed and lastly the foliage must be suitable for the survival of its larvae. This initial attraction to the plant could be due to the chemical composition of the plant (Raymomd, 1998).

When both vertebrates and invertebrates feed and damage a plant, it was observed that plant defences were induced (Glyphis and Puttick, 1989) and the amount of secondary compounds released depended on the degree of damage and on the plants species. Herbivory in natural communities could be high, reduce growth and reproduction in plants and influence competition of plants in the entire community (Coley, 1983). Plants have developed defences against some herbivores; for example, shrubby species developed morphological structures to survive herbivory, for example, thorns. Other plants without these structures often produce a large variety of secondary compounds or defence compounds. These compounds are closely related to predatory pressures that the insect may encounter (Faina *et al.*, 1999), for example, the compounds may limit the insect to the food available. The cost of defence and the value of the plant part in different anti-herbivore mechanisms have evolved and their allocation within a plant have been in response to the risk of discovery by herbivores (Feeny, 1976; Rhoades and Cates, 1976).

2.4.2. Phenolic compounds and nutrient availability

The relationship between carbon and nitrogen in plants always influences the allocation of energy to either growth or chemical defences. This has led to the formation of two major hypotheses that were proposed to

predict the effects of secondary metabolite concentrations in plants (Byrant, 1983).

Plants have to allocate available energy to growth and defence. Therefore, the balance between carbon and nutrient availability determines carbon-based secondary compounds in plants. For example, if there is less danger for plants, more resources will be allocated for growth. Conversely, if there is danger for the plant then more resources will be allocated as defences. This is the carbon-nutrient balance hypothesis (Byrant, 1983). The concentrations of carbon-based secondary compounds involving phenolic compounds will increase in cases where carbohydrates accumulate in excess of growth demands (Koricheva *et al.*, 1998). Therefore, plants whose growth is not limited by nitrogen should invest most of their available carbon into growth. Plants limited by nitrogen should have more carbon, which is then used for the production of carbon-based defences (Briggs, 1990).

In contrast, growth is largely limited by water and nutrients and allocation of compounds depend mainly on available carbohydrates. This is the growth-differentiation balance hypothesis. (Koricheva *et al.*, 1998). Therefore differentiation and production of carbon-based secondary compounds dominate when the factors other than photosynthesis are sub-optimal for growth, for example, under nutrient limitations.

Carbon availability could also alter allocation patterns of defences (Briggs, 1990). If the plants are grown in a nitrogen-deficient environment, the plant will produce relatively low concentration of secondary metabolites, for example, tannins. Therefore environments with low resource availability should be characterised by plants with high levels of secondary compounds and slow growth rates (Glyphis and Puttick 1989). This theory is supported by the South African shrub-land data in a Mediterranean climate zone. In this area, a few species had high levels of phenolics but more species had low levels of phenolics. This was also reported for other ecosystems with very low levels of soil nutrition, such as tropical forests (Glyphis and Puttick 1989).

2.4.3. Phenolic compounds and oviposition

Castellanos and Espinosa-Garcia (1997) showed that secondary metabolite concentration and the types of compounds and mixtures affected

the numbers of eggs laid by *Sitophilus granaries* on cereal. Diets with high concentrations of secondary metabolites had fewer eggs (less oviposition) compared to diets with less concentrated secondary metabolites (Castellanos and Espinosa-Garcia 1997). Also Clarke *et al.* (1998) using the oviposition of *Gonipterus scutellatus* on seven *Eucalyptus* species found that, out of the chosen species *G. scutellatus* preferred three eucalypt species for oviposition (which were susceptible to *G. scutellatus*). The remainder of the species were found to be resistant against defoliation by *G. scutellatus*. This means that species susceptible to *G. scutellatus* are those that are preferred by the pest.

CHAPTER 3

3.1 MATERIALS AND METHODS

3.1. Trial details

The *Eucalyptus* species used in this experiment were selected from a tree breeding trial in Draycott (KwaZulu-Natal) ($28^0$35'S, $29^0$37'E). This site is 1580 m above sea level with a Mispah soil type; the topsoil has a depth of 10-35cm (P. Govender pers. comm.). Twenty *Eucalyptus* species were planted in this trial during 2000 but only nine were evaluated in this project. These eucalypts were selected to cover a range of susceptibilities to defoliation by the eucalypt snout beetle, *Gonipterus scutellatus.* They included *E. macarthurii* Deane, *E. smithii* Baker, *E. andrewsii/campanulata* Maiden, *E. benthamii, E. fastigata* Maiden, *E. cypellorcarpa* Johnson, *E. dunnii* Maiden, *E. badjensis and E. nobilis.* The trial had three replications of a 4x5 rectangular lattice design. Plots consisted of 25 trees. Species were allowed to coppice in this trial after harvesting.

About 10 to12 fully expanded leaves were collected every second month from February 2002 to September 2002. During September juvenile, medium and mature leaves were collected. The leaves were stored in a minus 70°C fridge and then freeze-dried for 3 days using an Edward freeze drier (Mobdulyo). These leaves were then grounded with a mortar and pestle. The powder was then sieved with a 0.08 unit sieve to ensure uniform particle size. An amount of 0.05 g of the dry weight (DW) of this powder was used to extract the phenolics. Each tree was replicated six times, each plot three times and there were three replicates per species.

3.2 Total soluble phenol extraction

Methanol/acetone/water (in the ratio 7:7:1) was used as solvent 1 for the initial extraction and methanol and chloroform (in the ratio 1:1) was used as the second solvent. Dried leaf powder (0.05g) and 1ml of solvent 1 were mixed in an Eppendorf tube and the contents vortexed for one minute. The suspensions were placed overnight at room temperature on a Labcon 1077k rotating shaker (150 rpm). The suspension was centrifuged at 10^4 g for one minute in a microcentrifuge 7200g (Denver Instrumental Company, USA) to

remove plant debris. The supernatant was retained and stored on ice and the solvent (1ml) (methanol/acetone/water) was added to the same Eppendorf tubes, vortexed and placed in a shaker for 30 minutes. The suspension was centrifuged and this was repeated three times. All the supernants were pooled together and reduced to 1ml under vacuum.

Distilled water was added to the supernatants to separate the chlorophyll from the water-soluble supernatant. The tubes were centrifuged at 10 x 10^3g for one minute to obtain a clear supernatant. If any chlorophyll was detected, more water was added and centrifuged repeatedly. The final supernatant, adjusted to 1ml, was retained in an Eppendorf tube for the estimation of the concentration of total soluble phenolic compounds.

3.2.2. Estimating concentration of total soluble phenolic compounds

An Elisa plate was used to measure the concentration of total soluble phenolics. Distilled water (170µl) was added into an Elisa plate to which 5µl of the leaf extract was added. A blank, consisting of identical composition but replacing the sample with water served as a control. Sodium carbonate 20 % (50µl) was mixed into the solution and 25µl of Folin-Ciocalteu's reagent (SIGMA, Aldrich, Johannesburg, South Africa) was added as a colorimetric indicator (Sauvesty *et al.*, 1992). The samples were mixed and air bubbles were removed. Each plate was incubated at 40^0C in an incubator for 30 minutes. A Multiskan Ascent V1.24 Version 1.3.1. Spectrophometer was used for absorption determinations and the readings were obtained at a wavelength of 690nm. Each extract was replicated three times (therefore each leaf powder was extracted six times). A Standard curve using gallic acid (SIGMA, Aldrich, Johannesburg, South Africa) as standard (0 – 40mg) was done and the Standard curve of r^2 = 0.998 and y = 1.3527 was used. Results were expressed as equivalent milligrams of gallic acid per gram of dry weight (DW). The carbohydrate data used in the discussion was obtained from a co-worker, Rorisang Moleki, who was also using the same sample as above to check for carbohydrate content in the leaves.

3.3 Statistical analysis

Analysis of Variance (ANOVA) was carried out using SAS to test for statistically significance differences within and between total soluble phenols. Concentration was also investigated using ANOVA in SAS to look at differences between and within months and species. Where there were statistical significant differences non-significant groups were derived by Least Significant Differences (LSD's).

CHAPTER 4

4. RESULTS

4.1 Total Soluble Phenolics within February, April, July and September 2002.

There were significant differences in the amount of total soluble phenols between the nine *Eucalyptus* species in February (ANOVA: $F_{1, 8}$ = 19.02; $P < 0.0001$) with a range of 3.39 to 9.04 Eq. mg of gallic acid/g DW. Although there were significant differences in the amount of total soluble phenols there were species, which were not significantly different from each other (Table 1). *Eucalyptus andrewsii/campanulata, E. smithii and E. benthamii* were not significantly different from each other. The second cluster with species that were not significantly different from one another included *E. smithii, E. benthamii* and *E. fastigata*. This was followed by *E. benthamii, E. fastigata* and *E. dunnii*, which were not significantly different from each other. The next cluster was composed of *E. fastigata, E. dunnii, E. nobilis* and *E. macarthurii. Eucalyptus nobilis, E. macarthurii, E. badjensis* and *E. cypellocarpa* formed the last cluster with species not significantly different from each other

During April, there were significant differences in the amount of total soluble phenols between the species (ANOVA: $F_{1, 8}$ = 19.68; $P < 0.0001$) and their range was 4.75 to 9.29 Eq. mg of gallic acid/g DW. Although there were significant differences between the species, a large cluster of species not significantly different from each other was formed (Table 2). These species included *E. andrewsii/campanulata, E. benthamii, E. smithii, E. dunnii* and *E. nobilis*. In the next cluster of non-significantly different species, the above

species were part of the cluster except for *E. andrewsii/campanulata* and *E. benthamii* but also included *E. fastigata*. The third cluster was composed of *E. nobilis, E. fastigata* and *E. macarthurii.* The last group only has two species, which are not significantly different from each other and these are *E. badjensis and E. cypellocarpa.*

During July, there were also significant differences in the amount of total soluble phenols (ANOVA: $F_{1, 8} = 16.36$; $P < 0.0001$) and the range of the species was 6.042 to 12.309 Eq. mg of gallic acid/g DW (Table 3). Similar to February and April, *E. andrewsii/campanulata* and *E. smithii* were part of the first cluster with species not significantly different from each other and were no significantly different *E. nobilis* and *E. dunnii.* The second cluster, overlapped with the first one, and had *E. andrewsii/campanulata, E. smithii* and *E. dunnii* including *E. benthamii. Eucalyptus badjensis* was also not significantly different from *E. benthamii.* A cluster overlapping with the previous one was formed by *E. badjensis, E. cypellocarpa, E. macarthurii* and *E. fastigata*.

During September, there was a significant difference in the amount of total soluble phenols between juvenile, intermediate and old leaves (ANOVA: $F_{1, 3} = 217.71$; $P<0.0001$). There was also a significant difference in the amount of total soluble phenols within the species (ANOVA: $F_{1, 8} = 37.39$; $P<0.0001$). As in February, April and July, September had clusters that were formed by species that were not significantly different from each other (Table 5). For juvenile leaves there are three clusters formed. The first one was made up of *E. nobilis, E. dunnii, E. smithii, E. andrewsii/campanulata, E. benthamii* and *E. badjensis.* The second cluster only omitted *E. nobilis* and incorporated *E. cypellocarpa*. The last cluster was made up of *E. benthamii, E. badjensis, E. cypellocarpa, E. macarthurii* and *E. fastigata*.

For intermediate leaves, overlapping was evident between the first cluster composed of *E.* andrewsii/campanulata, and *E. smithii* and the second cluster composed of *E. smithii* and *E. dunnii.* In addition, the third cluster does overlap with the second one as they both share *E. dunnii* but the third cluster also had *E. nobilis, E. benthamii* and *E. macarthurii.* The fourth group was between *E. benthamii, E. nobilis, E. badjensis* and *E. macarthurii. Eucalyptus badjensis* and *E. macarthurii* formed another cluster overlapping with the

previous one and lastly *E. badjensis* was significantly different from *E. cypellocarpa.*

For the older leaves, there were no significant differences between *E. dunnii, E. smithii, E. andrewsii/campanulata* and *E. badjensis*, which form the first cluster. *Eucalyptus nobilis, E. benthamii, E. cypellocarpa* and *E. macarthurii* were not significantly different from each other.

4.2 Total soluble phenolics between February, April, July and September.

When the focus was on total soluble phenols between February, April and July, the leaves of *E. andrewsii/campanulata, E. macarthurii* and *E. benthamii* showed an increase in the amount of soluble phenols from February to September although the increase was not significant between February and April. For *E. smithii* there was an increase in the amount of soluble phenols from February, April, July and September. For *E. fastigata, E. dunnii, E. nobilis, E. badjensis* and *E. cypellocarpa* there were (no significant differences) increases or decreases in the amount of total soluble phenols during February, April and July (Table 6). This is illustrated in Figure 1. In all the nine-eucalypt species, there was a significant increase from July to September and September had the highest levels of total soluble phenols. This is illustrated clearly in Figure 2.

The carbohydrate data extracted from R. Moleki is for discussion purposes only and does not form part of the results.

CHAPTER 5
DISCUSSION

Each *Eucalyptus* species has its own unique total soluble phenolic content. *Eucalyptus andrewsii/campanulata, E. smithii, E. benthamii and E. dunnii* had the lowest levels of total soluble phenolics whilst *E. nobilis, E. macarthurii, E. badjensis, E. fastigata* and *E. cypellorcapa* had higher concentrations. The differences in the total soluble phenolic content may be due to differences in genetic composition of each individual species (Coley, 1986).

Differences were also observed for total soluble phenolic content within each species during February and April, but were more significant during July and September. Total soluble phenolic contents in the nine *Eucalyptus* species observed were lower during February, increased gradually during April and July, and were more concentrated during September. These differences might be due to a number of factors, which can either be biotic or abiotic (Feeny, 1975). Such abiotic factors may include rainfall (water availability), nitrogen availability (soil nutrients) and light intensity, which can affect the levels of phenols in a species (Haukioja *et. al.*, 1985). If we take into consideration that the research site was a summer rainfall area then we can assume that there was less rainfall from March towards the winter months and therefore water became a limiting factor for the trees. Our findings agreed with those of Byrant *et. al.*, (1983) on the carbon/nutrient balance hypothesis and the growth-differentiation balance hypothesis (Koricheva *et. al.*, (1998).

The carbon/nutrient balance hypothesis predicts that carbon based secondary compounds will increase in cases where carbohydrates accumulate in excess of growth demands and therefore factors which reduce photosynthesis more than growth will lead to a decrease in the carbon pool that is responsible for increasing carbon based secondary compounds (Byrant *et. al.*, 1983). This means that the manufacturing of carbon-based secondary metabolites depends on the growth of the plant, which in turn is controlled by the available carbon pool. If there is excess carbon available after growth then this is focused on production of carbon-based secondary metabolites. On the similar note the growth-differentiation balance hypothesis stipulates that growth is largely, limited by water and nutrients and differentiation depends mainly on available carbohydrates (Koricheva, *et. al.*, 1998). Therefore differentiation and hence the production of carbon based secondary compounds dominate when factors other than photosynthesis are suboptimal, for example, during low rainfall conditions. In this case the limiting factor maybe water but this depends on the poor site conditions and also low carbon pool.

The variations in the concentration of total soluble phenolics may also be affected by biotic factors such as defoliation by insects and browsers (Rhoades & Cates, 1976). When defoliation of the foliage occurs plants

secrete and release plant secondary metabolites on and around the site where the defoliation is taking place (Rhoades & Cates, 1976). By doing this, the plant is using the secondary metabolites as chemical defences to act against the agent that may be causing the defoliation. As a result, the browsing agent may stop and may not attempt browsing on the plant again. This is supported by Haukioja *et. al.*, (1985) study, where they correlated the foliage chemistry with herbivore damage. The results showed that there was a positive correlation between herbivore damage and foliage chemistry.

In this study, there was a continued increase in the concentration of total soluble phenols within the nine eucalypt species. *Gonipterus scutellatus* was observed to browse on the foliage of these eucalypts, and one can argue that the concentration of the total soluble phenols in these trees was affected by the defoliation event and therefore the concentration increased. To substantiate this view, we need to correlate the time when the total soluble phenols increased with the infestation of the beetle (both larvae and adults).

According to Tooke's (1953), in the first generation beetle, oviposition commences in September to November and larvae start emerging in December. Oviposition by the next generation of beetles commences in January, reaches a maximum in February, and decreases in March. Hence one can expect the concentration of total soluble phenols to be higher during periods when larvae and adults were present. During September, the concentration is very high and this agrees with our findings. In contrast, during the months of February, the concentration is low but we expect it to be high if we use the above concept. To explain the contrast in our results with the above findings, we can argue that since the larvae were feeding primarily on the juvenile shoots may be the phenolic concentration of the leaves were not as high.

Another aspect to focus on when observing the concentration of total soluble phenolics is the allocation of carbohydrates by the plant. This might have an impact on the amount of secondary metabolites because higher plants accumulate compounds which are produced from primary plants metabolism, for example, carbohydrates and secondary metabolites (phenolics) (Feeny, 1975). Also the availability of secondary metabolites depend on the availability of carbon due to the fact that if carbohydrates

accumulate in excess of that required for growth, then these carbohydrates will be used for the production of carbon-based secondary metabolites (Briggs, 1990).

As shown by my co-worker, Rorisang Moleki, carbohydrate content increased during the year between the species. In addition, the amount of available carbohydrates was less than that of the total soluble phenolics. This means that the trees were putting more effort in secondary metabolites production compared to primary compounds. There are many factors affecting the production of secondary metabolites and their concentration. If these factors and their effects on secondary metabolites can be studied more thoroughly then a technique for screening *Eucalyptus* species can be developed. Also more work needs to be done on the effects of these secondary metabolites on the life stages of *G. scutellatus*. This information can then be combined to formulate screening techniques that can be used to test for resistance of eucalypt species against *G. scutellatus*.

The preliminary results of this experiment were presented in the form of a poster at the SAAB 2003 conference held in Pretoria.

Table 1. Mean concentration of total soluble phenolics and carbohydrate content in leaves of nine *Eucalyptus* species during February 2002. (Carbohydrate data presented with permission from R. Moleki, 2004)

SPECIES	PHENOLIC CONTENT (mg of gallic acid/g of DW)*	CARBOHYDRATE CONTENT (% bricks)*
E. andrewsii/campanulata	3.39 [a]	0.67[a]
E. smithii	4.27 [ab]	0.86[a]
E. benthamii	5.12 [abc]	0.89[a]
E. fastigata	5.99 [bcd]	2.23[a]
E. dunnii	6.65 [cde]	1.67 [b]
E. nobilis	7.30 [def]	2.30 [c]
E. macarthurii	7.93 [def]	1.60 [b]
E. badjensis	8.91 [f]	0.94[a]
E. cypellocarpa	9.04 [f]	2.48 [c]

(* Number with the same letter within a column are not significantly different; 95% confidence limits)

Table 2. Mean concentration of total soluble phenolics and carbohydrate content in leaves of nine *Eucalyptus* species during April 2002. (Carbohydrate data presented with permission from R. Moleki, 2004)

SPECIES	PHENOLIC CONTENT (mg of gallic acid/g of DW)*	CARBOHYDRATE CONTENT (% bricks)*
E. andrewsii/campanulata	4.75 [a]	0.72 [a]
E. benthamii	5.08 [a]	1.00 [a]
E. smithii	6.09 [ab]	0.90 [ab]
E. dunnii	6.14 [abc]	1.91 [c]
E. nobilis	6.36 [abcd]	1.21 [b]
E. fastigata	6.82 [bcde]	0.98 [ab]
E. macarthurii	7.43 [cdef]	0.92 [ab]
E. badjensis	10.66 [g]	1.16 [ab]
E. cypellocarpa	9.29 [g]	1.06 [ab]

(* Number with the same letter within a column are not significantly different; 95% confidence limits)

Table 3. Mean concentration of total soluble phenolics and carbohydrate content in leaves of nine *Eucalyptus* species during July 2002. (Carbohydrate data presented with permission from R. Moleki; 2004)

SPECIES	PHENOLIC CONTENT (mg of gallic acid/g of DW)*	CARBOHYDRATE CONTENT (% bricks)*
E. nobilis	6.04 [a]	1.20 [ab]
E. dunnii	6.72 [ab]	2.34 [e]
E. smithii	7.29 [abc]	1.02 [a]
E. andrewsii/campanulata	7.67 [abcd]	1.14 [ab]
E. benthamii	8.49 [bcde]	1.43 [bc]
E. badjensis	9.43 [def]	10.9 [ab]
E. cypellocarpa	9.93 [efg]	2.35 [e]
E. macarthurii	11.39 [gh]	1.64 [cd]
E. fastigata	12.31 [h]	1.89 [d]

(* Number with the same letter within a column are not significantly different; 95% confidence limits)

Table 4. Mean concentration of phenolics in leaves of nine *Eucalyptus* species during September for juvenile, intermediate and old leaves.

SPECIES	PHENOLIC CONTENT (mg of gallic acid/g of DW)*		
	Juvenile	**Intermediate**	**Old**
E. nobilis	6.04 ^{a}	16.390 ^{cd}	21.984 ^{ef}
E. dunnii	6.72 ^{ab}	13.348 ^{bc}	15.538 ^{abc}
E smithii	7.29 ^{ab}	10.580 ^{ab}	12.117 ^{a}
E. andrewsi/campanulata	7.67 ^{ab}	7.149 ^{a}	15.101 ^{ab}
E. benthamii	8.49 ^{abc}	16.016 ^{cd}	22.529 ^{efg}
E. badjensis	9.43 ^{abc}	17.993 ^{def}	15.561 ^{abcd}
E. cypellocarpa	9.93 ^{bc}	21.984 ^{g}	22.973 ^{efgh}
E. macarthurii	11.39 ^{c}	16.936 ^{cde}	22.998 ^{efgh}
E. fastigata	12.31 ^{c}	20.251 ^{efg}	20.605 ^{e}

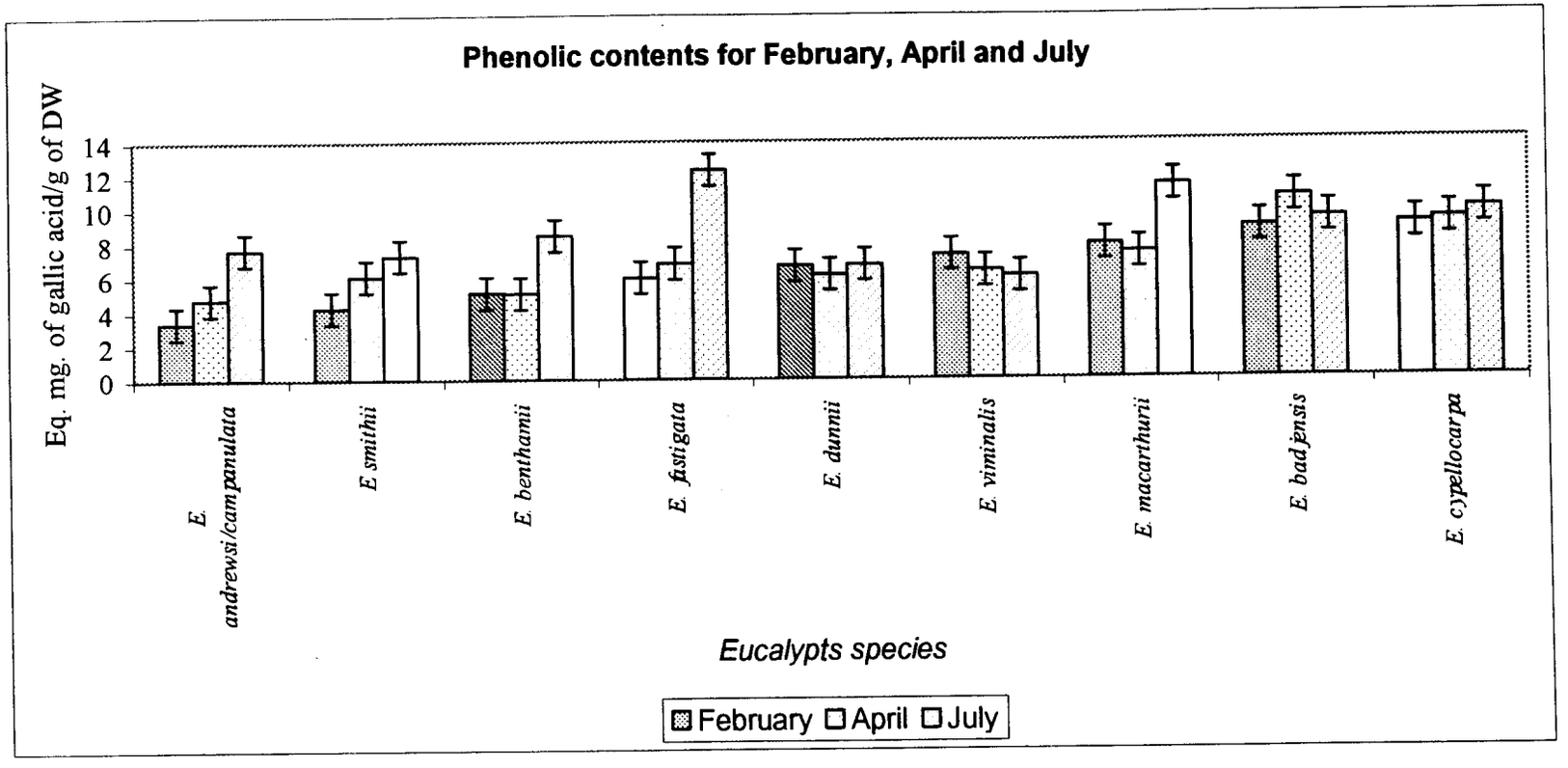

Figure 1. Showing mean concentration of total soluble phenolics in leaves of nine *Eucalyptus* species during February, April and July 2002 respectively.

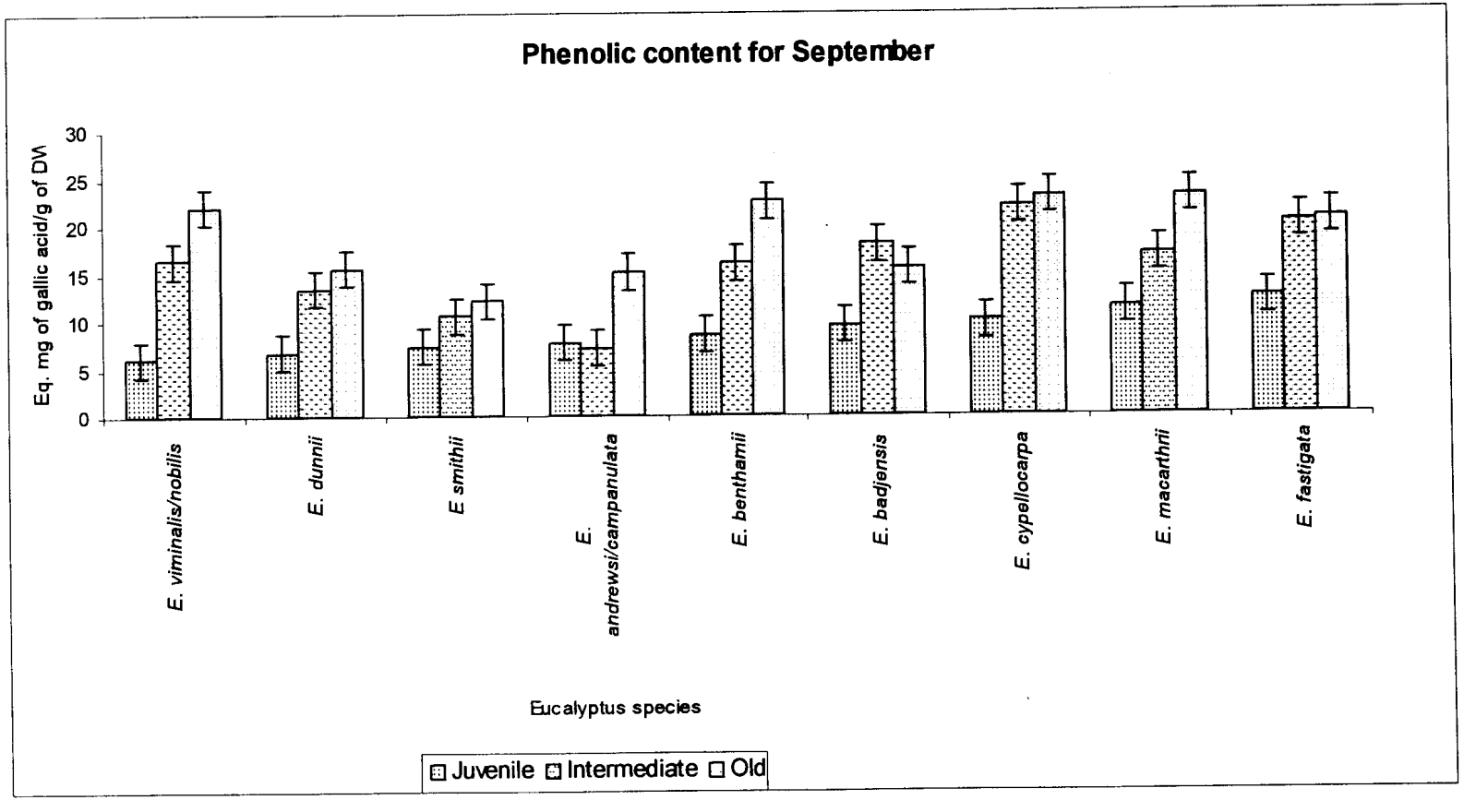

Figure 2. Showing mean concentration of total soluble phenolics in leaves of nine *Eucalyptus* species during September 2002 for juvenile, intermediate and old leaves.

Table 5. Total soluble phenolics and carbohydrates concentration in nine species of *Eucalyptus* leaves during February, April, July and September 2002. (Carbohydrate data presented with permission from R. Moleki, 2004).

SPECIES	TOTAL SOLUBLE PHENOLICS (Eq. Mg of gallic acid/g of DW)*				CARBOHYDRATES (% bricks)*			
	February	April	July	September	February	April	July	September
E. andrewsii/campanulata	3.39[a]	4.75[a]	7.67 [b]	7.41	0.67[a]	0.72 [a]	1.14 [ab]	1.5
E. smithii	4.27[a]	6.09[ab]	7.29 [b]	8.94	0.86 [a]	0.90 [a]	1.02 [a]	1.42
E. benthamii	5.12[a]	5.08[a]	8.49 [b]	12.26	0.89 [a]	1.00 [a]	1.43 [b]	2.24
E. fastigata	5.99[a]	6.82[a]	12.31 [b]	16.28	2.23 [c]	0.98 [ab]	1.89 [d]	1.39
E. dunnii	6.65[a]	6.14[a]	6.72[a]	10.04	1.67 [b]	1.91 [c]	2.34 [e]	0.89
E. nobilis	7.30[a]	6.36[a]	6.04[a]	11.22	2.30 [c]	1.21 [b]	1.20 [ab]	1.25
E. macarthurii	7.93[a]	7.43[a]	11.39 [b]	14.16	1.60 [b]	0.92 [ab]	1.64 [cd]	1.23
E. badjensis	8.91[a]	10.66[a]	9.43[a]	13.71	0.94 [a]	1.16 [ab]	1.09 [ab]	2.59
E. cypellocarpa	9.04[a]	9.29[a]	9.93[a]	15.96	2.48 [c]	1.06 [ab]	2.35 [e]	1.29

*Number with the same letter across the row between the columns are not significantly different; 95% confidence limits) The September results represents the averages for juvenile and intermediate leaves and are used for comparisons only hence there are not statistical differences.

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