Inducing condensed tannin production in *Colophospermum mopane*: Absence of response to soil N and P fertility and physical damage

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Abstract

The effects of available soil N and P and the effect of simulated browsing (leave removal) on foliar condensed tannin (CT) concentration were tested on young *Colophospermum mopane* (J. Kirk ex Benth) J. Leonard (Mopane) plants. Although clear differences in growth occurred between different levels of soil N, no differences in foliar CT concentration were found. Changes in available soil P and physical damage did not affect the plant growth or chemical composition. The complete absence of response of CT concentration to physical damage and soil nutrients may be related to the age of the trees in this study. It is proposed that existing theories on the interaction between soil properties and carbon based defences in trees are expanded, to include the potential responses of young trees to different soil nutrient levels.

Introduction

Even in dry, herbivore rich savannas, vegetation quantity is usually not limiting herbivore densities (The green world theory, Hairston et al., 1960). Therefore, plant species may reduce herbivore pressure through chemical and physical defences (Boege, 2004; Karban and Myers, 1989; Mole, 1989 and references therein). One form of chemical defence is the production of condensed tannins (CT). Tannins are estimated to be the fourth most abundant biochemical produced by vascular plants (Hernes and Hedges, 2000) and are important in ecosystem processes (Kraus et al., 2003, 2004a; Northup et al., 1995; Sjoberg et al., 2004).

The high costs associated with production of tannins are thought to impose a selective penalty on plants, but these are thought to be traded off against improved herbivore defence. Two types of tannins exist: hydrolysable and condensed. Low levels of hydrolysable tannins in forage may increase nutrient intake by temporal binding of proteins (Bernays et al., 1989; Class et al., 2003; Mole, 1989) whereas CT can reduce protein availability (Bernays et al., 1989; Mangan, 1988; Mole, 1989; Robbins et al., 1987; Soest, 1987) and may influence forage selection (Belowsky and Schmidtz, 1994; Class et al., 2003; Cooper et al., 1988; Matson et al., 2004). This makes understanding the factors that influence tannin production by plants important to understand ecosystem functioning.

Tannins are part of a larger chemical group, the polyphenols. The variation in polyphenol concentration of plants has been related to potential leaf age (Coley, 1988) and environmental factors, such as soil properties (Osier and Lindroth, 2001), temperature stress (Rivero et al., 2001), light intensity (Dudt and Shure, 1994), and herbivory (Furstenburg and Van Hoven,

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1992; Kraus et al., 2003; 2004b; Osier and Lindroth, 2001 and references therein), but these relations are not clearly understood, and different studies show different results (Styles and Skinner, 1997). Although it may be generally believed that chemical defences can be induced, and result in lower herbivore pressure, this theory is strongly debated (Koricheva et al., 2004; Nykanen and Koricheva, 2004).

A number of hypotheses have been proposed, which explain changes in foliar phenolic compound production in response to soil nutrient levels and herbivory. Coley et al. (1985) and Coley (1988) link differences in the concentration of CT and fibres between species to intrinsic growth rates, leaf longevity of plants, and herbivore pressure. These authors hypothesise that the concentration of CT increases with increasing leaf longevity, decreases with increasing growth-rate and increases with increasing herbivore pressure. The carbon-nutrient balance hypothesis (Bryant et al., 1983) predicts a shift from N-based to C-based defences in a situation where soil nutrient levels become more limited, because of the relative low cost of carbon, resulting in an increase of phenolic compound concentration. In response to herbivory, they predict that plants respond to browsing by producing shoots with well-developed chemical defences.

The recent process-based 'protein competition model of phenolic allocation' by Jones and Hartley (1999) is an extension of the carbon-nutrient balance hypothesis. This model explains relative phenolic compound production of plants through an internal chemical balance between N demand for protein production and phenolic compound production. In this model, phenylalanine controls the production of polyphenols and proteins, which in turn is regulated by soil N and P availability. According to the model, phenolic compound production increases with decreasing soil N and P. Jones and Hartley (1999) furthermore predict an increase in the production of phenolic compounds in response to physical damage to the plant as a result of induced phenylalanine ammonia lyase concentration.

The presented study is part of a broader study on the effects of herbivores on Savannah woodland structure and chemical composition, with a focus on *Colophospermum mopane* (J. Kirk ex Benth) J. Leonard (Mopane) woodlands, which cover an area of 550,000 km^2 in Southern Africa (Mapaure, 1994). Mopane is used for many domestic purposes such as firewood, construction, and ropes (Madzibane and Potgieter, 1999; Mashabane et al., 2001). Although mopane leaves are rich in proteins, deterrent tannin levels in mopane are also high and effectively prevent continuous browsing by many herbivores for most of the year (Styles and Skinner, 1997). Only African elephants (*Loxodonta africana*) (Ben-shahar, 1996; Ben-Shahar and Macdonald, 2002; Smallie and O'Connor, 2000), Eland (*Tragelaphus oryx*) (Styles and Skinner, 1997), and some insects are known to regularly use mopane as a food source. Also, seasonal browsing by the Greater Kudu (*Tragelaphus strepsiceros*) and other, smaller ruminants (Styles and Skinner, 1997) have been reported to occur. Cattle cannot survive the dry-season on mopane leaves alone because of low intake rates (Ludeman, 1966; Timberlake, 1995), but when mixed with maize, urea and bone meal, mopane can be used as 'bush meal' for cattle to survive dry periods, making it an important food source to reduce livestock losses in the dry season (Timberlake, 1995).

Mopane trees were grown in a greenhouse experiment on soils with different levels of available N and P, to test how foliar CT concentration in mopane is affected by soil nutrients and herbivory. To test the effects of severe browsing by herbivores on foliar CT levels, leaves were removed from the plants after 4 months of growth and left to regrow foliage for 2 months. Based on work by Bryant et al. (1983), Coley (1988), Coley et al. (1985) and Jones and Hartley (1999), the following hypotheses were formulated: Increased soil N levels result in a decrease of leaf CT concentration; An increase in soil P results in a decrease of CT concentration of leaves; Physical damage by removal of leaves induces CT production which results in an increase of CT concentration in leaves.

Materials and methods

Plant material and growing conditions

Seeds of mopane were obtained through the Skukuza nursery, in Kruger National Park, and transported to the Netherlands. The one-seeded

pods of mopane have a thick husk, and in order to increase the germination success, this protective outer layer was removed from all seeds. The peeled seeds were soaked for 24 h in soil saturated with water to initiate the germination process, after which they were placed at 20 °C for 1 week to germinate. By then approximately 90% of the seeds had germinated, and the seedlings were transferred to plastic plant containers (diameter 15 cm, height 20 cm) with two seedlings per pot, to compensate for the potential loss of seedlings after transplanting. The seedlings were allocated one of four soil treatments described below. All treatments were based on the same initial mixture of 1 unit of black soil added to 7 units of coarse sand, to which powdered nitrogen fertiliser ($Ca(NO_3)_2 \cdot 4H_2O$) or phosphorous fertiliser (P_2O_5) was added. The lowest nutrient levels were based on concentrations found in East African savannas (Ludwig et al., 2004). Twelve pots were filled with untreated sand/soil mixture (Treatment N−P−; 3.25 g N m^{-3} and 2.1 g P m^{-3}). The three other series of 12 pots contained an additional 35.75 g N m^{-3} (Treatment N+P−; 39.00 g N m^{-3} and 2.1 g P m^{-3}), an additional 17.4 g P m^{-3} (Treatment N−P+; 3.25 g N m^{-3} and 19.5 g P m^{-3}), or both (Treatment N+P+; 39.00 g N m^{-3} and 19.5 g P m^{-3}). The levels of nutrients applied were adapted from those used in a field experiment by Ludwig et al. (2001). As anticipated, a number of plants died after transplanting, leaving only one plant per pot. Therefore it was decided only to include the most successful (tallest) plant for all pots in this study. After 2 months, the smaller of two plants was removed from each pot, taking care to minimise disturbance to the rest of the substrate and to the other plant. A latin-squares design randomly distributed the treatments. Plants were grown in daylight (from May to November) with the photoperiod extended to 12 h with greenhouse lights. The automated greenhouse control system monitored air temperature and humidity throughout the experiment. Temperature was kept at 25 °C during the day, and 20 °C at night, air humidity was kept over 70%, and plants were watered once a day.

Sampling

During the growth, basic physical parameters, such as stem length and number of leaves, were recorded to determine the effect of soil nutrients on plant growth. After 4 months, the plants were large enough to allow sampling of leaves for chemical analysis.

Furstenburg and Van Hoven (1992) found an increase in foliar CT concentration in *Acacia nigrescense*, within 30 to 100 minutes after browsing by giraffes commenced. This elevated concentration of foliar CT remained for 40–66 h. Therefore, in the current study sampling was done in phases. Except for a few small plants of which all leaves were collected, and a control group, of which no leaves were collected, all mature leaves on one side of the main-stem were collected after 4 months of growth ($t = 0$). The leaves on the other side of the main-stem were removed after 1 day for half of the remaining plants ($t = 1$), and for the other half after 4 days ($t = 4$). After 2 months sampling, most plants had re-sprouted, and all fully developed leaves were collected of all previously sampled plants ($t = 60$), as well as of the control group, of which no leaves had been removed before (Control). After harvest all leaves were dried at 70 °C for 48 h, and stored for analysis.

Chemical analysis

Condensed tannins concentration was determined for all sample sets. Condensed tannins was extracted from ground leaves following (Hagermann, 1988; Hagerman, 2002) and the concentration was determined with the improved acid butanol assay (Porter et al., 1986). Purified Quebracho tannin was used to standardise the measurements (Waterman and Mole, 1994). Because the reactivity of tannins to the acid butanol assay is species–specific, the obtained concentration is a relative measure of concentration, and cannot be used to calculate absolute concentrations (Waterman and Mole, 1994).

N and P concentration was determined for the samples of $t = 0$. A mixture of sulphuric acid, selenium and salicylic acid was used for destruction of the samples (Novozamsky et al., 1983) after which N and P concentration were measured with a Skalar San-Plus auto analyser. Some of the plants were small, and not all analyses could be performed for all samples, therefore differences in the number of samples available for statistical analysis occurred between treatments.

Statistical analysis

A Shapiro–Wilk's W test for normality was performed on arcsine transformed data of the CT, N, and P concentration of leaves, N/P ratio, and CT/N ratio, as well as on the number of leaves and stem length, for the individual treatments. These factors did not deviate from normality. Therefore we used a factorial ANOVA to test for differences in leaf CT, N, P, N/P ratio, and CT/N ratios between treatments at $t = 0$, with soil N (low and high) and soil P (low and high) as factors. Tukeys' unequal n HSD test was used to discriminate groups for which differences occurred.

Differences in foliar CT concentration between $t = 0, 1, 4, 60$ were determined using a one-way ANOVA. A one-sided t-test was used to test whether the foliar CT concentration of the control group is lower than the foliar CT concentration of samples from $t = 60$.

Results

Effect of soil treatments

The growth (measured through the number of leaves and stem length), and foliar N concentration were higher for plants growing on soils with an increased soil N than those growing on soils with low N (Table 1, Figure 1). This effect was not influenced by soil P concentration (Table 1, Figure 1). The foliar P concentration was not influenced by soil nutrients (Table 1). The N/P ratio in plants growing on high N soils was more than double that of the N/P ratio of plants growing on soils with lower N concentration (Table 1). Foliar concentration of CT was not affected by the amount of N or the amount of P in the soil (Table 1).

Effect of leaf removal

There was no difference in foliar CT concentration between plants damaged by removal of leaves, and the control group (Table 2), although the foliar CT concentration of leaves did increase with time (Table 2).

Discussion

Effect of soil nutrients

We did not find any differences in foliar CT concentration for mopane seedlings grown on soil with different N and P (Table 2). These results do not agree with results found in other studies on other plant species. These predicted a decrease of phenolic compounds with an increase of soil nutrients (Coley et al., 1985; Jones and Hartley, 1999). This relationship was recently confirmed by Kraus et al. (2004b), who found significant

Table 1. Average foliar condensed tannin (CT) concentration (quebracho tannin equivalents in mg g^{-1}), N (mg g^{-1}), P (mg g^{-1}), N/P ratio, and CT/N ratio in mopane leaves after growing seedlings for 4 months ($t = 0$) on four different soil nutrient levels. (N−: Low soil N, N+: High soil N, P−: low soil P, P+: High soil P)

Treatment	N− P−	N− P+	N+ P−	N+ P+
n	7	9	8	11
CT	381[a]	430[a]	400[a]	335[a]
95% Conf. Int.	260–526	314–564	282–538	243–443
N	13.66[a]	13.97[a]	24.83[b]	23.72[b]
95% Conf. Int.	10.24–17.55	10.91–17.42	20.46–29.62	20.05–27.69
P	2.17[a]	2.27[a]	1.82[a]	1.61[a]
95% Conf. Int.	1.64–2.78	1.79–2.81	1.37–2.34	1.24—2.02
N/P ratio	6.37[a]	6.29[a]	13.68[b]	15.02[b]
95% Conf. Int.	5.08–7.81	5.14–7.54	11.93–15.52	13.47–16.65
CT/N ratio	0.385[a]	0.332[a]	0.163[a]	0.141[a]
95% Conf. Int.	0.214–0.605	0.191–0.512	0.066–0.304	0.062–0.251

Groups (a or b) were discriminated with a Tukeys' unequal N HSD test, after significant differences ($P < 0.05$) were detected using a factorial ANOVA on ArcSin transformed data, with factors: Soil N (low and high) and soil P (low and high).
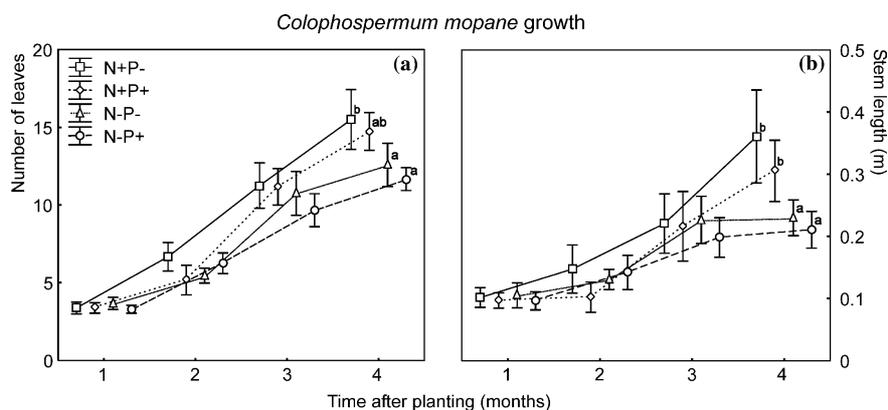
Figure 1. Growth of *Colophospermum mopane* seedlings on different soil nutrient levels during the first 4 months after planting, measured by (a) the average number of leaves and (b) the average main stem length. Initial sampling took place four months after planting ($t = 0$). Averages were calculated for all plants grouped by treatment. The graphs of individual treatments are off-set along the time axis for clarity. Groups (a or b) were discriminated with a Tukeys' unequal N HSD test, after significant differences ($P < 0.05$) were detected using a factorial ANOVA, with factors: Soil N (low and high) and Soil P (low and high).

Table 2. Average foliar condensed tannin (CT) concentration (Quebracho tannin equivalents in mg g^{-1} dry weight), 95% confidence interval, and number of samples of mopane leaves, harvested at different times as described in the methods section

Sample	Date	CT	95% Conf. Int.	n
$t = 0$	September 11th	383[a]	334–435	35
$t = 1$	September 12th	385[a]	312–467	15
$t = 4$	September 15th	429[a]	353–511	16
$t = 60$	November 10th	589[b]	528–653	35
Control	November 10th	552[b]	470–640	8

The first samples ($t = 0$, t in days) were collected after 4 months of growth. Groups (a or b) were determined using a one-way ANOVA in combination with an unequal N HSD *post hoc* test. The difference between samples collected at $t = 60$ and the control group was tested using a Tukeys' *t*-test, using a 95% significance threshold.

increases of foliar total phenol and CT concentrations in response to reduced soil fertility for one year old trees grown under controlled conditions. Kraus et al. (2004b) also found significant changes in the ratio between CT and N, in response to fertilisation. In the present study this difference did not occur (Table 1). This could indicate that neither P nor N were limiting for growth in this study. However, the strong differences in growth for plants grown on different N levels (Figure 1) indicate that N limitation occurs for the low N treatment. Although there is a trend of lower number of leaves and shorter stems for plants grown on soils with lower P concentration, these differences are not significant (Figure 1). This indicates that the amount of P available to the plant may not be limiting

growth, even when no P is added to the original soil mixture.

Koerselman and Meuleman (1996) and Verhoeven et al. (1996) suggested using the ratio between leaf N and P as an indicator of the type of nutrient limitation, where a ratio of more then 16 indicates P limitation, and a ratio of less then 14 indicates N limitation, based on wetland studies in temperate climates. Ludwig et al. (2001) found that an N/P ratio of 12 or higher indicates P limitation in an open savanna. This suggests that variation exists in N/P ratio thresholds for different ecotypes. In this study we found an increase of N/P ratio from 6.45 for plants grown on N poor soils, to 15 for plants grown on N rich soils, which was not influenced by soil P concentration (Table 1). Again, this suggests that

the P levels in this study were probably not limiting for mopane.

Effect of leaf removal

Physical damage to the plants by removal of leaves did not influence CT concentration in the remaining leaves (Table 2). The concentration of foliar CT in leaves which had re-grown after complete defoliation, collected at $t = 60$, is significantly higher then the concentration in samples collected at $t = 0, 1, 4$, with t in days after the first sampling date (Table 2). This difference cannot be attributed to the effect of removal of leaves: When comparing the foliar CT concentration in leaves that re-grew after plants were completely defoliated ($t = 60$) with those from the control group (plants which had not been defoliated) there is no difference in concentration ($P = 0.29$). The increase of CT over time is probably the result of plant aging. Young plants often have a different physiology than mature plants. The young plants invest a lot of energy in the development of biomass. As described in the protein competition model (Jones and Hartley, 1999), growth results in allocation of C for protein production, instead of polyphenol production. This would result in lower phenol concentration in young plants compared to older plants, and may dominate the effect of soil properties on the production of phenolic compounds.

Bryant et al. (1983) suggested that high tannin levels in young boreal forest trees have evolved in response to the high risk of browsing by hares. Based on this, combined with the theory that CT concentration is negatively correlated to the potential growth rate of a species (Coley, 1988; Coley et al., 1985), we propose that juvenile plants may exhibit higher levels of tannins than mature plants for slow growing species, or species which invest a lot of energy in development of root systems, before developing aboveground biomass. Fast growing species can reduce biomass loss from browsing by increasing vertical growth. These plants therefore allocate C to growth instead of to the production of C-based defences. Therefore these species will exhibit lower levels of CT in the juvenile form than older species. When we apply this hypothesis to mopane, we notice that under ideal growing conditions, mopane plants can reach a main stem length of up to 0.5 m in 4 months, and even nutrient limited plants reach an average height of 0.2 m in 4 months (Table 1). Under field conditions this tree can reach up to 4 m height within 8 years (Sharma et al., 1989). It can therefore be considered to be a fast growing species. And although an increase of foliar CT concentration in time occurs, the effect of nutrient limitation on the tannin concentration is absent, which is in line with the hypothesis presented here.

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Section editor: H. Lambers