Chrysanthemoides spp from southern Africa analyzed for tannins in terms of predictions made from Optimal Defense Theory

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Abstract: Three Asteraceous shrub species of the genus *Chrysanthemoides; C.monilifera pisifera, C. monilifera monilifera* and *C. incana;* growing in two experimental plots (irrigated and non-irrigated) at Elsenberg Agricultural Research Centre, were analyzed for total polyphenolics, condensed tannins as well as tannin biological activity. In all species polyphenolic concentrations were low; less than 5 mg CEQ g^{-1} dry weight (0.5% of d.w.). No tannins, condensed or hydrolyzable, were found in any of the species. The absence of tannins, the low polyphenolic levels (concentrations in excess of 2% d.w. are thought to be necessary to deter herbivore browsing; Swain, 1979) and the general palatability of this genus in southern Africa leads to the conclusion that utilization of these plants as a perennial browse shrub will not be limited by chemical defenses. On a theoretical level, predictions from Rhoades' (1979) Optimal Defense Theory as well as related hypotheses (eg. Feeny, 1976; Coley, 1985) are questioned. In particular the prediction that species from nutrient poor habitats should have greater defense expenditure (than species from nutrient rich environments) is questioned in context of Campbell's (1986) argument that the low quality food nutrient poor environments offer ~~are~~ is in itself an anti-herbivore "defense".

Introduction

Until recently secondary compounds found in plants have been regarded as waste products of primary metabolism with no possible survival value to the plant. This view has changed dramatically in the last decade or two with intense research into plant-herbivore interactions. There is by now a general consensus that secondary plant compounds play a significant role in determining patterns of plant utilization (Janzen, 1979).

Several studies seems to have established that unpalatability in non-toxic plants is due to polyphenolic concentration (Rhoades, 1979). The important anti-herbivore compound found in this family of chemicals is tannin. Tannins are any substances capable of precipitating the gelatin of animal hides as an insoluble compound, so changing the hide to leather which is resistant to putrefication. Tannins are divided into two main groups according to chemical structure, tannin action and water solubility: the proanthocaynins (condensed tannins) and hydrolyzable tannins. The former are found in all classes of plants, including ancient classes such as *Equisetum*; in Angiosperms they are mainly confined to woody species, but are found in some grasses and herbs. The hydrolyzable tannins, on the other hand, occur only in dicotyledons; they

are two to five times more effective as protein precipitants on a weight basis than proanthocyanins (Swain, 1977).

Tannins bind to proteins forming complexes which render them unable to perform their function. Thus, for example, in the stomach enzymes responsible for digestion can not function. Goldstein and Swain (1965) demonstrated that tannins prevented enzyme activity *in vitro*. A plant thus protected is nutritionally unavailable to herbivores. The respective functions of the two types of tannin are thought to be different (Cooper & Owen-Smith, 1985).

Hydrolyzable tannins serve to inactivate the digestive enzymes of herbivores; while condensed tannins are attached to the cellulose and fibre-bound proteins of cell walls, thereby defending plants against microbial and fungal attack (Swain, 1979). Cooper and Owen-Smith (1985) suggest that the unpalatability of some woody plants to ruminant herbivores is associated primarily with the concentration of condensed tannins rather than with polyphenolics assayed in terms of their protein-precipitation properties (i.e. mainly hydrolyzable tannins). Therefore Cooper and Owen-Smith (1985) propose that this unpalatability is a consequence of the ruminants dependance upon microbial fermentation of cell walls for part of their energy needs. Thus, hydrolyzable tannins are primarily a defense against insect attack where as condensed tannins defend cell walls against fungal and

pathogen attack. From this perspective ungulates are third parties to the chemical warfare between higher plants and pathogenic micro-organisms, that is, defense against ungulates is incidental to this warfare (Cooper & Owen-Smith, 1985).

There is no definitive evidence for the mode of tannin action being antidigestive, all evidence being correlative (Bernays *et al.*, 1989). Bernays *et al.* (1989) further suggest that the other possible functions and metabolic costs of tannins have not been fully investigated, i.e. if other elements (e.g. nitrogen and phosphorus) are limiting it may be no loss to have carbon tied up in tannin. Despite this there is a general agreement that these compounds are generally effective as non-lethal feeding deterrents against a wide range of organisms from insects to mammals (Glyphis and Puttick, 1988), but they are relatively expensive to manufacture, i.e. in terms of carbon; at least two percent (Swain, 1979), and up to 50% (Gibbs, 1974), of plant dry weight may be tannins. Tannins occur in a variety of tissues of many plant species, especially in those which become woody during growth. There are often higher concentrations in lignified, woody tissues.

Most plant communities face a vast array of herbivores ranging from minute insects to large ungulates who consume between 10 and 20% of the annual production (Bazzaz *et al.*,

1987). Because of this impact herbivores are important selection agents and a plant has to defend itself from attack in order to survive. But defense has its costs and several studies have shown that overall allocation of resources to defense is negatively correlated to plant growth. Thus there should be a trade-off between factors related to growth (and reproduction) and components related to defense. This in essence is Rhoades' (1979) "Optimal Defense Theory" (O.D.T.), or more formally: defenses are costly, in terms of fitness, and thus less well defended plants should do better than more highly defended plants in the absence of herbivores.

To illustrate this concept of costly defenses Crawley (1983) undertook the following experiment. Two morphs of wild ginger, *Asarum caudatum*, differing in palatability to a slug, *Agriolimax columbianus*, were selected. When the two morphs were grown together in the absence of the slugs, the palatable morph produced 1.36 times more seeds. But, in the presence of the slugs the palatable morph produced only half as many seeds as the unpalatable morph.

Trade-offs are a consequence of different plant functions; growth, defense and reproduction; competing for limited resources within the plant. The economic concepts of costs-and-benefits have been used to explain different allocation patterns. For example, reproduction has

physiological cost requirements of the limited resources which otherwise would support vegetative growth, but reproduction has the obvious benefit of evolutionary fitness. The most widely used measure, or currency, of resource allocation is biomass. For example, the amount of biomass allocated to reproductive organs relative to vegetative parts. The patterns of biomass allocation seem similar in most plants, but analysis shows different nutrients in vegetative and reproductive organs. That is, there are different patterns of allocation for different resources. This is especially important in trade-offs between nitrogen based defenses and those based on carbon.

There are two principle chemical defense systems with differing metabolic costs. Tannins are non-toxic digestibility reducing defenses needed in relatively large amounts (> 2% d.w.; Swain, 1979) whereas toxins such as alkaloids and glucosinolates are needed in much smaller quantities (Gulmon & Mooney, 1986). Even though the toxins are more expensive in construction and maintenance costs than tannins, their effectiveness at low concentrations makes them a cheaper (in terms of carbon and energy, but not in potentially scarce resources such as nitrogen) defense against herbivore attack. But, specialist herbivores are able to co-evolve with toxins and given time will render this defense useless (except against generalist attack). On the other hand digestibility reducing compounds are

extremely difficult to adapt to and are effective against generalist as well as specialist herbivores (Janzen, 1979).

This has led to the ideas of "quantitative" and "qualitative" defenses as well as "apparent" and "non-apparent" plants (Feeny, 1976). "Quantitative" defenses such as tannins, resins and silica are dosage dependent and are often present in high concentrations. "Qualitative" defenses such as alkaloids, cyanogenic compounds and others are characteristically present in low concentrations; these compounds protect against generalist herbivores, but provide little protection against specialist attack. Quantitative defenses are characteristic of "apparent" plants where-as qualitative defenses are more typical of "non-apparent" plants. "Apparent" plants are k-strategists that are widespread and longlived and thus prone to both generalist and specialist attack. "Non-apparent" plants are r-strategists, uncommon in both space and time; usually these plant have few specialist enemies, but need protection from generalist herbivores (Feeny, 1976). Thus in a nutshell, plants, such as *Chrysanthemoides* spp., which are "easy to find" in evolutionary time will spend more on defense (than plants less common in time) and tend to employ defenses against which it is difficult to evolve immunity.

Crawley (1983), in a criticism of Feeny's (1976) hypothesis, suggests that apparency is immeasurable. Whether

or not a plant is hard to find depends as much on the sensory ability and powers of dispersal of the herbivore as on the spatial and temporal abundance of the plant. A specialist herbivore on a scarce plant in bound to be good at finding hosts. Furthermore, there is no such thing as a typical generalist herbivore (Crawley, 1983).

In addition to variation in defense allocation between different types of plant life strategies, variation in defensive investment can be correlated to environmental resource availability (Coley *et al.*, 1985). Coley's (1987) "productivity" hypothesis includes these environmental pressures which may modify plant response to herbivory. Thus in nutrient poor environments where leaf cost is high, species are thought to be better protected than are species from nutrient rich environments conducive to growth. That is, species from nutrient rich environments are more likely to regrow new leaves than defending them against defoliation since in such habitats regrowth is cheaper than defense (up to a point of course). An extreme example of this is the grasses which have evolved under conditions of continuous grazing pressure, they allocate so much carbon and nitrogen to growth that production in undergrazed conditions is substantially reduced (Bazzaz *et al.*, 1987).

Variation in resource allocation to defense within a species seem to follow trends opposite to those observed for

between species variation. These differences seem to mainly due to spatial and temporal variation in resource availability. That is, within a species resource allocation to growth appears to have the highest priority where-as allocation to defense occur under conditions of increased resource levels (above normal), thus only excess resources are invested in defenses (Bazzaz *et al.*, 1987). For example, increasing resources such as light and nutrients (i.e., creating conditions conducive to increased growth) leads to a decrease in defense investment (Mooney *et al.*, 1983). Thus, where-as species adapted to low resources have greater defense allocation than species adapted to rich environments, within a species individuals in resource limited environments invest less in defense than conspecifics under richer conditions (Bazzaz *et al.*, 1987).

Finally, defense allocation can vary temporally, as in a seasonal flush of resources or as a response to defoliation. An example from Gulmon and Mooney (1983) illustrates the complex patterns of resource allocation over time. In a deciduous tree leaf-growth is rapid initially (when storage resources are used) followed by a period where both photosynthetic rate and allocation to leaf-growth is high; towards midseason leaf-growth declines and by the end of the season both photosynthesis and leaf-growth are zero. Defending young leaves is more expensive (in terms of total seasonal growth) than building up defenses after the initial

concentration in the leaves. Le Roux (unpubl.) found that *R. spinosa* responded to greater defoliation intensity by increasing tannin concentration; *O. sinuatum*, taxonomically close to the *Chrysanthemoides* genus, had very low polyphenolic concentrations (less than 0.5% of dry weight) and no detectable condensed tannins.

Mediterranean ecosystems have characteristically low nutrient levels, especially in South Africa and Australia. Glyphis and Puttick (1988) found relatively high phenolic levels in all twelve fynbos species tested. For all species (except one annual, *Heamanthus* spp) total phenolics exceeded two percent dry weight which Swain (1979) proposes as the minimum amount to deter herbivores. ODT (Rhoades, 1979) and more specifically Coley's (1987) "productivity" hypothesis predicts high defense expenditure in nutrient poor ecosystems. A number of other studies also report high polyphenolic levels associated with low nutrient soils (Glyphis and Puttick, 1988).

These conclusions sound reasonable, but rest on an assumption of equal herbivory across all ecosystems. In other words, herbivory pressure in ecosystems such as the fynbos is treated as equal to herbivory in tropical ecosystems where much of plant defense theory is formulated. The thought that fynbos and tropical ecosystems have similar herbivory pressure is inconceivable given the great

differences in animal diversity in these two ecosystems. Edward (cited in Grubb, 1990) separates "neutral" and "specific" defenses. Thick cell walls may have supportative, anti-disease as well as anti-herbivory functions. In contrast, certain classes of secondary chemicals, such as alkaloids and essential oils, come closer to being specific defenses against herbivores. That is, the defensive value of some feature may be a secondary consequence of the feature's primary function. Thus, for example, cell walls may be thickened as a consequence of sclerophylly (being primarily a response to low soil nutrients; Beadle, 1966); this in turn would confer some anti-herbivore protection to the leaf as compared to a soft leaf. Could it be that the low nutrient fynbos ecosystem has never been able to support a herbivore guild large enough to justify the high investment in polyphenolics Glyphis and Puttick (1988) found? If this is so, an explanation other than anti-herbivore defense is needed to explain the high polyphenolic concentrations of fynbos plants.

A similar argument is followed by Campbell (1986), who tested the "productivity" hypothesis prediction of greater investment in anti-herbivore defenses in plants from nutrient poor soils with reference to spinescence. In fact, as Campbell (1986) found, fynbos has a very low incidence of spinescence. Spines in the fynbos are mostly confined to leaf spinescence and more likely to be a consequence of

characteristic fynbos traits such as small, evergreen sclerophyllous leaves. Campbell (1986) suggests that the low level of spinescence in the fynbos is a consequence of the low levels of herbivory in the fynbos (historically as well); which in turn is a result of the low nutritional value of fynbos leaves. In other words, the poor quality food fynbos offers herbivores is in itself a herbivore deterrent.

Thus, not all researchers agree with Feeny (1976) and Rhoades (1979) and there are other dissenting voices. Swain (1979) is of the opinion that using tannins or toxic compounds such as alkaloids for defense have about the same metabolic cost. It is therefore more likely that the distribution of defensive compounds in plants is dependent on the biochemical evolution of the taxa concerned than on their apparency.

Bernays *et al.* (1989) point out that distribution, abundance or species richness can be variously be correlated with amounts or general types of tannin. For example, Feeny (1970) noted more damage and more lepidopteran species occurred on oak at times of the year when tannin concentration was at its minimum. But, there are problems with ecological correlative evidence, because interrelated factors are difficult to quantify and separate (Bernays *et al.*, 1989).

It could be that some environmental factor such as seasonality is responsible for the correlation. That is, it could be spring: larvae are hatching, but abundant resources could allow the plant to grow new leaves rather than defending them. There are even some cases of a positive effect of tannins: for example there is some evidence that tannins prevent the absorption of alkaloids into the bloodstream (Bernays *et al.*, 1989). Despite this, it is clear that on the whole an increase of tannins in the diet of herbivores lead to negative effects such as decreasing weight gain, this has been shown for livestock (Donelly, 1983) and many other animals (Cooper & Owen-Smith, 1985). Thus, the theory of apparency has no direct evidence but rather correlations supporting and some even contradicting it (Bernays *et al.*, 1989).

Overall Bernays *et al.* (1989) conclude that tannin effects are very different under different circumstances of dose, species of vertebrate, and whether the tannin is hydrolyzable or not. Deterrence is widespread but not universal. Even high levels of tannins may have no measurable effects on food acceptability to certain animals that habitually feed on tannin-containing foods. Further, low to moderate concentrations of tannins may even have stimulatory effects in many animal species. But, in general relatively high concentrations of tannins are thought to

have negative effects on diet assimilation in most herbivores and thus such plants are usually the least preferred.

The Karoo is a vast semi-arid shrubland covering much of the Cape province. It is largely unsuitable for crops due to the lack of water and thus the Karoo is mainly used for grazing. Traditionally sheep and goats have been used to stock these ecosystems.

The south-western parts of the Karoo receives its precipitation during the winter months and consequently there is a summer drought period. During this dry summer season available fodder is at a minimum especially at the peak of summer, in January and February. This obviously has dire consequences for meat production and thus researchers from Elsenberg Agricultural Research Centre are investigating perennial shrubs with potential as dry season fodder. Two sub-species of *Chrysanthemoides monilifera* (L.) Norlindh, *C. monilifera monilifera* (west coast bietou), and *C. monilifera pisifera* (south coast bietou), as well as a second species, *C. incana* (Burm. f.) Nordlindh (vaal bietou), are among the species being investigated. Sub-species *C. monilifera monilifera* is a more arid adapted plant from the west coast with smaller leaves that *C. monilifera pisifera* which occurs along the wetter south coast. *C. incana* is associated with sandy soils, usually

coastal dunes. *Chrysanthemoides spp* are Asteraceous shrubs fairly common in the south-western Cape; both sheep and goats find it palatable.

There is much debate on the relative importance of grasses and shrubs in the Karoo and south-western Cape, some researchers (eg. Roux, 1980 and Roux & Vorster, 1983) contend that the Karoo was originally covered by more grassland than it presently is, which through disturbance by European-style pastoralism, has been replaced dwarf shrubs, i.e. Karoo invasion. In recent years this assertion has been challenged (eg. Hoffman, 1988; Hoffman & Cowling, 1990; and Hoffman, Barr & Cowling, 1990) and it has been suggested that the Karoo is a dynamic, everchanging ecosystem largely determined by erratic rainfall events. In fact, Hoffman (1988) suggests that present management systems applied in the Karoo are inappropriate and rely on untested and untestable theory. Thus, a sound management strategy is lacking for the Karoo and given the unpredictable nature of grazing and browsing in this biome, the cultivation of indigenous browse shrubs such as *Chrysanthemoides* species could be a way of decreasing this unpredictability.

In the south-western Cape sheep farming is slowly replacing wheat cultivation (Stock, pers. comm.). Planting potential browse plants, such as *Chrysanthemoides* species, in place of wheat may be a way to maximize veld carrying

capacity. In other words, over the dry summer season experienced in the south-western Cape, farmers and their livestock may benefit from browse that has been specifically planted for this purpose, instead of relying solely on existing vegetation. Allowing livestock to browse on cultivated vegetation during the dry season and on indigenous vegetation for the rest of the year, may not only maximize veld carrying capacity but also afford the indigenous vegetation greater protection since it would not be browsed in the dry season (plants under stress are often more vulnerable). Exploiting indigenous species before exotics such as *Atriplex* species is a sound conservation strategy, i.e minimizing the potential problems of invader species.

The following questions were addressed in this study. Firstly, to determine if any of the three species produced tannins and if so, which the most, which the least? Secondly, did tannin production show a temporal aspect and did the plants differ in tannin production under different defoliation treatments? Thirdly, did tannin production differ in response to changing resource availability, for example under conditions of irrigation?

Methods and Materials:

Study site and species description

Field research was conducted at Elsenberg Agricultural Research Centre (33° 56' S, 18° 52'), approximately 15 km south of Stellenbosch. The average annual rainfall is approximately 987 mm with most precipitation occurring between April and August. The dry season contrasts sharply with the relatively wet rainy season since it coincides with the hot summer. Maximum temperatures in summer can reach as high as 37.4°C while winter temperatures can be as low as 1.4°C, although frost is rare.

Several shrubs with potential as browse for goats and sheep are being investigated by researchers at Elsenberg. Their aim is to find a perennial shrub which would have nutritious leaves during the long dry summer. Included among the plants being investigated are three members of the Asteraceous family, *Chrysanthemoides incana* (Burm.f.) Nordlindh, *C. monilifera monilifera* and *C. monilifera pisifera* (L.) Nordlindh.

These three plants were selected since they are indigenous to the southern Cape where-as the other species being investigated at Elsenberg are exotic *Atriplex* species

from northern America. All three of the Asteraceous species selected are perennial shrubs with large, slightly fleshy leaves. *C. incana* is usually a prostrate spiny shrub, although new shoots are soft and spineless with small leaves (± 20 mm). *C. incana* is found on sandy soils, especially along coastal dunes. The species *C. monilifera* has a wide range and six fairly distinct geographical races are recognized and each have been given subspecies status (Coates-Palgrave, 1988). *C. monilifera monilifera* occurs in the southwestern Cape and is associated with relatively dry habitats where-as *C. monilifera pisifera* is usually found in more mesic habitats along the Cape south coast from southwestern Cape to Transkei. The most obvious morphological difference between these two subspecies is that *C. monilifera monilifera* has smaller leaves than *C. monilifera pisifera*.

At Elsenberg the browsing potential of these plants is being investigated in terms of their production under different defoliation and irrigation regimes. Production is quantified as biomass of regrowth after defoliation (about 3-4 months later). To this end *C. monilifera* (both ssp) and two of the *Atriplex* spp were planted in two plots (in a grid with plants about 1.5 m apart) towards the end of October 1988. A year later in September 1989 *C. incana* and a further two *Atriplex* spp were added to the grid in five rows (Figure 1). The two plots are identical in terms of the

positioning of the species. The plots are about 100 m apart down a gentle slope; the upper plot has a permanent irrigation system (18 mm over 3 hrs twice a week during the dry season, i.e. plants in this plot were never water stressed) while the lower plot is not irrigated. Within each plot plants are cut down to 30cm and 60cm, in alternate rows (Figure 1). The shrubs are clipped evenly all over using a curved metal rod as a guide which can be set to 30cm or 60cm on a pole placed in the centre of the shrub (Figure 2). For the production experiment all clipped material is collected and both wet and dry mass is determined.

In this type of experimental design control plants are not necessary and have thus not been included. In the present study, which was not conceived of at the start of the production experiment, a control would have been appropriate. But, the usefulness of knowing whether these potentially important browse species contained tannins outweighed these concerns.

Field studies for the present study were undertaken during 1991 and two collections were done. The first collection of leaves occurred on 4 July 1991, a day before the production experiment harvest. A second collection was done on 20 August 1991, about six weeks after the first collection. Since this period coincides with the Cape winter

	30	60	30	60	30	60	30	60	30	60	30	60 cm
	1	2	3	4	5	6	7	8	9	10	11	12
1	OO	OO	OO	OO	OO	CP	OO	OO	OO	OO	OO	OO
2	CM	OO	CP	OO	CM	OO	CP	OO	OO	CM	OO	OO
3	OO	OO	OO	CP	OO	CM	OO	OO	OO	OO	CM	CP
4	OO	CM	OO	OO	CP	OO	OO	OO	CP	OO	OO	OO
5	OO	OO	CM	OO	OO	CP	OO	OO	OO	CP	OO	OO
6	OO	CP	OO	CM	OO	OO	OO	OO	CM	OO	CP	OO
7	OO	OO	OO	OO	OO	OO	OO	CP	OO	CM	OO	OO
8	OO	CI	OO	OO	CI	OO	CI	OO	OO	CI	OO	OO
9	OO	OO	CI	OO	OO	CI	OO	OO	CI	OO	OO	OO
10	OO	OO	OO	CI	OO	OO	OO	CI	OO	OO	CI	OO
11	OO	OO	OO	OO	OO	OO	OO	OO	OO	OO	OO	OO

Figure 1 Plot design at Elsenberg. There were two plots like this, one irrigated (18 mm twice weekly in dry season) while the other plot was not irrigated. Alternate rows were cut to 30 cm and 60 cm. Plants sampled are marked; CP: *C. monilifera pisifera*, CM: *C. monilifera monilifera* and CI: *C. incana*. Plants marked OO were not sampled.

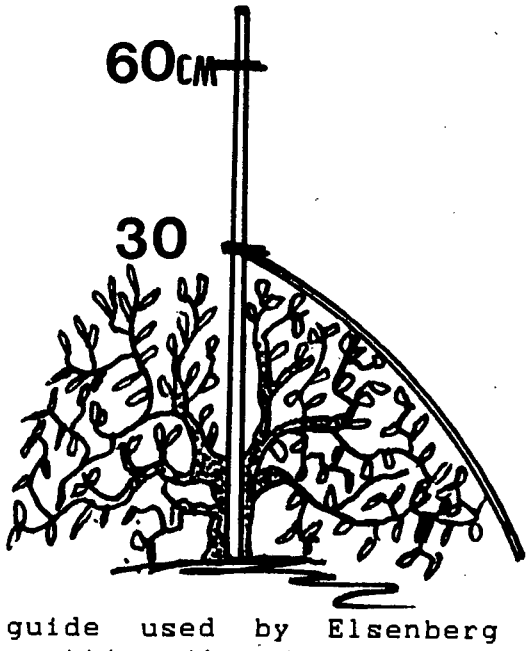

Figure 2 Clipping guide used by Elsenberg Agricultural Research Centre when cutting the shrubs to either 30 cm or 60 cm.

rains the irrigation system was switched off and both plots (irrigated and non-irrigated) received the same amount of rain. The present study might have been more informative if it had been undertaken over the dry summer season when the two plots would have had maximum difference in water input. This was not possible due to the structure of an academic year. Thus, in the present study any differences in polyphenolic concentrations between plants from either plot can only be attributable to long term consequences of different water regimes.

Approximately 20 young, mature leaves were collected from five plants in each treatment. That is, for each species in the irrigated plot five plants cut to 30cm and five plants cut to 60cm were sampled; the same procedure applied to the non-irrigated plot. Thus, in all 60 samples were collected. Six weeks later with the second collection a further 60 samples were collected using the same method. All samples were collected in the morning at an ambient air temperature of about 10 °C. The leaves collected were placed in brown paper bags and packed into an insulated freezer box with dry ice (-78.5°C). At the laboratory samples were frozen at -18°C for two weeks before being oven dried for 96 hrs at 55°C (as recommended by Hagerman, 1987). Each sample was then ground through a 40 mesh sieve in a Wiley Mill prior to chemical analysis.

In a second part of the study plants from sites across a geographic range were collected and frozen, dried and then ground through 40 mesh sieve. Only seven localities were sampled, namely a site between Bredasdorp and Struisbaai, along the Gouritz river, Cape Infanta, Yzerfontein, Clanwilliam dam, Piketberg and Wellington. Samples were taken from plants in farm fields, usually intensely grazed, and plants associated with roadsides were avoided since most of these are nursery reared plants planted along many roads.

Chemical analyses

All the extractions and chemical analyses were performed in the Botany Department of the University of Cape Town.

Total polyphenolics

The total polyphenolic concentrations of each plant in each of the treatments were quantified by the Prussian Blue assay (Price & Butler, 1977). Hagerman and Butler (1989) recommend this method since it is less sensitive to interference from proteins than other total polyphenolic assays such as Folin's assay.

Phenolics were extracted from each sample by shaking 60 mg of ground leaf material in 3 ml of methanol. This

suspension was then vacuum filtered through a Buchner funnel fitted with Whatman No. 1 filter paper. A further 3 ml of methanol was used to rinse the extraction test tube and also vacuum filtered. The combined filtrate (6 ml) was added to 50 ml of distilled water in an Erlenmeyer flask and polyphenolic concentrations were determined within an hour of extraction.

Three ml aliquots of 0.1 M $FeCl_3$ in 0.1 M HCl followed by 3 ml of 0.008 M $K_3Fe(CN)_6$ were added to each extract (56 ml). The reaction was allowed to proceed for 10 minutes after which the optical density (OD) of two replicates from each sample was measured in 1 cm glass cells at 720 nm on a Bausch & Lomb Spectronic 21 spectrophotometer, which had been zeroed with distilled water. The OD of two replicate blanks without plant extract, but otherwise identical in composition to the sample extracts, were measured as well. All OD values were compared to a catechin (Sigma Chemical Co.) standard. Sample readings are given as catechin equivalents (mg CEQ g^{-1}dry wt).

Condensed Tannins

Condensed tannins were determined by the Vanillin assay (Price *et al.*, 1978). Hagerman and Butler (1989) recommend this assay since it is thought to be specific for flavanol units even in the presence of hydrolyzable tannins or other

polyphenolics. Condensed tannins were extracted by shaking 200 mg of plant material with 10 ml of methanol for 20 minutes. The suspension was vacuum filtered through Whatman No. 1 filter paper and 1 ml aliquots of this filtrate dispensed into each of 4 test tubes (two experimental and two blanks).

Vanillin reagent was prepared daily by mixing equal volumes of 1% vanillin (w/v) in methanol and 8% 10 M HCl (v/v) in methanol. Both the extracts in the test tubes and the reagent were kept in a water bath at 30°C. Five milliliter aliquots of reagent and 5 ml of 4% HCl were added to the experimental and blank test tubes respectively. The reaction was allowed to proceed for 20 minutes after which samples were read at 500 nm in 1 cm glass cells on a Bausch & Lomb Spectronic 21 spectrophotometer. The mean OD of the blanks were subtracted from the mean OD of the experimental test tubes for each sample. A catechin (Sigma Chemical Co.) standard curve was constructed and results are expressed as catechin equivalents (mg CEQ g^{-1}dry wt).

Protein precipitating tannins

Protein precipitating tannins (an index of biological activity) were quantified by the radial diffusion assay of Hagerman (1987). Agarose protein gel plates were prepared by heating a 1% agarose (Sigma Chemical Co.) solution in buffer

A (50 mM acetic acid and 60 uM ascorbic acid adjusted to pH 5) to boiling while stirring. The solution was then cooled to 45°C in a water bath and the protein (0.1% (w/v) BSA [Sigma Chemical Co.]) added while gently stirring the solution. Aliquots of 9.5 ml were dispensed into 8.5 cm Petri dishes which were then cooled and stored on a level surface at 4°C. Four uniform 4 mm (diameter) wells were punched in the gel plates, approximately 1.5 cm apart.

Two ml of 70% acetone were added to 400 mg of plant tissue in capped polytop vials which were then rotated for 60 minutes. Twenty ul of each sample extract was then placed into a well with a micropipette. The Petri dishes were then covered and sealed with cling wrap and incubated at 25°C for 96 hours. Standard solutions of tannic acid (Merck Chemical Co.) up to a concentration of 125 mg in 1 ml of 70% acetone were mixed and 20ul of each solution dispensed into a well, in the same way as for the plant extracts. For each precipitation ring formed, two diameters at right angles were measured and the mean squared. These diameter squared values were then plotted against the standard solutions so as to obtain a standard curve of absorbance against mg tannic acid g^{-1} dry weight.

Statistical analyses

Three way analyses of variance were used to compare total polyphenolic concentration across irrigation, time (pre- versus post-harvest) and defoliation intensity for each species. Two way analyses of variance were used to compare total polyphenolic concentration for each species within each plot, the factors being time (before/after) and defoliation intensity. Tukeys multiple range tests were done where appropriate. All data manipulation was done on an IBM compatible Miad 100X computer using Quattro Pro (version 2) spreadsheet program and Statgraphics (version 4) for statistical procedures.

Results

Total polyphenolic concentrations of the leaves were calculated from the catechin standard curve for the Prussian Blue assay (Figure 3). All three species showed very low polyphenolic concentrations. There were significant ($p < 0.05$) differences between species in polyphenolic concentrations (Table 1). Overall, of the species investigated, *C. monilifera pisifera* had slightly higher polyphenolic concentrations than *C. monilifera monilifera* (Figures 4 & 5). *C. incana* had the lowest polyphenolic concentration (Figure 6). *C. monilifera monilifera* plants

from the second collection in the non-irrigated plot which were cut to 30 cm showed the maximum polyphenolic concentrations of all plants, 5.44 mg CEQ g^{-1} dry weight (0.54% of dry weight). In all

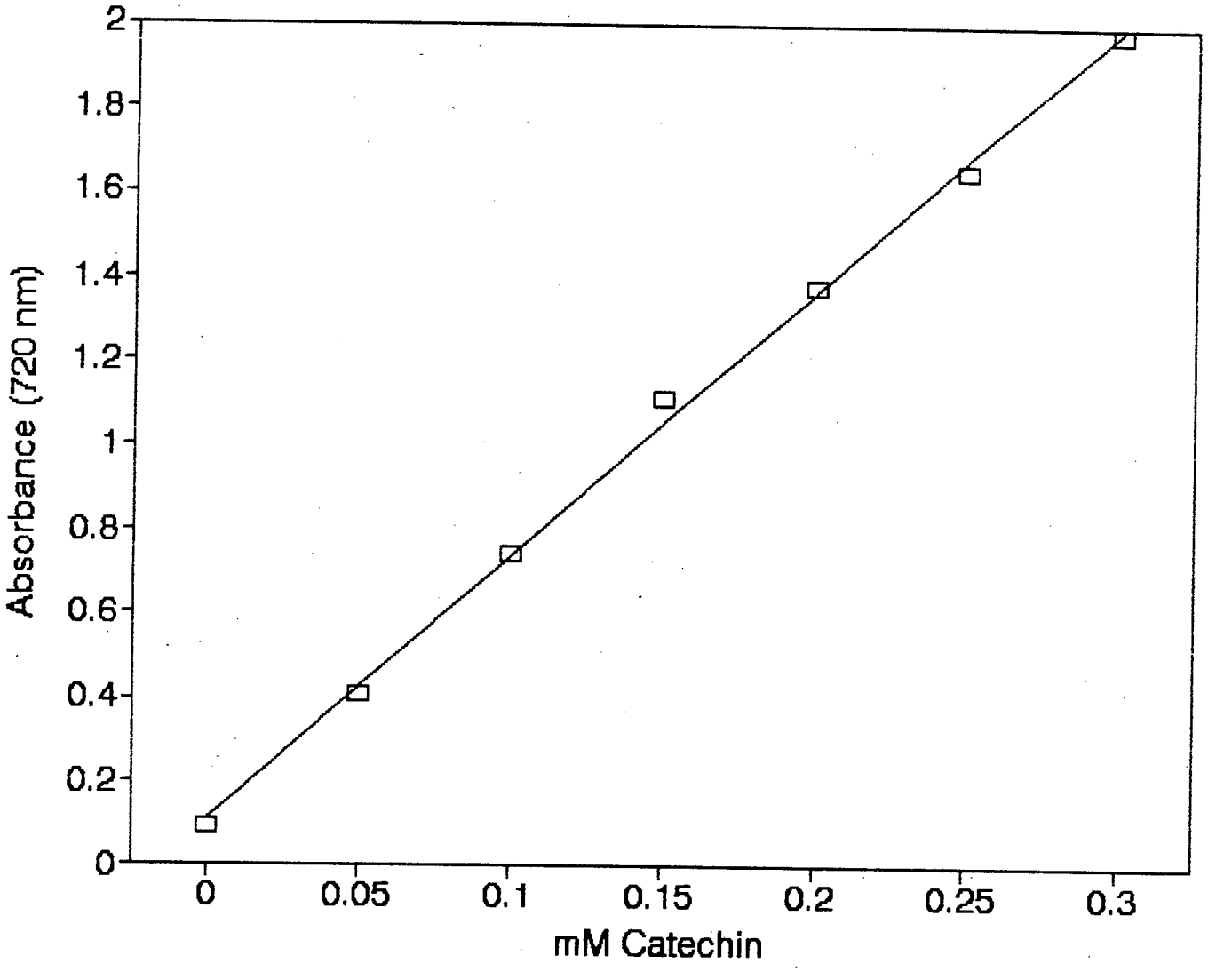

Figure 3 Catechin standard curve for the Prussian Blue assay for total polyphenolics. mg CEQ g^{-1}d.w. obtained by multiplying mM Catechin with the following formula: (1.74/60)*1000, the factor 1.74 was obtained as follows: (290.3 mg * 6 ml)/1000, Catechin's molecular weight is 290.3. r^2 = 0.99

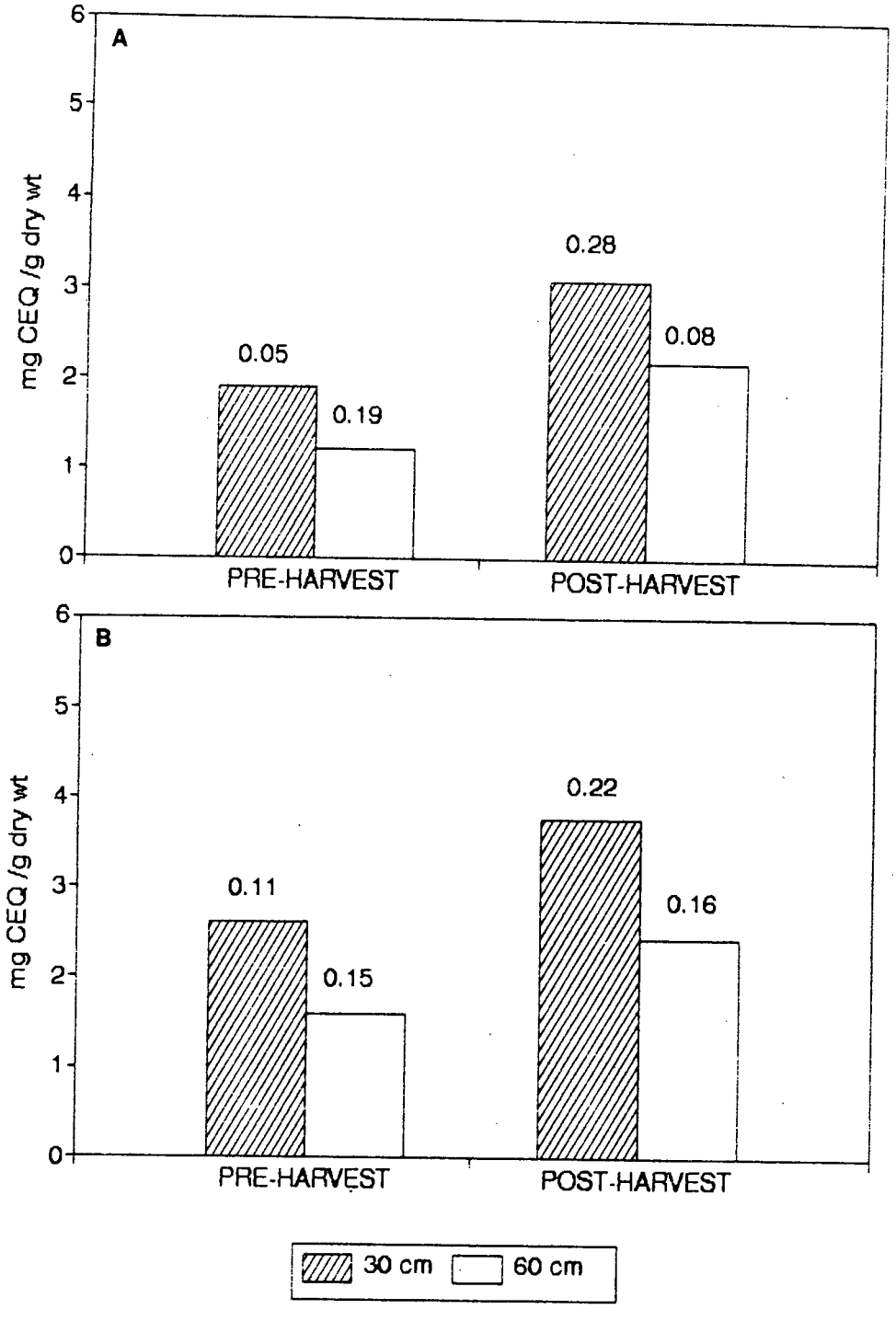

Figure 4 Total polyphenolic concentrations of *C. monilifera pisifera* defoliated to 30 cm and 60 cm in pre- and post-harvest collections, a) irrigated plot b) non-irrigated plot. S.E. values on top of bars.

I would have preferred these graphically presented as bars.

So would I

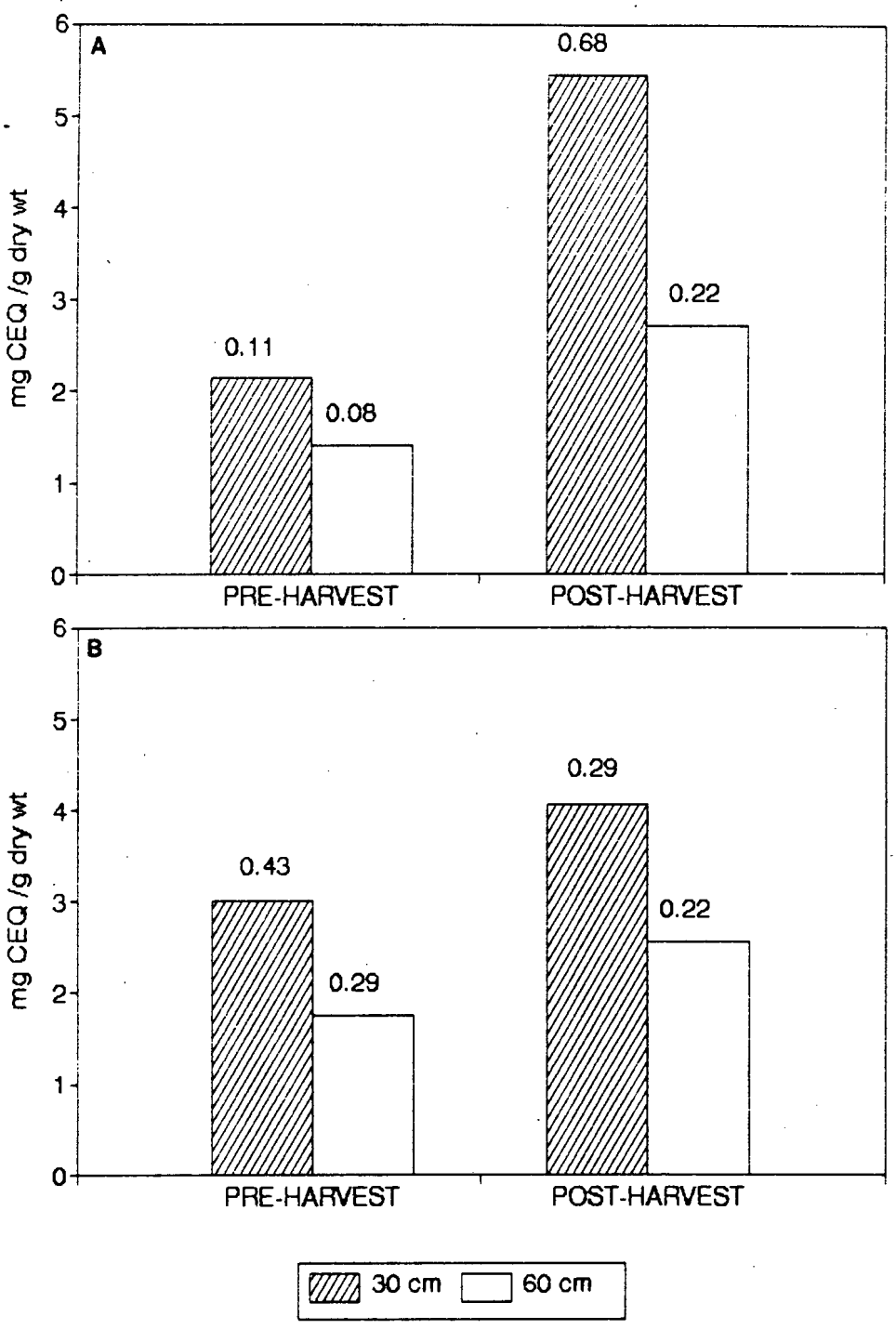

Figure 5 Total polyphenolic concentrations of *C. monilifera monilifera* defoliated to 30 cm and 60 cm in pre- and post-harvest collections, a) irrigated plot b) non-irrigated plot. S.E. values on top of bars.

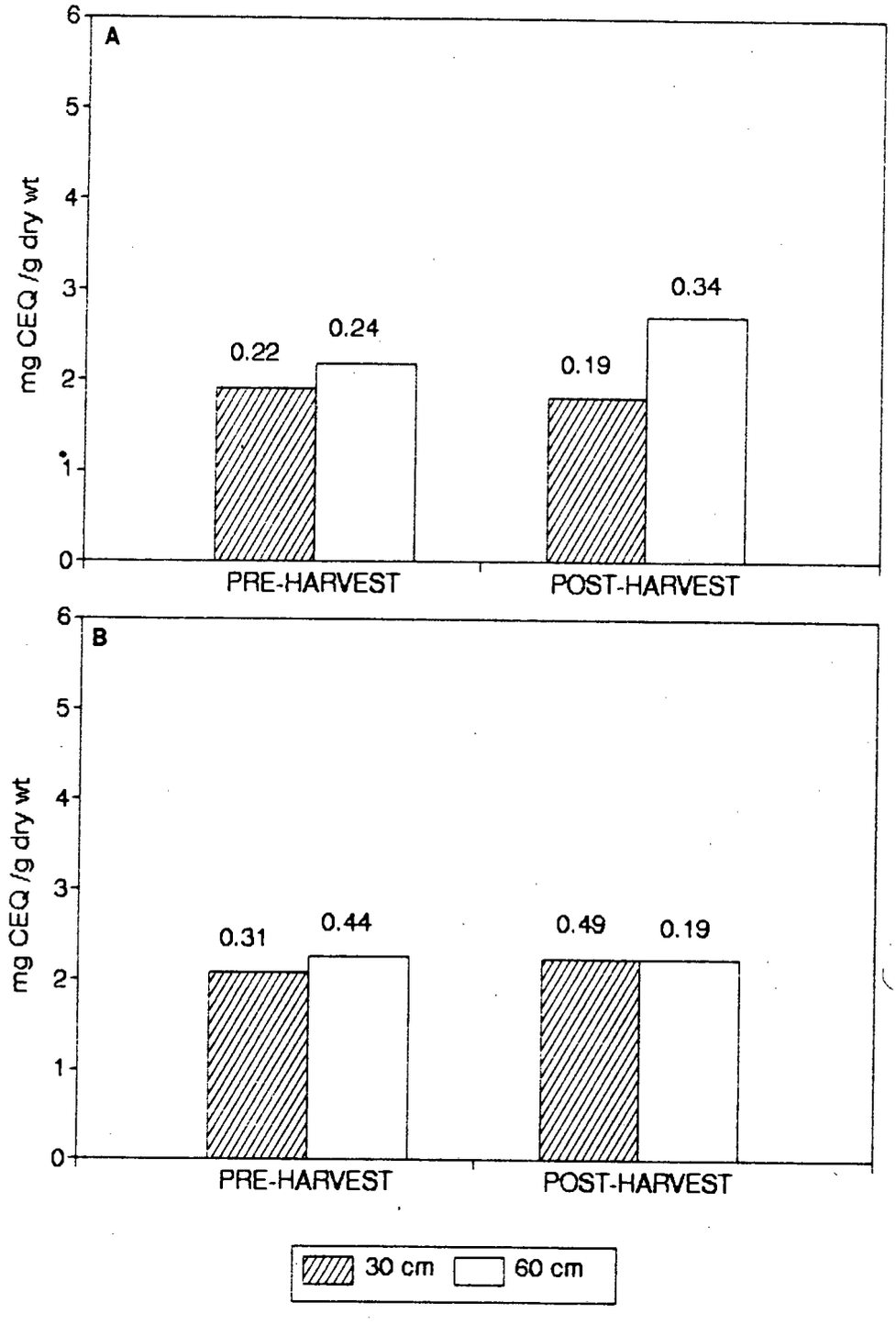

Figure 6 Total polyphenolic concentrations of *C. incana* defoliated to 30 cm and 60 cm in pre- and post-harvest collections, a) irrigated plot b) non-irrigated plot. S.E. values on top of bars.

Table 1 One-way analysis of variance comparing the three species, *C. monilifera pisifera* (CP), *C. monilifera monilifera* (CM) and *C. incana* (CI), with respect to total polyphenolics. Tukey's multiple range test shows where the difference lies, different letters signify heterogeneous groups, spp in ascending order of polyphenolic concentrations.

Spp	Tukey test	d.f.	F-ratio	Sig. level
CI	a	2	4.27	0.016
CP	b			
CM	c			

Table 2 Three-way analysis of variance comparing the following three factors: Time of collection (pre- & post-harvest); Irrigation (irrigated vs. non-irrigated plot) and Defoliation intensity for *C. monilifera pisifera*.

Factor	d.f.	F-ratio	Sig. level
Main effects	3	53.09	0.000
Time	1	74.54	0.000
Irrigation	1	17.59	0.002
Defoliation	1	67.14	0.000

Table 3 Three-way analysis of variance comparing the following three factors: Time of collection (pre- & post-harvest); Irrigation (irrigated vs. non-irrigated plot) and Defoliation intensity for *C. monilifera monilifera*.

Factor	d.f.	F-ratio	Sig. level
Main effects	3	22.68	0.000
Time	1	35.94	0.000
Irrigation	1	0.17	0.685
Defoliation	1	31.93	0.000

Table 4 Three-way analysis of variance comparing the following three factors: Time of collection (pre- & post-harvest); Irrigation (irrigated vs. non-irrigated plot) and Defoliation intensity for *C. incana*.

Factor	d.f.	F-ratio	Sig. level
Main effects	3	0.856	0.473
Time	1	0.413	0.531
Irrigation	1	0.062	0.808
Defoliation	1	2.092	0.157

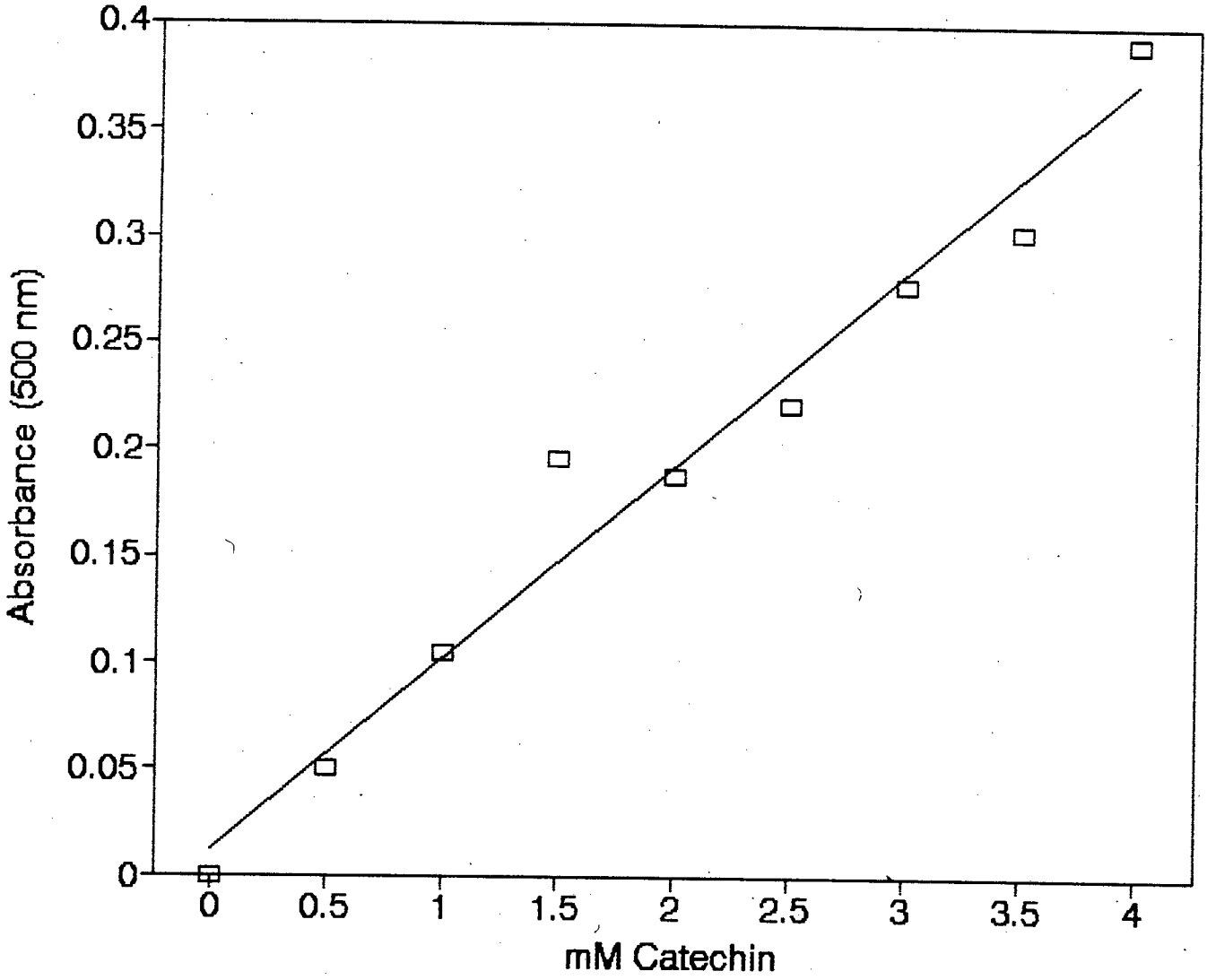

Figure 7 Catechin standard curve for the Vanillin assay for condensed tannins. $r^2 = 0.93$

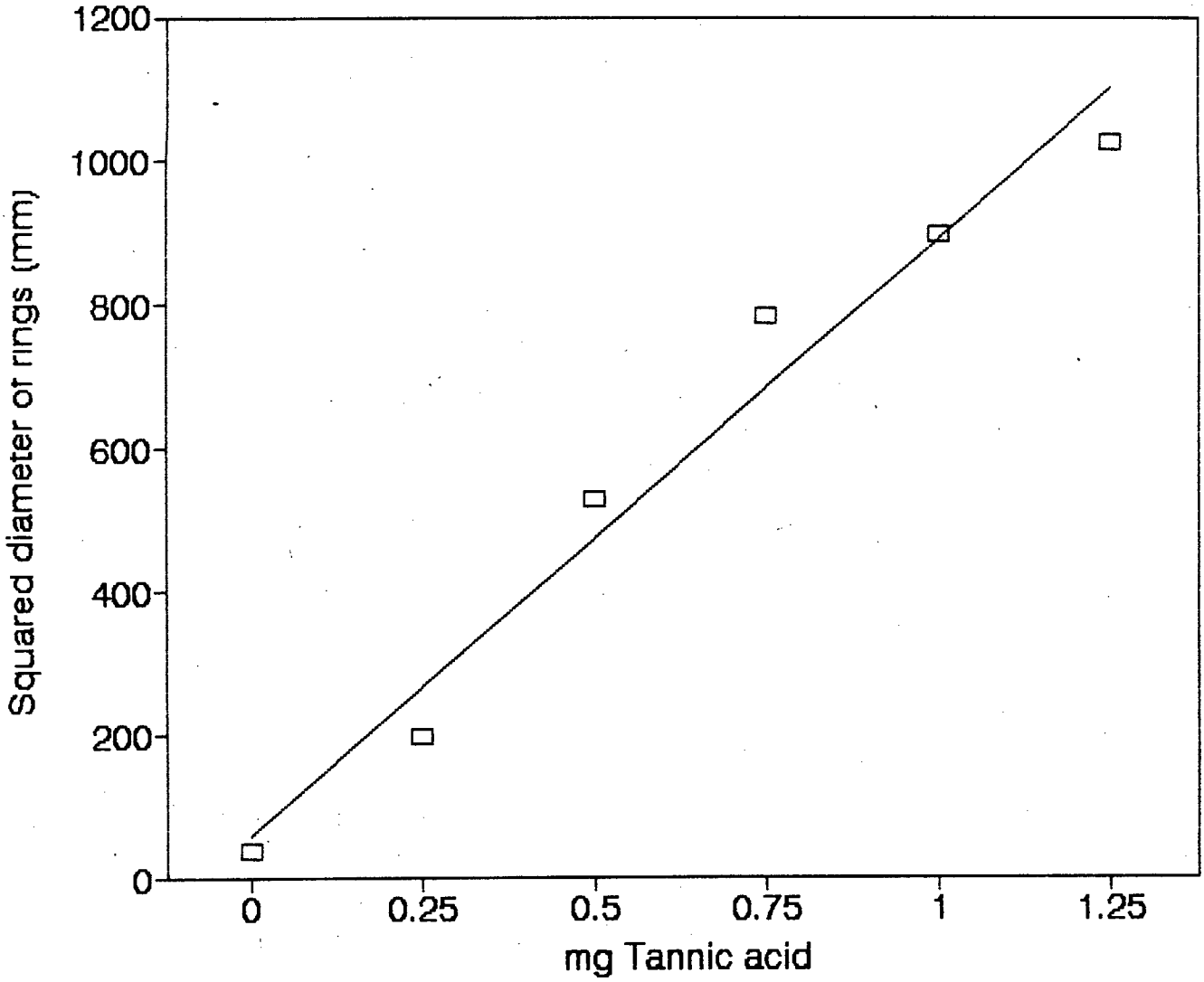

Figure 8 Tannic acid standard curve for the Radial diffusion assay testing protein precipitating ability of tannins. r^2 = 0.97

three plants polyphenolic concentrations were well below 2% dry weight, thought to be the minimum concentration needed to deter herbivores (Gulman & Mooney, 1986).

The effect of irrigation on polyphenolic concentrations was significant ($p < 0.001$) in *C. monilifera pisifera*, where plants on the irrigated plot had higher polyphenolic concentrations than plants on the non-irrigated plot (Table 2). The other two species, *C. monilifera monilifera* and *C. incana*, did not show significant ($p > 0.05$) differences between irrigation treatments (Tables 3 & 4).

In both *C. monilifera pisifera* and *C. monilifera monilifera* polyphenolic concentrations were significantly ($p < 0.001$) higher in the second collection (six weeks after harvest) than in the first collection (at harvest). *C. incana* showed no significant ($p > 0.05$) difference between collections.

C. monilifera pisifera and *C. monilifera monilifera* both responded to defoliation intensity by significantly ($p < 0.001$) increasing polyphenolic concentration in plants cut to 30 cm as compared to plants cut to 60 cm. *C. incana* showed no such response to defoliation (Figure 6).

A geographical survey of *Chrysanthemoides* spp from seven sites in the south-western Cape revealed very low

polyphenolic concentrations (all less than 5 mg CEQ g^{-1} d.w.). No tannins were found in any of the plants analyzed, although sample sizes were too small to test statistically (Table 5).

Table 5 Polyphenolic concentrations in *C. monilifera pisifera* (CP), *C. monilifera monilifera* (CM) and *C. incana* (CI) spp at seven sites in the south western Cape. Concentrations given in mg CEQ g^{-1} dry weight.

Locality	Species	Phenolic conc.
Bredasdorp	CI	2.70 mg CEQ g^{-1} d.w.
Gouritz river	CP	3.51
Cape Infanta	CP	3.08
Yzerfontein	CM	2.14
Clanwilliam dam	CM	3.94
Piketberg	CM	3.65
Wellington	CP	4.01

In summary, all three plants had very low polyphenolic concentrations. *C. monilifera pisifera* and *C. monilifera monilifera* responded similarly to the different treatments. Both increased polyphenolic concentrations in response to or time/season defoliation per se, i.e. six weeks after the initial harvest polyphenolic concentrations were on average one and a half times higher. Further, both subspecies responded to defoliation intensity by increasing polyphenolic concentrations in plants more intensely defoliated, i.e. plants cut to 30 cm had higher polyphenolic concentrations than plants cut to 60 cm. The only difference between the

subspecies was their response to irrigation, *C. monilifera pisifera* had significantly higher polyphenolic concentrations in the irrigated plot as compared to the non-irrigated plot. *C. monilifera monilifera* showed no such response. Although *C. incana* contained polyphenolics, concentrations did not vary in response to defoliation (time-wise and intensity) nor to irrigation.

No detectable results were evident in the Vanillin assay for condensed tannins and the radial diffusion assay for tannin protein precipitating ability. Standard curves for both assays were performed and since these proceeded as expected it was concluded that condensed and hydrolyzable tannins were absent or present at undetectable levels in the *Chrysanthemoides* spp assayed (Figure 7 & 8).

Is the 30cm clipping more "intense" than 60cm? When were they last clipped? What was the growth since then? Was the same amount of material being removed.

Discussion

All three species investigated are known for their palatability to browsers. In the present study all three species had polyphenolic concentrations well below 2% of their dry weight (*C. monilifera monilifera* at 5 mg CEQ g^{-1} d.w. or 0.5% d.w. had the highest concentration). The same was true for samples from across some of the genus' geographic range, no plant sampled had polyphenolic concentrations higher than 5 mg CEQ g^{-1}dry weight (0.5% d.w.). A polyphenolic concentration of at least 2% of dry

weight is thought to be necessary to act as a herbivore deterrent (Swain, 1977; Gulman and Mooney, 1986). In fact, in some plants polyphenolic concentrations can be very high, for example *Anogeissuis latifolia* whose young, red leaves consist of up to 50% tannin on a dry weight basis (Gibbs, 1974).

The low polyphenolic concentrations found in the three species by the present investigation are consistent with Le Roux (unpubl.) who found very low (less than 0.5% dry weight) polyphenolic concentrations in *Osteospermum sinuatum*, which is closely related to the *Chrysanthemoides* genus. The data for *C. incana* is not consistent with Glyphis and Puttick (1988) who found 4.9% total polyphenolics per dry weight in this species. This concentration value is an average for a year, during winter polyphenolic concentrations dropped to 2% of dry weight, in the present study *C. incana*'s concentration of polyphenolics did not change much from an average of about 2 mg CEQ g^{-1} dry weight (0.2% of dry weight). Thus, in the present study none of the species investigated had appreciable quantities of total polyphenolics, especially not in quantities that would confer protection against herbivores.

Given the low levels of polyphenolics in these three species and sub-species it is hardly surprising that condensed and hydrolyzable tannins were undetectable. The

Vanillin assay (Price *et al.*, 1978) is selective for condensed tannins in the presence of hydrolyzable tannin or other polyphenolics (Hagerman & Butler, 1989). The radial diffusion assay (Hagerman, 1987) is sensitive to the protein precipitating activity of tannins, both condensed and hydrolyzable; negative results in this assay implies that no biologically active tannins are present.

In other words, nontannnin phenolics must be responsible for the total polyphenolic concentrations measured using the Prussian Blue assay (Price & Butler, 1977). Hagerman and Butler (1989) do point out that this assay does not discriminate between tannin and nontannin phenolics, or for that matter between phenolics and other easily oxidized material such as ascorbic acid.

A surprising result from the present study is the precision with which phenolic concentrations (non-tannin phenolics) in *C. monilifera pisifera* and *C. monilifera monilifera* responded to defoliation events. In other words, the small amount of phenolics present responded to defoliation in a way consistent with Optimal Defense Theory (ODT) (Rhoades, 1979). That is, phenolic concentrations increased in response to defoliation per se as well as to defoliation intensity. The biological function of non-tannin phenolics is unknown (Gibbs, 1974), but it is unlikely to be defensive since these molecules are not toxic and have no

known biological activity. Furthermore, the phenolic concentrations found in all three species are too low for a "quantitative" defense, such as phenolics, to have any defensive value.

Thus, the non-tannin phenolics found by the present study must have some non-defensive function, if indeed it does have a function; e.g. it may simply be a metabolic waste product. Many simple low molecular weight phenolic compounds present in plants may be polymerized by oxidation to yield brown tannin-like substances containing quinonoid groups (Swain, 1977). These can also precipitate protein and cross-link to other polymers. They are often formed in necrotic cells after invasion by a pathogen, as shown by the browning which takes place in and around the area. The importance of enzyme-catalyzed browning reactions in plant defense has long been suggested, but there is little hard evidence of their value (Swain, 1977). In any event, higher concentrations than found in species from the present study are required for this possible function.

Thus, phenolics found in all three species were non-tannin in nature and in concentrations too low to have a defensive value to the plant. These data are consistent with the palatability status of these plants and it concluded that these species are not chemically defended against herbivore attack. All three species are fast growers, with

C. monilifera pisifera being a particularly rapid grower. In fact, *C. monilifera pisifera* is such a fast grower that it is used to stabilize sand dunes and highway embankments. Furthermore, this particular subspecies is a major alien invader in countries such as Australia, Sicily and California where attempts to control its spread has all but failed. *C. incana* is the only species in the present study with some anti-herbivore protection, it has spines although these do not protect the new regrowth since they occur on the older stems.

The general absence of anti-herbivore defenses, chemically and morphologically (e.g. spines), in the three species investigated would suggest that for these plants the replacement of leave tissue (lost to herbivory) is less costly than defending those leaves lost. Either that or these species have not evolved under major browsing pressure. Although historic and pre-historic records seem to indicate browsers across most of the *Chrysanthemoides* genus' geographic range (Skead, 1980), it would have been unlikely that their population densities reached the proportions of domestic browser density in modern agriculture. Furthermore, though debatable, the low nutritional quality of fynbos has probably never supported large herbivore populations. Thus, it seems likely that these species evolved under low to moderate browsing pressure which they counter with regrowth.

The three species' growth response to defoliation and irrigation has not yet been quantified and thus it is not possible to speculate on these plants' productivity. Early indications seem to be that all three species are more productive when cut to 60 cm as compared to plants cut to 30 cm, in fact several of the 30 cm plants are infected with some fungus which seems to invade the exposed cut stems after harvest, eventually killing the plant. Of nine *C. incana* planted in the irrigated plot four are almost dead. *C. monilifera pisifera* from the wetter south coast environments seems to be the fastest grower where-as *C. incana* from dry sandy habitats the slowest grower.

ODT (Rhoades, 1979) argues that since defenses are costly, in terms of fitness, less well defended plants should fare better than more highly defended plants in the absence of herbivores. Thus, not having to allocate resources to defense allows the plant to allocate all resources to growth and reproduction. According to ODT no defense allocation is only possible in the absence of herbivores. Therefore ODT implies that undefended plants such as *Chrysanthemoides* spp must have evolved in the absence of intense herbivory. This is consistent with Campbell's (1986) suggestion that the low nutritional value of fynbos is in itself an anti-herbivore "defense". I do not know of any study on the nutritional value of *Chrysanthemoides* spp leaves, but would venture to say that

given their palatability and acceptability to browsers these leaves must have some nutritional value.

The above is not consistent with Glyphis and Puttick's (1988) conclusion that since fynbos plants grow in poor soils they need to be better defended and therefore the high polyphenolic concentrations they found in fynbos species. It may be wise, in the case of fynbos, to first investigate the possibility that high polyphenolic concentrations may be the consequence of some factor other than herbivory. Bernays *et al.* (1989) warn about ecological correlative evidence and suggest that other possible functions and metabolic costs of tannins have not been fully investigated; i.e. if other elements (eg. N) are limiting it may be no loss to have carbon tied up in tannin.

In other words, is it reasonable to describe the high polyphenolic concentrations Glyphis and Puttick (1988) found as an anti-herbivore defense, given the possibility that here may never have been intense herbivory in the fynbos? Plants in the fynbos are nitrogen limited, but not carbon limited and it may be that fynbos species with high polyphenolic concentrations may be accumulating these carbon products because the lack of nitrogen does not permit the building of useful molecules (eg. proteins). Why don't *Chrysanthemoides* spp accumulate polyphenolics? Perhaps this genus is able to use excess carbon in structural material

(eg. lignin, cellulose) or it may be that excess carbon is accumulated in another form, i.e. not as polyphenolics.

The practical implications of finding no tannins in the three species investigated is obvious given their anticipated potential of browse for domestic ungulates. The only possible problem envisaged by myself is as follows: given that these species investigated may have evolved in the absence of intense herbivory they may not be adapted to a high degree of defoliation. There is some indication of this, in the present study many plants cut to 30 cm fared badly with the tips of cut branches becoming infected with some fungus.

Thus in conclusion, the two southern African species from the genus *Chrysanthemoides* showed no tannins and very low polyphenolic concentrations. The shrub is therefore chemically unprotected (lethal toxins are also assumed to be absent since these species are palatable) and it is thus suited for use as browse. The absence of a chemical or morphological (eg. spines) defense may indicate that this plant evolved in the absence of intense herbivory and as such may be vulnerable to a high degree of defoliation. On a theoretical level the absence of anti-herbivore defenses in this genus; which characteristically grows on nutrient poor soils; is not consistent with the predictions of Optimal Defense Theory (Rhoades, 1979) and associated hypotheses

(eg. Feeny, 1976; Coley, 1987). But, the above findings are consistent with the idea that the low quality food available in nutrient poor environments is in itself a herbivore deterrent (Campbell, 1986). A question raised in the present study asks whether the high polyphenolic concentrations Glyphis and Puttick (1988) found in the fynbos species they analyzed, can be reasonably described as an anti-herbivore defense, given the possibility that here may never have been intense herbivory in the fynbos? Finally, it is speculated that fynbos species with high polyphenolic concentrations may be accumulating these carbon products because the lack of nutrients (especially N) does not permit the building of useful molecules (eg. proteins); i.e. excess carbon accumulates in the form of polyphenolics.

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