Polyphenolic production in response to defoliation in three karoo shrubs: Osteospermum sinuatum, Pteronia pallens and Ruschia vulnerans.

Honours Project 1990

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ABSTRACT

Three karooid shrubs (*Osteospermum sinuatum*, *Pteronia pallens* and *Ruschia vulnerans*) were subjected to three defoliation intensities viz. none, forty and eighty per cent, to determine whether tannin and total polyphenolic contents change in response to damage. Results showed that *R. vulnerans* had the highest concentrations of polyphenols and tannins in the above ground plant parts of the all the study species. Lower concentrations of total polyphenols, but no tannins were found in *P. pallens* and *O. sinuatum*. Defoliation resulted in an increase in the total above-ground plant tannin and polyphenolic concentrations in the 40% and 80% treatments of *R. vulnerans*, whereas the inverse occurred in *P. pallens*. There was no response to defoliation treatments in terms of polyphenolic production in *O. sinuatum*.

INTRODUCTION

"One way to avoid being eaten is to make one-self inedible. This simple principle is widely used by both plants and animals. However, in the case of plants being the primary producers in the food chain and unable to run away, they remain in place, ready to be eaten" (Smidt-Nielsen, 1986).

It was first suggested by Stahl in the late 1800's, that some of the chemical properties of plants may have evolved for protection against attack by herbivorous animals (Rhoades, 1979). The idea was further taken up by Fraenkel (1959) who pointed out that insect host-finding and gustatory behaviour was largely under the control of plant "secondary substances" i.e. compounds thought to have no metabolic function, hence commonly referred to as secondary metabolites (Rhoades, 1979). However, during the past two decades an enormous amount of research has been published on the role of these secondary metabolites in plant-animal interactions (Feeny, 1970; Swain, 1977; Rosenthal & Janzen, 1979; Cooper and Owen-Smith, 1985; Glyphis, 1985; Glyphis and Puttick, 1988). Herbivores exert a major impact on plants, across both ecological and evolutionary time scales in that they can increase plant mortality and may remove biomass that might be allocated to growth or reproduction. Consequently, plants have evolved an extraordinary array of secondary metabolites (over 10 000 known low molecular-

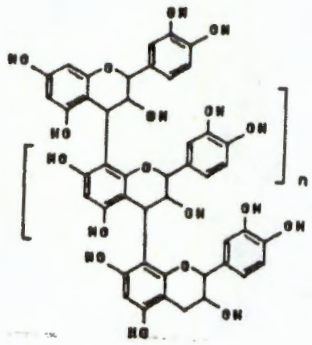
are thought to
weight compounds) which act as antiherbivore defenses. These compounds appear not to be waste products, as was first thought, nor do they serve any other known function in the plant (Swain, 1977; Coley et al. 1985).

It has been established that secondary substances are derived from the shikimate pathway which is directly dependent upon the activity of phosphoenol-pyruvate (PEP) (Mooney, 1972). Secondary metabolites derived from this pathway, include a wide variety of compounds including structures such as simple phenols, polyphenolics (tannins), lignins, aromatic amino acids and flavanoids (Mooney, 1972). Numerous plant-animal interaction studies have shown that tannins play a major role in deterring insect and ungulate herbivory of plants (Feeny, 1970; Cooper and owen-Smith, 1985; Glyphis, 1986; Glyphis and Puttick, 1988; Karowe, 1989). However they are generally known to be non-toxic (Feeny, 1970). Observations with cattle and sheep have indicated that tannin concentrations higher that 2% d.w. deter feeding by herbivorous vertebrates (Swain, 1979).

Tannins occur widely in the plant kingdom, especially in the Angiospermae (Watt and Breyer-Brandwijk, 1932) and are associated with woody plants in particular (Glyphis, 1985). Tannin concentrations may reach 40% of dry weight in the bark of some species of *Quercus* and 15% of dry weight in tea leaves (Meyer et al, 1960). Tannins are known from almost

all organs and tissue types and in the leaves they tend to be concentrated in the pallisade cells (McNair, 1965).

Tannins are generally defined as naturally occurring water-soluble polyphenolic compounds capable of binding to and /or precipitating water soluble proteins, a property which is paramount in plant-herbivore interactions. Consequently much effort has been directed at determining the chemical structure and function of this group of secondary metabolites (Bate-Smith, 1973; Hadock et al., 1987; Mole and Waterman, 1987; Mole et al., 1989; Hagerman and Butler,1989). Based on chemical structures, tannins are divided into two types, condensed and hydrolysable. These differ in their component subunits and bonding between these (Figure 1). Condensed tannins (proanthocyanidins) are flavanoid polymers, with carbon-carbon bonds joining the individual flavanoid monomers. Condensed tannins are not susceptible to hydrolysis but can be oxidatively degraded in strong acid to yield anthocyanidins. On the other hand, hydrolysable tannins are gallic or hexahydroxydiphenic acid esters of glucose or other polyols (Hagerman and Butler, 1989). Both condensed and hydrolysable tannins interact with proteins to form soluble or insoluble complexes (Hagerman and Butler, 1989). However, due to their different chemical structures, condensed and hydrolysable tannins are perceived as having different biological functions. Condensed tannins are suggested to function by



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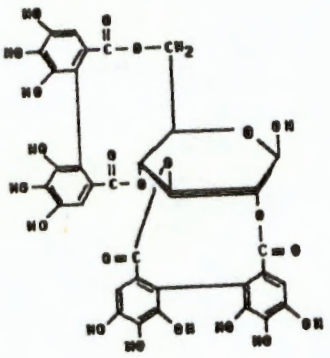

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Figure 1. Representative structures of tannins and tannin subunits: **1** is the condensed tannin (proanthocyanidin) from *Sorghum*, with polymer length of 6 - 7; **2** is a simple hydrolysable tannin (ellagitannin) (ex Hagerman & Butler, 1989).

complexing the proteins binding to cellulose and hemicellulose in plant cell walls thereby preventing the breakdown of these polymers by fungal and bacterial extracellular hydrolases. Since ruminant herbivores are dependent upon microbial fermentation of cellulose and hemicellulose for a significant fraction of their energy needs, the resultant decline in microbial fermentation of plant material will reduce the nutritional value of the plant species to ungulates (Swain, 1979; Cooper and Owen-Smith, 1985). On the other hand, hydrolysable tannins act to inhibit digestive enzyme activity of herbivores and are more astringent than condensed tannins. It has been suggested that they play a major role in reducing the growth and development of many specialist herbivorous insects and are suggested to have a lesser effect on generalist browsing and grazing ungulates (Cooper and Owen-Smith, 1985; Karowe, 1989).

A major goal in the study of plant herbivore interactions is to develop an understanding of why plant species differ in their commitment to defense and hence in their susceptibility to herbivores (Coley et al., 1985). It is generally known that even though most species of higher plants have qualitatively similar resource requirements for growth and reproduction, they differ in the way they use resources to carry out three essential functions - reproduction, defense and growth (Bazzaz et al., 1987).

Therefore it would be more appropiate to consider the resource allocation phenomenon in relation to plant physiology per se. According to the Optimal Defense Theory (ODT) (Rhoades, 1979), organisms evolve and allocate defenses in such a way as to maximize individual fitness. Since defenses are costly, less well defended individuals have higher fitness than more highly defended individuals when enemies are absent and the reverse applies when enemies are present. Also within an organism, defenses are allocated in direct proportion to the risk of the particular tissue and the value of that tissue in terms of fitness loss to the organism resulting from attack on that tissue. It would therefore be expected that valuable tissues, in terms of their contribution to the plant's fitness, would be allocated more defenses than to less valuable tissues. Finally, the ODT suggests that defenses are costly and are a function of the total energy and nutrient budget of the organism. Defense is also negatively related to energy and nutrient allocation in the organism to other contingencies i.e. growth and reproduction.

To date, few studies have focussed on plant-animal interactions in South African ecosystems (Cooper and Owen-Smith, 1985; Glyphis, 1985; Glyphis and Puttick, 1988; Ras, 1990). Despite the fact that most of the karoo veld is extensively used for grazing for sheep and other indigenous herbivores such as rodents (e.g. Parotomys krantsii) (Milton

et al. in press) and other ungulates, little work has assessed the interaction and dynamics of plant secondary compounds production and herbivory. Therefore the main objective of this study was to test whether the patterns of allocation to defense in three widespread karroid shrubs were consistent with the predictions made by the Optimal Defense Theory. The study was designed to answer the following four questions.

1. Do the relative tannin-polyphenol levels relate to the grazing status of each species?
2. What is the relative response of the plant in terms of tannin-polyphenol production to different defoliation treatments?
3. Can the relative allocation of energy to defense be explained in terms of the trade off between this function and growth within each species ?
4. Are valuable plant tissues (in terms of the plant's fitness) allocated higher levels of tannin-polyphenols than less valuable tissues?

METHODS

Study site and species description

Field studies were carried out at the Tierberg Karoo Research Centre (33° 10'S, 22° 17'E) on the southern edge of the Great Karoo, 20 kilometers north of the Swartberg mountain range (Plate 1). The average annual rainfall in this area is 167±7mm. Rains are most likely to fall between February and May (ca 75mm) and droughts most often occur between September and January. Summer minimum and maximum temperatures range between 12°-16°C and 29°-34°C respectively. The winter minimum and maximum temperatures range from 3°-5°C and 16°-21°C respectively (Milton et al. in press).

Three species, *Osteospermum sinuatum* (Asteraceae), *Pteronia pallens* (Asteraceae) and *Ruschia vulnerans* (Mesembryanthemaceae) were selected on the basis of their varying degrees of palatability. *Osteospermum sinuatum* is a dwarf deciduous shrub with orthophyllous and fleshy leaves and it is one of the most palatable shrubs in the karoo (Plate 2) (van der Heyden and Stock, in press). *Pteronia pallens* is a dwarf evergreen shrub with sclerophyllous leaves and is highly unpalatable to sheep (Plate 3) (Watt & Breyer-Brandwijk, 1932). *Ruschia vulnerans* is a dwarf evergreen shrub with succulent leaves, which is unpalatable but non-toxic to sheep (Plate 4)(Stock pers. comm.).

Plate 1. Study site at the Tierberg Karoo Research Centre.

Plate 2. *Osteospermum sinuatum.*

Plate 3. *Pteronia pallens.*

Plate 4. *Ruschia vulnerans.*

Experimental design

The field studies were initiated at the end of April 1990. Within each species (*P. pallens*, *O. sinuatum*, *R. vulnerans*) five replicates of plants of similar size were subjected to three defoliation treatments:

1. control - no clipping of plant leaf and twig material,
2. 40% of plant leaf and twig material clipped, and
3. 80% of plant leaf and twig material clipped.

Thereafter, the remaining leaf and stem tips were spotted with paint to distinguish between new and old growth at the time of harvesting.

Harvesting of plant material

Plant shoots were harvested at the end of June 1990. Harvesting was carried out between 7:30 and 10:00am while the air temperature was less than 8°C. Plants were clipped at the base of the stem and immediately placed in brown paper bags and then into an insulated box and refrigerated without freezing within a couple of hours. Plant material was stored at 4°C during its transportation to the University of Cape Town. At the earliest opportunity (within 48 hours), the new plant growth was separated from the old plant material. Plant material was oven dried for approximately 96 hours at 50°C to a constant mass. The plant material was further separated into three categories

(old stem-, old leaf- and new leaf material), of which the dry weights were determined. The separate plant parts were shredded through a Wiley Mill and ground through a 40 mesh sieve and stored in a desiccator until analyses could be carried out.

Extraction and analysis of plant material

All plant extractions and analyses were carried out in the Botany Department of the University of Cape Town.

Total polyphenols

The total polyphenolic concentrations within each plant category was determined using the Prussian Blue assay (Price and Butler, 1977). This technique is recommended by Hagerman and Butler (1989) since it is less susceptible to interference from proteins than other total polyphenolic assays (e.g. Folin assay).

Assay method: Ground plant material (60mg) was shaken constantly for 60s in 3ml of methanol, then poured into a Buchner funnel (containing Whatman No 1 filter paper), with the suction already turned on. The extraction vessel was rinsed with a second 3ml aliquot of methanol and filtered under vacuum. The combined filtrate (6ml) was mixed with 50ml of water in an Erlenmeyer flask and the polyphenol content determined within an hour.

Three milliliters of 0.1 M $FeCl_3$ in 0.1 N HCl was added to the extract (56ml) followed immediately (within 5 seconds) by addition of 3 ml of 0.008M $K_3Fe(CN)_6$. This was done at 1 minute intervals to allow for spectrophotometric readings of the samples at equivalent intervals after initiation of the reaction. In this way, small increase in OD with time exposure of $FeCl_3$ to methanol could be avoided (Price and Butler, 1977).

The OD of three replicates from each sample extract (thereby alleviating spectrophotometric error) was read after 10 minutes in 1-cm glass cells at 720nm on a Bausch & Lomb Spectronic 21 spectrophotometer, which had been zeroed with distilled water. Blanks of identical composition, but omitting the plant extracts, were also determined (3 replicates).

Standard Curve: A standard curve of OD versus commercial catechin up to 1mM was constructed. Sample readings were expressed as catechin equivalents.

Sigma Chemical Co.

Condensed Tannins

These were determined using the Vanillin assay (Price et al, 1978), recommended by Hagerman and Butler (1989). It is thought to be specific by selectively determining flavanol units (chemical structure of condensed tannins) in the

presence of hydrolysable tannins or other polyphenolics (Hagerman and Butler, 1989).

Assay method: Ground plant material (200mg) was extracted with 10ml of methanol in capped, rotating polytop vials (No 8) for 20 minutes and poured into a Buchner Funnel (containing Whatman No 1 filter paper) with the suction already turned on. One milliliter of the filtrate was dispensed into each of 6 test tubes (three experimental and three blanks). The extracts in the test tubes were placed in a water bath and allowed to warm to 30°C.

Vanillin reagent was prepared daily by mixing equal volumes of 1% vanillin (w/v) in methanol and 8% concentrated HCl (v/v) in methanol. The reagent was kept at 30°C and was added in 5ml aliquots at 2-minute intervals to the 1-ml aliquots of the samples. Five milliliters of 4% concentrated HCl in methanol (v/v) (prepared daily and previously warmed to 30°C) was added to the blanks, at 2-minute intervals. In this way the reading of the set of experimental tubes and the subsequent reading of the blanks were carried out over equally timed periods for each plant extract.

The test tubes remained in the water bath for 20 minutes whereupon samples were read at 500nm in 1-cm glass cells on a Bausch & Lomb Spectronic 21 spectophotometer.

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Protein precipitating tannins

The protein precipitating tannins (index of biological activity) were quantified in each plant category using the radial diffusion assay (Hagerman, 1987). This assay is recommended (Hagerman and Butler, 1989) for its simplicity and it assumes that the precipitated complex provides an index of the amount of biologically active tannin in the sample. Furthermore the simplicity of the assay allowed for a large number of samples to be analysed simultaneously.

Assay method: A 1% (w/v) solution of agarose (Sigma Chemical Co.) was prepared in buffer A by heating the suspension of agarose to boiling while stirring. The

solution was cooled to 45°C in a water bath, and the protein [0.1% (w/v) BSA (Sigma Chemical Co.)] added while the solution was gently stirred. Aliquots (9.5ml) were dispensed into Petri dishes (8.5 cm diameter) and allowed to cool on a level surface. The plates were stored at 4°C to prevent bacterial growth. Uniform wells were punched in the plates with a punch 4.0mm in diameter. The wells were placed 1.5cm apart so that there were approximately 4 to 5 wells per plate.

Plant tissue (400mg) was extracted in 2ml 70% acetone for an hour at room temperature in rotating capped polytop vials. An aliquot of 20 μl of plant extract was added to each well with a micropipette. Petri dishes were covered and sealed with cling wrap and incubated at 20° for 96h. The diameter of each ring was then measured. For each ring, two diameters at right angles to one another were measured to minimize errors due to nonuniform ring development (Plate 5).

Standard Curve: Concentrations of tannic acid up to 1mg/ml were used to construct a standard curve. Tannin concentrations within the plant parts were calculated from the square of the average of the two diameters from the tannic acid standard curve (Plate 6).

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Standard Curve: Concentrations of tannic acid up to 1mg/ml were used to construct a standard curve. Tannin concentrations within the plant parts were calculated from the square of the average of the two diameters from the tannic acid standard curve (Plate 6).

(a)

(b)

(c)

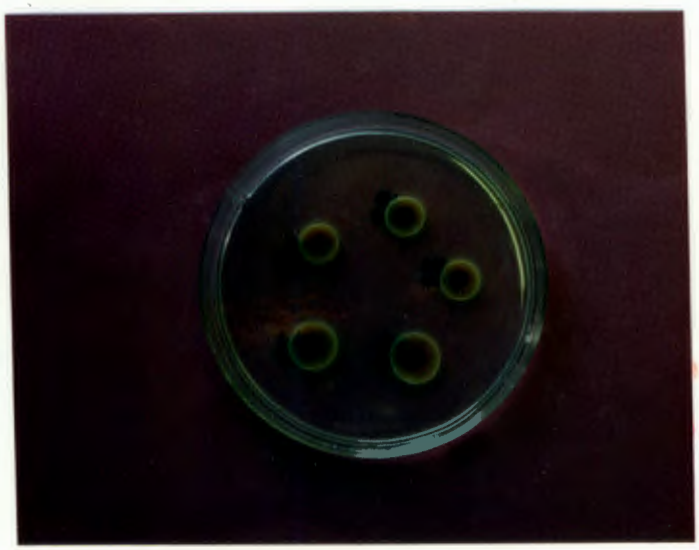


Plate 5. Estimation of protein precipitating tannins by measuring the two diameters of the rings formed from the extracts on the protein gel plates: (a) no ring formation in *O. sinuatum*; (b) no ring formation in *P. pallens*; (c) ring formation in *R. vulnerans* indicating the presence of protein-precipitating tannins.

Regrowth ratios

The regrowth ratio was calculated to determine the relative difference between species in terms of regrowth after defoliation. The following formula was used:

$$\text{Regrowth ratio} = \frac{\text{Dry mass of new and twig material}}{\text{Dry mass of old leaves and stems}} \times 100$$

Statistical analyses

Two way analyses of variance were used to compare the production of polyphenolics, condensed tannins and protein precipitating tannins across defoliation treatments between and within species. One way analysis of variance was used to compare the effects of defoliation on polyphenolic production (each chemical component) within each species. This was followed by Tukeys multiple range tests to determine where the significant differences lie.

RESULTS

Total polyphenolics

The concentrations of total polyphenols in the above-ground plant parts of each species were calculated from the catechin standard curve (Figure 2). Polyphenolic concentrations varied significantly among species ($p < 0.0001$) (Figure 3, Table 1). *R. vulnerans* had the highest polyphenolic concentrations of the the study species (Figure 3). It was estimated that the total polyphenolic content

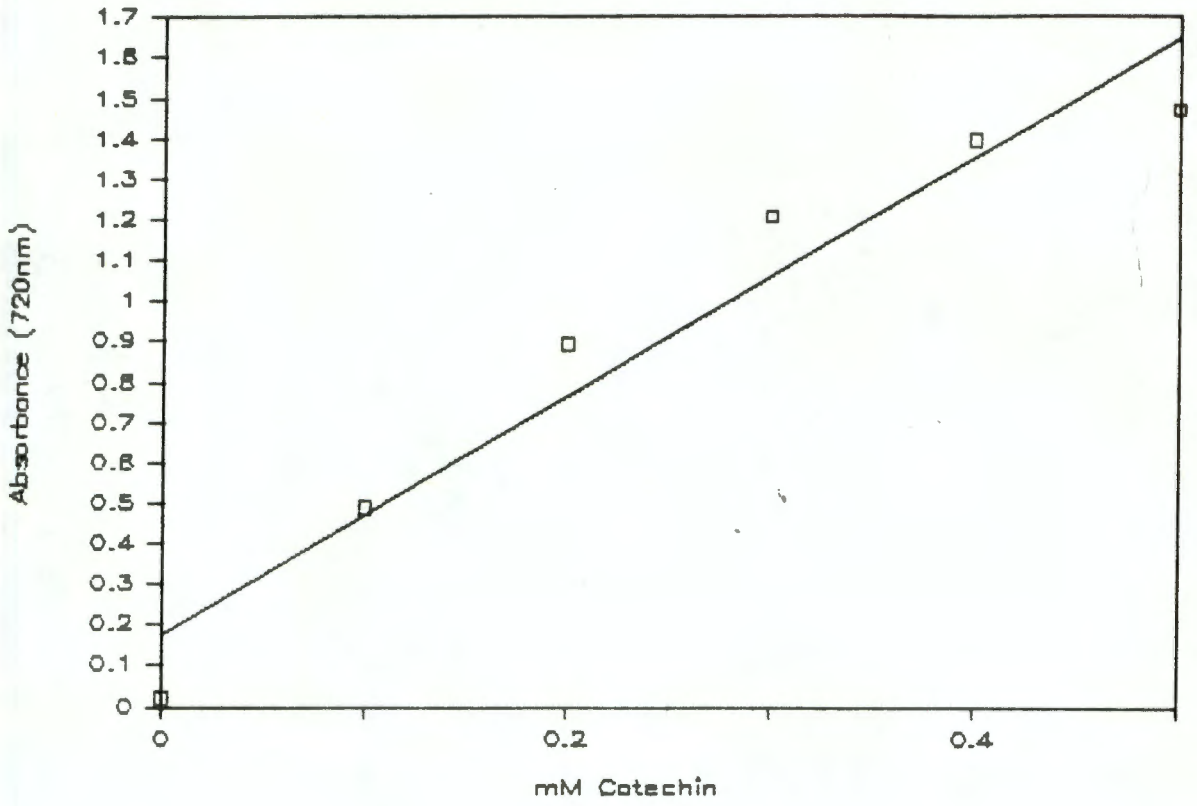


Figure 2. Catechin standard curve for total polyphenolics.

Table 1. Two way ANOVA where total polyphenols of new leaf, old leaf and old stem tissue are compared across species and treatments. F values shown together with significance level. Significance test ?

	Species	Defoliation
	F value	F value
Total polyphenols		
new leaf	150.42****	1.237
old leaf	220.43****	4.62*
old stem	188.12****	1.39

* $p < 0.05$
**** $p < 0.0001$

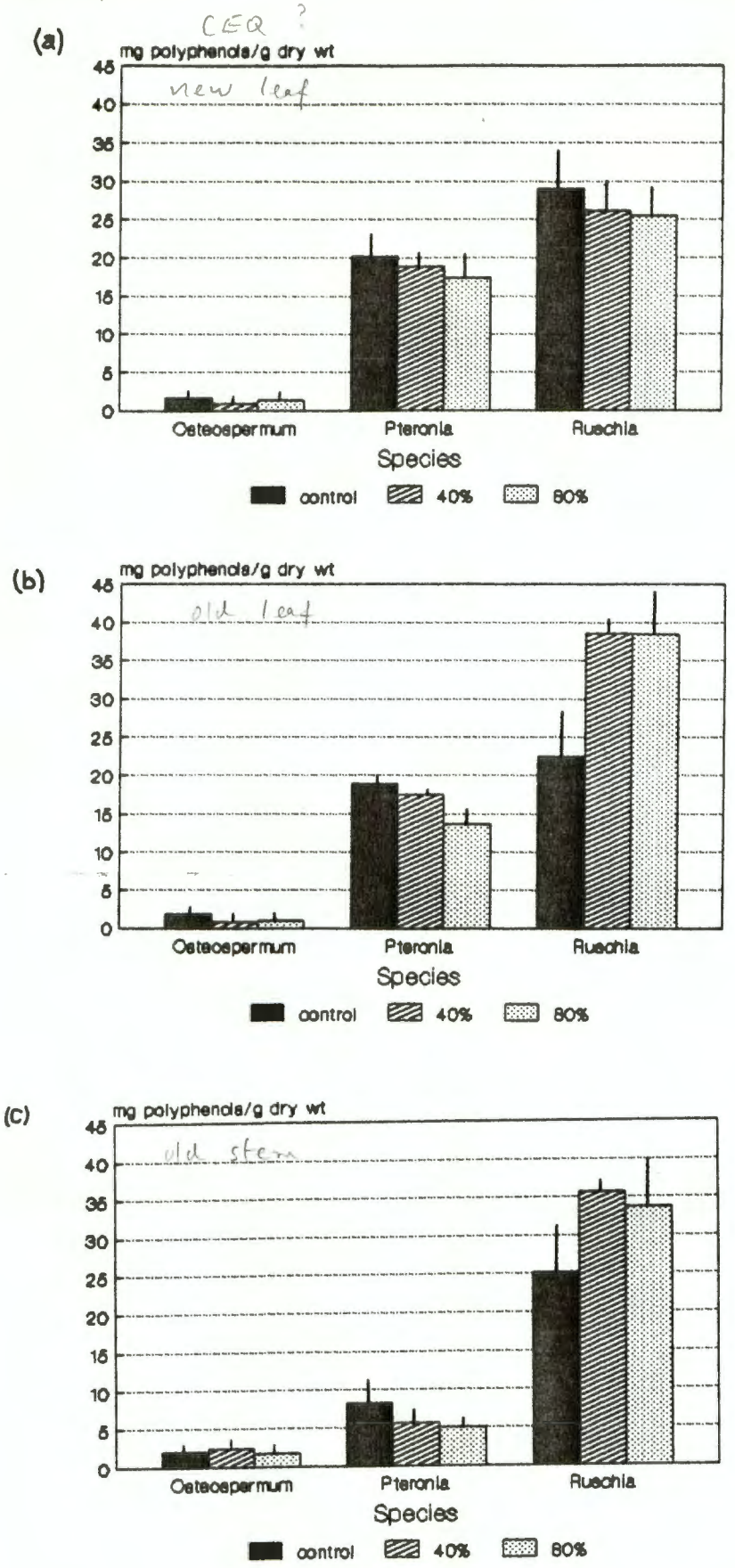


Figure 3. Total polyphenolic concentrations in each organ of *R. vulnerans*, *P. pallens* and *O. sinuatum*, for each defoliation treatment: (a) new leaf tissue; (b) old leaf tissue; (c) old stem tissue.

within the plant parts of this species ranged from 2.68 to 3.31 CEQ % d.w. In contrast total phenolic concentrations in the organs of *P. pallens* and *O. sinuatum* ranged from 0.633 to 1.88% and 0.12 to 0.21 CEQ % d.w. respectively.

The effects of defoliation intensity on the total polyphenolic production within each species (Figure 4) showed that plant responses were significantly different in *R. vulnerans* and *P. pallens* (Table 2). Within *R. vulnerans*, the 40% defoliated plants had significantly higher ($p < 0.01$) total polyphenol content (100.34mg) than the control plants (76.6mg). Despite the similar increase in polyphenolic content in the 80% plants (97.62mg), this increase was not significantly ($p > 0.05$) different from the control plants (Table 2). The lack of significance can be attributed to the large standard errors of the sample means within both these plant treatments due to the low number of replicates. Other factors such as small sample size, and slight variation in the time at which sample readings were taken (ranging between 10-12 minute intervals after reactions were started), could also account for this phenomenon. In contrast, within *P. pallens*, defoliation resulted in a significant decrease ($p < 0.01$) in total polyphenolic content of the 80% plant (35.47mg) relative to the control (47.61mg) (Table 2). Although the 40% plants did not differ significantly ($p > 0.05$) from the control plants, they too exhibited a lower production of

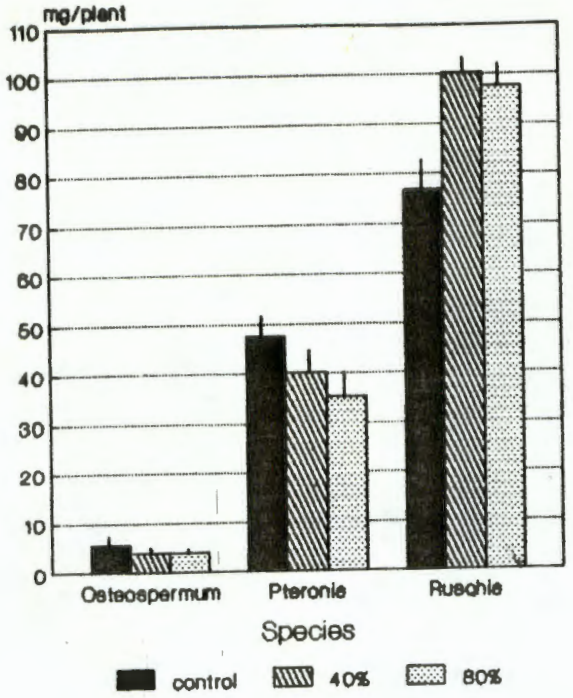


Figure 4. Total plant polyphenolic content of *R. vulnerans*, *P. pallens* and *O. sinuatum* for each defoliation treatment.

As the defoliated plant is smaller (presumably), then concentrations must be much higher. Surely, it is amts that are important w.r.t. palatability.

Table 2. Two way ANOVA where total polyphenolics (amount of) of each species are compared across defoliation treatments and organ categories. F values shown together with significance level. Letters indicate homogenous groups derived from Tukeys multiple range test.

	Defoliation	Organs
	F value	F value
Total polyphenols		
R. vulnerans	6.069**	3.540
C a		
40 b		
80 a b		
P. pallens	7.52**	86.84****
C c		
40 c		
80 d		
O. sinuatum	1.35	3.399
C n		
40 n		
80 n		

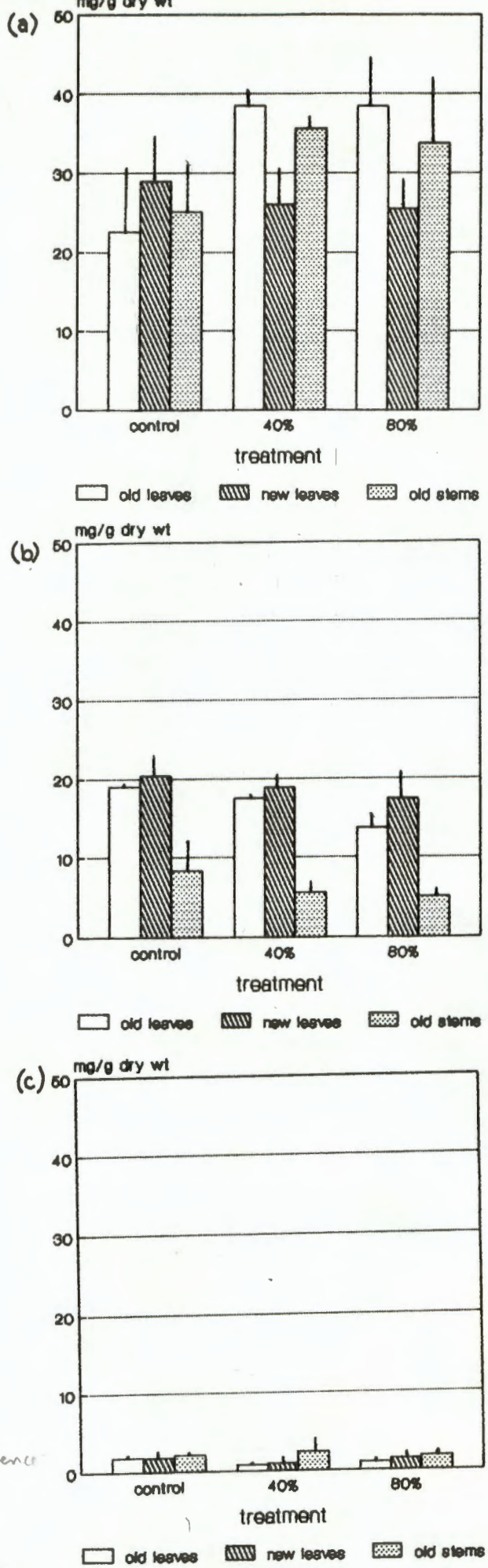
** p < 0.01
**** p < 0.0001

Total polyphenolics
- sum of all different types of polyphenolics
or - total amt of polyphenolics in each plant?

polyphenolics than the control plants (40.08mg) (Figure 4). On the other hand, defoliation treatments had no effect on the polyphenolic production in *O. sinuatum* (Figure 4).

In terms of the distribution of total polyphenolics in the different organs of *R. vulnerans*, there was no significant difference in the polyphenolic concentrations between the organ categories (Table 2). However, in response to defoliation treatments, there was a significant increase in polyphenolic content in the old leaves of the 40% and 80% defoliated plants (38.5mg and 35.3mg respectively) relative to the controls (22.5mg) (Figure 5a, Table 3). Although polyphenol levels in the old stem material in the three defoliation treatments did not differ significantly, there were simililar responses to that of the old leaves in the 40% and 80% defoliated plants (Figure 4).

The distribution of polyphenolics in the new and old leaves (18.8mg and 16.7mg) of *P. pallens* were significantly higher than in the old stems (6.3mg) (Figure 5b, Table 2). There was a significant ($p < 0.01$) decline in polyphenolics in the old leaf material of the 80% defoliated plants (13.7mg) relative to both the control (18.9mg) and 40% (17.4mg) defoliated plants (Table 3). However the relative polyphenolic content in the new and old leaves remained relatively constant in all the defoliation treatments.



R. vulnerans

P. pallens

O. sinuatum

What is the difference between this & Fig 3? Looks like the same data sent to me.

Figure 5. Total polyphenolic concentrations in all the plant parts for each defoliation treatment: (a) *R. vulnerans*; (b) *P. pallens*; (c) *O. sinuatum*.

Table 3. One way ANOVAS comparing total phenolics of each organ across defoliation treatments for *R. vulnerans*, *P. pallens* and *O. sinuatum*. F values are shown together with significance level. Letters indicate homogenous groups derived from Tukeys multiple range test.

		Total polyphenols		
		R.vulnerans	*P. pallens*	*O.sinuatum*
		F value	F value	F value
new leaf	C a 40a 80a	0.464	0.880	1.129
old leaf	C b 40c 80c	8.633*	16.774**	2.423
old stem	C d 40d 80d	3.097	1.830	0.355

* $p < 0.05$
** $p < 0.01$

O. sinuatum showed no significant differences in polyphenolics between different organ categories of all treatments (Figure 4). Furthermore, the concentrations of polyphenolics remained relatively constant within each organ category following defoliation (Figure 5c). Again, I have this concentration / content problem. Fig 4 is content (mg/plant); Fig 5 is conc. (mg/g dwt). Surely, with 80% defoliation, one (at least) of these must change.

Condensed tannins

The condensed tannin content of the plant parts of each species were calculated from the catechin standard curve (Figure 6). Condensed tannins were present in only one of the three species, *R. vulnerans* (Figure 7).

There was a significant difference between the 40% and the control treatments with respect to the total content of condensed tannins per plant ($p < 0.05$, Table 4). Although the 80% treatment plants exhibited the highest quantities of condensed tannins (total of the three organs), this increase was not found to be significantly different ($p > 0.05$) from both the control and 40% treatment plants (Figure 8). This can be attributed to the large standard errors of the sample means within these treatments due to low replication. As with the Prussian blue assay for the total phenolics, slight variation in assay conditions (fluctuating water bath temperature and time intervals at which readings were done) could also have contributed to this phenomenon.

Or perhaps there really is no difference - see Fig 8

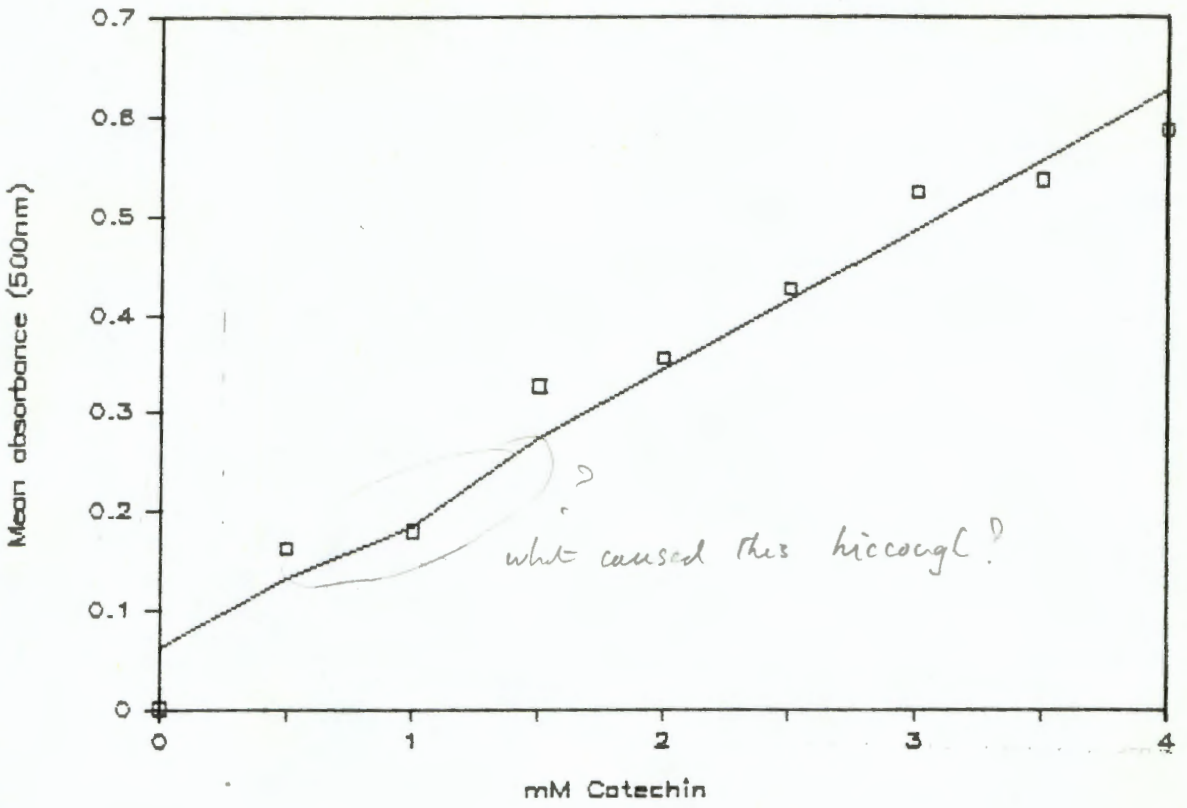


Figure 6. Catechin standard curve for condensed tannins.

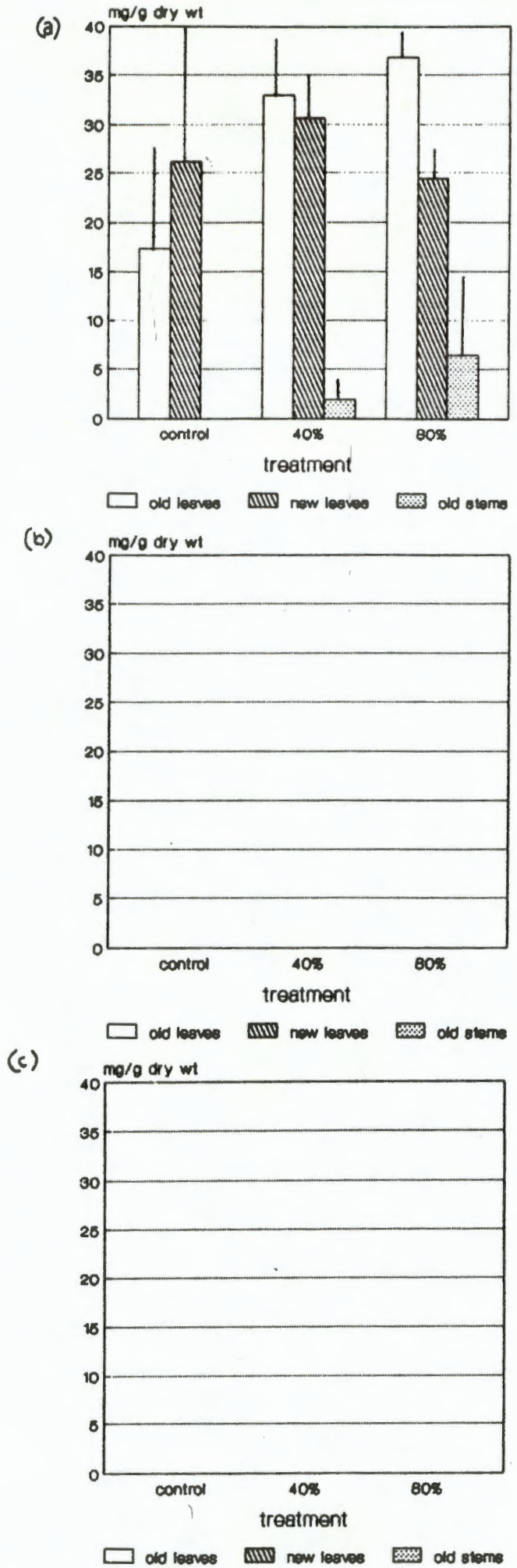


Figure 7. Condensed tannin concentrations in all the plant part for each defoliation treatment: (a) *R. vulnerans*; (b) *P. pallens*; (c) *O. sinuatum*

Table 4. Two way ANOVA comparing condensed tannins of *R. vulnerans* between defoliation treatments and organ categories. F values are shown together with significance level. Letters indicate homogenous groups derived form Tukeys multiple range test.

	Condensed Tannins
	F value
Defoliation	4.086*
C [a]	
40 [b]	
80 [b a]	
Organs	38.7****
new leaf [a]	
old leaf [a]	
old stem [b]	

* p < 0.05
**** p < 0.0001

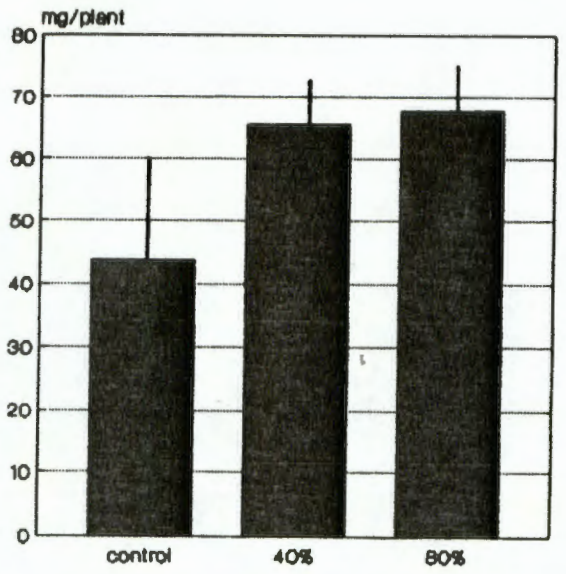


Figure 8. Total plant condensed tannin concentrations in the three defoliation treatments of *R. vulnerans*.

concentrations

Table 5. One way ANOVAS condensed tannins of each organ between defoliation treatments for *R. vulnerans*. F values shown with significance level. Letters indicate homogenous groups derived from Tukeys multiple range test.

		Condensed tannins
		F value
new leaf		0.44
	C [a]	
	40[a]	
	80[a]	
old leaf		8.24*
	C [b]	
	40[c]	
	80[c]	
old stem		2.38
	C [a]	
	40[a]	
	80[a]	

* $p < 0.05$

Condensed tannin concentration differences between organ categories of this species showed that new and old leaf material had significantly ($p < 0.0001$) higher concentrations than old stem material (Figure 7, Table 4). This enormous difference is interesting since the concentration of condensed tannins within the old stem constitutes only 10% of the polyphenolic concentrations found in this organ (an estimate derived from the means taken across the three plant treatments). Nevertheless the concentrations of condensed tannins in new and old leaves constituted a very high proportion of the polyphenolic concentrations (>85%) found in those organs (Figures 5 and 7).

In response to defoliation, concentrations of condensed tannins in the old leaves increased significantly ($p < 0.05$, Table 5) in the 40% and 80% plants relative to the control. The concentration of condensed tannins in new leaves remained unchanged in response to defoliation. The apparent increase in the concentration of condensed tannins in old stems with increasing defoliation intensity (Figure 7), was not statistically significant ($p > 0.05$, Table 5).

Protein precipitating tannins

Estimated concentrations of protein precipitating tannins were derived from the tannic acid standard curve (Figure 9). Protein precipitating tannins were only found to occur in *R.*

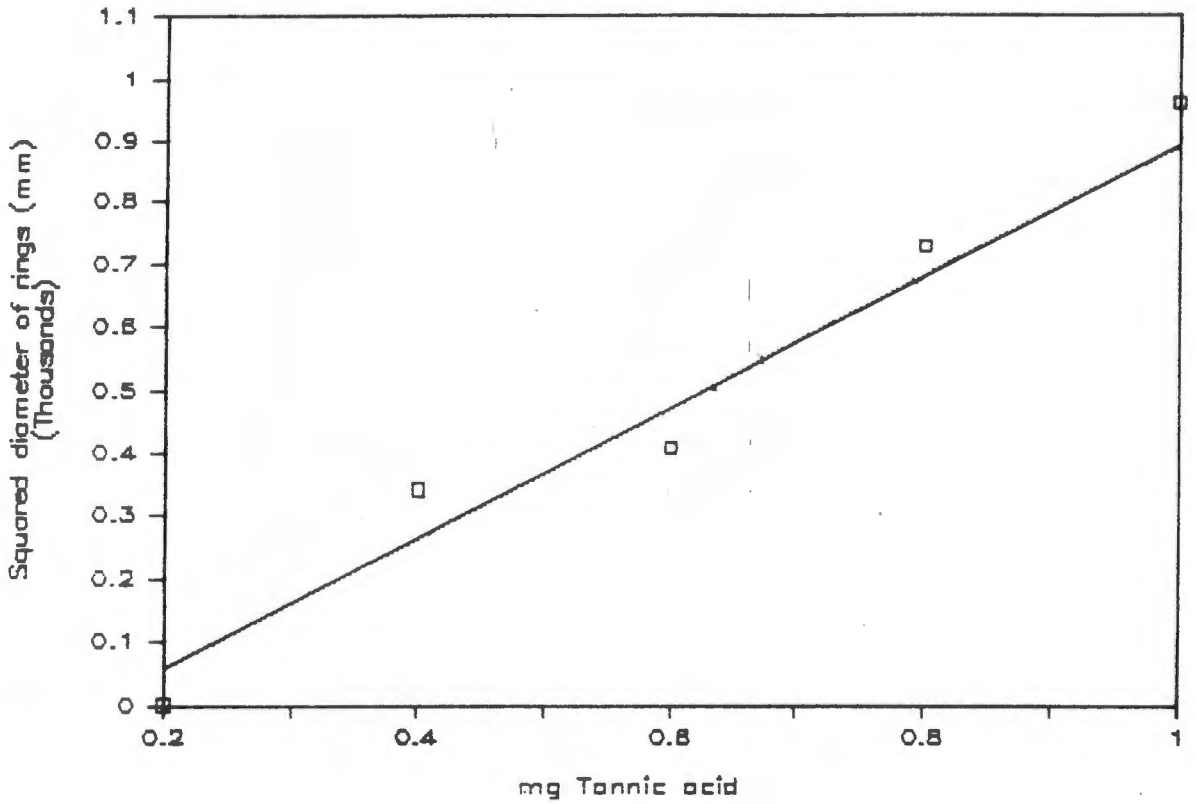


Figure 9. Tannic acid standard curve for protein-precipitating tannins.

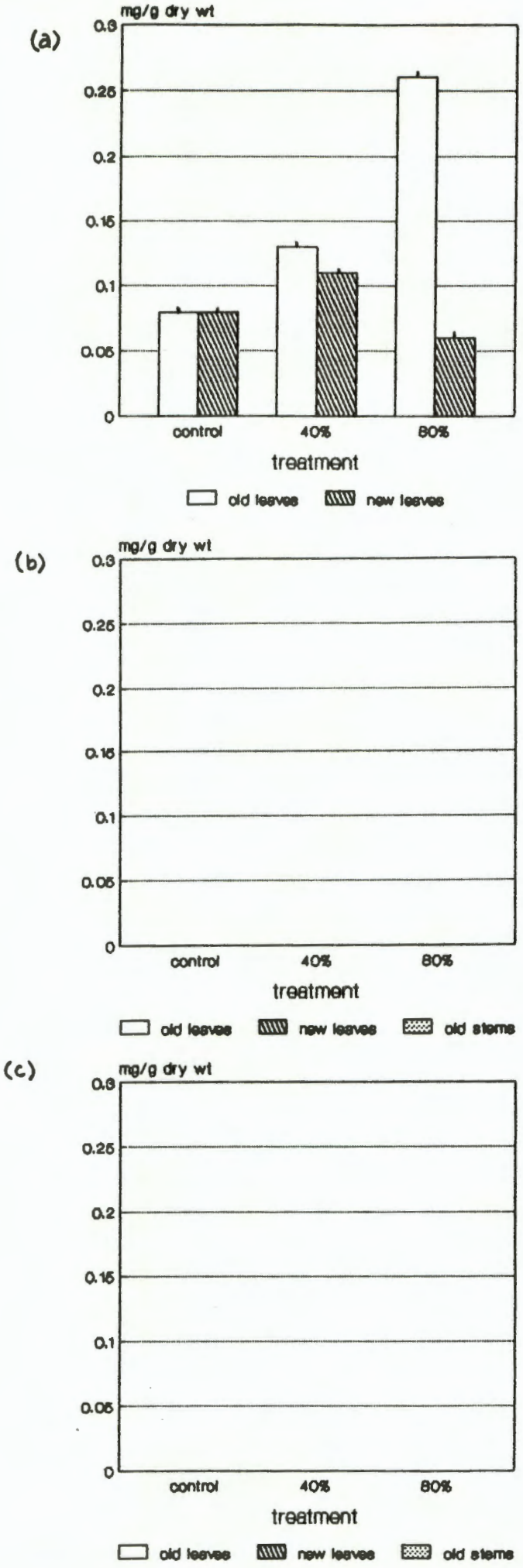


Figure 10. Protein-precipitating tannin concentrations in all the plant parts for each defoliation treatment: (a) *R. vulnerans*; (b) *P. pallens*; (c) *O. sinuatum*.

vulnerans (Figure 10). Old leaves had significantly higher concentrations of protein precipitating tannins than new leaves ($p < 0.01$). Regarding the total plant protein precipitating tannin concentrations, the 80% plant had significantly higher concentrations than both the control and 40% plants. There was no significant difference ($p > 0.05$) in protein precipitating tannins between the control and 40% defoliated plants (Table 6).

Despite the fact that no direct measurement of hydrolysable tannins was made, it can be inferred that *P. pallens* and *O. sinuatum* have little or even no tannin polyphenolics, since no condensed nor protein precipitating tannins were found in their respective plant extracts. In *R. vulnerans*, the condensed tannin component constituted a large proportion of the total polyphenolics. Furthermore, the protein precipitating tannins showed patterns similar to those found for condensed tannins between and across defoliation treatments (Figures 7 and 10). Hence it may be inferred that the protein precipitating effect quantified in this study was due mainly to the condensed tannin component.

Regrowth ratios

The regrowth ratios for the three treatments of plants within each species are shown in Figure 11. There were significant differences between the regrowth ratios of the three defoliation treatmnents ($p < 0.0001$) of *R. vulnerans*

Table 6. Two way ANOVA comparing protein-precipitating tannins of *R. vulnerans* between defoliation treatments and organs. F ratios shown together with significance levels. Letters indicate homogenous groups derived from Tukeys multiple range test.

	Protein-precipitating tannins
	F value
Defoliation	7.357*
C [a]	
40 [a b]	
80 [b]	
Organs	17.082**
new leaf [a]	
old leaf [b]	

* $p < 0.05$
** $p < 0.01$

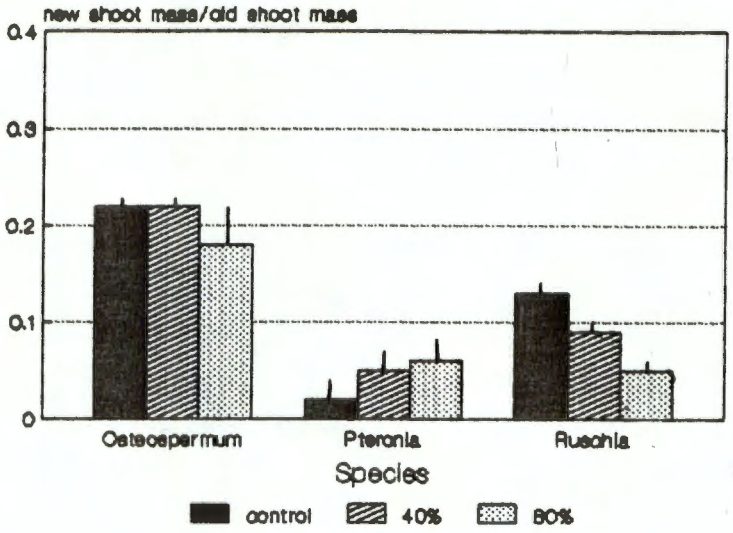


Figure 11. Regrowth ratios for *O. sinuatum*, *P. pallens* and *R. vulnerans* in response to defoliation treatments.

Table 7. One way ANOVAS comparing regrowth ratios for *O. sinuatum*, *P. pallens* and *R. vulnerans* between defoliation treatments. F values shown together with significance level.

	Defoliation
Regrowth ratio	F value
R. vulnerans	240.52****
P. pallens	3.60
O. sinuatum	0.582

**** $p < 0.0001$

(Table 7). The regrowth ratios decreased with increasing defoliation intensity. This pattern coincides well with production of total polyphenolics of the defoliation treatments. In contrast, *P. pallens* and *O. sinuatum* did not show significant differences between the regrowth ratios across the defoliation treatments (Table 7). However, within *P. pallens*, increasing defoliation treatments resulted in an increase in plant regrowth. As in the case of *R. vulnerans*, this pattern coincides with the production of total polyphenolics across defoliation treatments.

DISCUSSION

It has been established in this study that the palatability status is not entirely related to the tannin polyphenol levels of the study species. *P. pallens*, known for its unpalatability and toxicity to animals, was found to possess no tannin polyphenols in its above-ground organs. However, concentrations of other unidentified phenols were detected, particularly in the new and old leaf material. It has been suggested that acyl-glucosides are the major chemical deterrent agent in this species (Heywood et al., 1977). *R. vulnerans*, known to be generally unpalatable, but non-toxic to sheep showed relatively high tannin polyphenolic concentrations in the above-ground organs which relates well to the unpalatable status of this species. The high polyphenolic levels and the presence of thorns indicate that

this species employs both structural and chemical defenses to deter herbivores. In *O. sinuatum*, the absence of tannin polyphenolics and low concentrations of other unidentified phenolics in the above-ground organs, coincides with palatability status of this species. Therefore this variation in the type of defense compounds found within species suggests that the biochemistry of defense compounds is constrained by phylogeny.

Defoliation had a significant effect on the production of polyphenolics of *R. vulnerans* and *P. pallens*, the two species which showed the presence of chemical defense compounds. Within *R. vulnerans*, the 40% and 80% defoliated plants exhibited elevated levels of polyphenolics, condensed tannins and protein-precipitating tannins, in comparison to the control (Figures 4, 8 and 10). This finding supports the hypothesis that due to the high cost involved in chemical defense compounds, plants would be more heavily defended when they are subjected to herbivory than when enemies are absent (Rhoades, 1979). Similar findings have been reported with respect to the effects of defoliation treatments on polyphenolic production in *Popoulus* and *Acer* species (Baldwin and Schultz, 1983), *Betula pubescens* spp. *tortuosa* (mountain birch) (Tuomi et al.,1988) and *Portulacaria afra* (spekboom) (Ras, 1990). In contrast to these findings, *P. pallens* showed an inverse relationship between defoliation intensity and polyphenol production

(Figure 4). These contrasting patterns re-emphasize the notion that resource allocation should be considered in terms of the continuous trade off between reproduction, defense and growth. It is hypothesized that the optimal defense investment should increase as the potential growth rate of the plant decreases. This argument is based on the prediction that as potential growth rates become more limited by resource availabilty, replacement of resources lost to herbivores become more costly. Furthermore, a given rate of herbivory (grams of leaf removed per day) represents a larger fraction of the net production of a slow-grower than that of a faster growing species. Hence the relative impact of herbivory increases as inherent growth rates decline (Coley et al., 1985). In the case of *R. vulnerans*, the trade off between allocation to growth and defense is very evident in Figures 4 and 11, i.e. there is a notable decrease in regrowth which coincides well with the increase in defense compounds in the 40% and 80% defoliated plants. In contrast, the 40% and 80% defoliated plants of *P. pallens* resulted in increased plant regrowth (Figures 4 and 11). Hence it can be argued that within P. pallens, production of chemical defense compounds decreases at the expense of energy allocated to growth to compensate for defoliation. This hypothesis is further supported by the findings for *O. sinuatum* (Figures 5 and 11), which is a rapid regrower and showed no allocation of resources to defense compounds in response to defoliation.

I reserve judgement on these interpretations until the content/concentration confusion has been resolved.

The distribution of polyphenols within the plant parts in response to defoliation were shown to differ *for R. vulnerans* and *P. pallens*. Within *R. vulnerans*, defoliation treatments resulted in an increase in polyphenolics, condensed tannins and protein precipitating tannins in both the old leaves and old stems, whereas in the new leaf material it remained relatively constant (Figures 5,7 and 10). However, in response to defoliation within *P. pallens*, the polyphenolic content within the old leaves decreased significantly relative to the new leaves. Furthermore, the polyphenolic content within the new and old leaves were consistently higher than in the old stem material across defoliation treatments (Figures 3 and 5).

It has been suggested that the value of a particluar organ is the contribution that it makes to the fitness of the plant (or its genes) make to the future populations (Harper, 1989). According to the ODT, it is hypohtesized that plants allocate more defense compounds to valuable tissues, than to less valuable tissues (Rhoades, 1979). Since leaves are the primary energy trapping organs of plants, they can be considered to be highly valuable in terms of their contribution to the plant's fitness. However, based on the amount of chemical defense compounds produced within the new and old leaf categories itself, studies have shown there to be a clear distinction between

the value of new and old leaves to the plant (Feeny, 1970; Meyer and Montgomery, 1987). Since photosynthetic activity decreases with the age of the leaf, it is argued that the value of new leaves increases relative to old leaves if a proportion of the fixed carbon is currently directed to the production of new leaves. In such plants, the new leaves would be relatively more worth defending than old leaves (Harper, 1989). On the other hand, it is argued that if the rate of new leaf growth is not dependent on current assimilates; but the plant invest current assimilates in accumulating reserves for a subsequent season (storage pool), then there would be no obvious difference between the value to the plant of assimilates produced by a leaf when it is young and when it is old. Instead the value of a leaf will depend much more on the relationship between leaf age and leaf longevity. If the plant's older leaves continue to assimilate, even at a reduced rate, and are acting as a nutrient sink from which nutrients can be mobilized for future growth, they are may be worth defending (Harper, 1989).

Photosynthetic studies have shown *R. vulnerans* to have unusually low carbon assimilation rates throughout the year (Phillips, pers comm.), which would explain their slow growth rates. Van der Heyden (pers comm.) found that this species has slow regrowth rates and does not re-allocate carbohydrates from storage pools following defoliation. It

can be inferred from this information that carbon assimilated during photosynthesis, however low, is not allocated to the production of new leaf material, but is possibly utilized for maintenance of respiratory carbon substrates and for the production of chemical defenses. Therefore, as hypothesized by Harper (1989), new leaves would not be considered to be more valuable than older leaves in *R. vulnerans*. Instead it can be argued that the old leaves, which continuously assimilate carbon, although at low rates, are more valuable in terms of the plant's fitness and is therefore worth defending.

The relative increase in the polyphenolic concentrations in the old stem material of *R. vulnerans* in response to defoliation may be related to the fact that a major proportion of the plant's TNC pool is stored in the stems (van der Heyden, pers comm). Hence the old stem material can be considered to be valuable in terms of its contribution to plant fitness, in that it stores a major proportion of the resources needed for future growth, defense and reproduction. On the other hand, the stems of *P. pallens* form a relatively lesser important storage organ for TNC than for *R. vulnerans*. Consequently the stems would be less likely to have a large degree of protection which would explain the relatively lower polyphenolics in the stems of *P. pallens* relative to *R. vulnerans* (Figure 3).

P. pallens showed greater regrowth responses to defoliation than *R. vulnerans*, which can be explained by the high carbon assimilatory rates of this species throughout the year (Phillips pers. comm.). Van der Heyden (pers. comm.) found that *P. pallens* generally has a lower concentration of TNC in storage organs than *R. vulnerans*. It can be argued that production of new leaf material is highly dependent upon the current assimilates of the plant. Consequently the new leaf material can be considered to be more valuable in terms of its contribution to plant fitness, than the old leaves. This explains the decline of the chemical defense compounds in the the old leaves of the defoliated plants. It can be inferred that assimilated energy is directed to new leaf production and the defense of these new leaves to herbivores.

Growth form and leaf longevity differences between *R. vulnerans* (evergreen succulent), *P. pallens* (evergreen sclerophyll) and *O. sinuatum* (drought- deciduous perennial), may be important factors influencing the type of defense compounds (or the amount) within these three species. It is generally argued that leaf longevity increases in slow-growing plants of resource limited environments (Coley et al., 1985), since nutrients are conserved and recycled within the plant, instead of being lost to the environment during rapid leaf turnover. It is suggested that defense compounds, such as polyphenols and fibre (quantitative

defenses) are expected to be present in high concentrations in long-lived leaves for the following reasons. The initial structural cost of quantitative defenses are high, however, their continued maintenance costs within the plant are small since they are fairly inactive metabolically. However, due to their immobility, they are retained in senescent leaves and lost to the plant upon leaf death. Therefore these immobile defenses would be advantageous in long-lived leaves which have more time over which to spread these fixed costs (Coley et al. 1985). Data from 41 tree species in a neotropical forest support this hypothesis in that significant increases in polyphenols and fibre contents were observed in leaves as leaf longevity increases (Coley, 1983). Similarly, in this study, the relatively high levels of polyphenols in *R. vulnerans* and *P. pallens* correlates with their leaf longevity. It should be borne in mind that these species experience low soil water level during most part of the year. On the other hand, *O. sinuatum* which escapes the summer drought by shedding its leaves, have very neglible amounts of quantitative defenses.

The results of this study support the predictions made by the optimal defense theory in that the level of resource allocation to defense is at a dynamic equilibrium with resources allocated to other contingencies such as growth and reproduction. It was shown that species exhibiting inherently slow growth rates imposed by resource limitation,

have relatively higher levels of chemical defense (*R. vulnerans*) than species exhibiting inherently faster growth rates (*O. sinuatum*). The patterns of polyphenolic production in response to defoliation ovbserved in *R. vulnerans* coincides with the results obtained from a similar study carried out on *Portalacaria afra* in the Valley Bushveld area of the eastern Cape (Ras, 1990). Both *R. vulnerans* and *P. afra* are evergreen succulents growing in environments where water limits plant growth rates. Consequently it was found that defoliation treatments resulted in an increase in polyphenolic levels within the above-ground plant parts in both these species. Similarly in boreal forests and arctic tundra, inherently slow-growing woody species that have limited capacity for compensatory growth were shown to be less palatable to a wide variety of herbivores in comparison to more rapidly growing species that have a greater capacity for compensatory growth (Bryant et al., 1983).

Although limited, there is accumulating evidence including this and other South African and international studies, which support the theory that a limited capacity to aquire resources and an inherently slow growth rate limit a plant's ability to compensate for herbivory through growth. Consequently slow-growers opt for producing high levels of defense compounds in their above ground parts. It would be interesting to test this hypothesis for fynbos species which

grow in unusually nutrient poor environments and exhibit inherently slow growth rates.

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