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Variation in polyphenolic content of *Athrixia phylicoides* (L.) (bush tea) leaves with season and nitrogen application

F.N. Mudau^{a,*}, P. Soundy^b, E.S. du Toit^b, J. Olivier^c^a Department of Plant Production, University of Limpopo, Private Bag X 1106, Sovenga, 0726, South Africa^b Department of Plant Production and Soil Science, University of Pretoria, Pretoria 0002, South Africa^c Department of Geography and Environmental Studies, University of South Africa, PO Box 392, UNISA 0003, South Africa

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Abstract

The high concentrations of polyphenols present in leaves of bush tea (*Athrixia phylicoides* L.), a popular herbal beverage with medicinal properties, were examined in wild and cultivated populations to determine their magnitude of variation with season and application of nitrogenous fertilizers. Concentrations of total polyphenols in leaves of wild plants were lowest in March, April and September and highest in June and July, with nitrogenous fertilizer applications below 300 kg ha⁻¹ N further elevating polyphenol concentrations in leaves of cultivated plants grown under restricted lighting. These findings, which contradict the Carbon/Nutrient balance hypothesis, conclude that the most suitable conditions for cultivating bush tea to obtain plants with an optimal leaf polyphenol content are those of reduced light intensity during winter and in soils supplemented with a nitrogenous fertilizer.

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1. Introduction

Athrixia phylicoides (L.) (bush tea) is a small Asteraceae shrub with thin woolly stems, dark green pointed leaves and small daisy-like flowers (Roberts, 1990) that vary in colour from shades of pink to purple depending on soil type and geographic area of occurrence (Van Wyk and Gericke, 2000). *A. phylicoides* is a popular herbal tea with medicinal and aphrodisiac properties (Roberts, 1990; Mabogo, 1990). It is claimed that tea protects against cancers of the lungs, skin, liver, pancreas, and stomach (Anon, 1992) and reduces the risk of heart attack by lowering cholesterol levels and the adhesiveness of blood platelets (Stensveld et al., 1992). The predilection by humans to herbal teas has been attributed to their high concentrations of polyphenols (Owour et al., 2000; Venkatesan et al., 2004) known to possess a broad range of beneficial biochemical and physiological properties (Hirasawa et al.,

2002). One polyphenol antioxidant reported in green tea is epigallocatechin-3-gallate (EGCG), which has been found to reduce free radicals and inflammatory prostaglandins (Katiyar and Mukhtar, 1996).

Various agronomic practices and processing techniques (Fernando and Roberts, 1984) have been applied to enhance concentrations of total polyphenols in green tea (Owour et al., 1991; Owour and Odhiambo, 1994; Owour et al., 2000). Application of nitrogenous fertilizers which increase photosynthetic rate (Haukioja et al., 1998), non-structural carbohydrate accumulation (Wanyoko, 1983) and biosynthesis of carbon based secondary metabolites (Haukioja et al., 1998) have been reported to enhance total polyphenol concentrations in cultivated green tea leaves (Owour et al., 1991; Owour et al., 2000). However, it is unknown whether such agronomic practices would also improve leaf polyphenol concentrations in bush tea, the primary indicator of antioxidant potential (Hirasawa et al., 2002). Consequently, we examined wild and cultivated bush tea populations to establish the most suitable time and growing conditions to harvest plants for optimum polyphenol content.

* Corresponding author.

E-mail address: mudafn@ul.ac.za (F.N. Mudau).

2. Materials and methods

2.1. Wild populations

200 g leaf samples were collected at monthly intervals between January and December 2003 from a wild population of *A. phyllicoides* located in close proximity to Muhuyu Village (24°N 50'E, 31°S17'E) and the samples air-dried under shaded conditions.

2.2. Cultivated populations

A. phyllicoides cuttings were collected from a wild population in Venda (Muhuyu village, Limpopo Province, South Africa), dipped into a rooting hormone (Seradix® No. 2), and allowed to establish in seed trays under mist irrigation. Following rooting, cuttings were transplanted into 1 l bags containing a standard commercial potting medium of pine bark, sand and styrofoam bead mix (1:2:1 vvv) with an added wetting agent (Aquatrols, Cherry Hill, N.J) at 0.2 kg m⁻³. The transplanted cuttings were then placed into a hardening chamber maintained at 20°C and plants irrigated with an automated sprinkler, which supplied 350 mm of water per day. The physical and chemical characteristics of the growing media determined according to methods described in Hanlon et al. (1994) were as follows: Electrical conductivity 0.9 dS m⁻¹, pH 4.7 with the pine bark containing 1.2 mg kg⁻¹ NO₃-N, 0.1 mg kg⁻¹ P and 1.3 mg kg⁻¹ K. After 3 months of growth, juvenile plants containing approximately 25 leaves were transplanted into larger 20 l bags positioned beneath 50% shade nets in a commercial nursery in Louis Trichardt (23°N 50'E, 30°S17'E). A randomized complete block design (RCBD) with 6 treatments each replicated 8 times was adopted. The treatments comprised 0, 100, 200, 300, 400 and 500 kg N ha⁻¹ applied as limestone ammonium nitrate. Four consecutive nitrogen trials were conducted, one in each season, namely autumn (March to May), winter (June to August), spring (September to November) and summer

(December to February). At the end of each season, 200 g leaf samples were harvested at random from the replicated populations in each nitrogen treatment and the samples were freeze dried.

2.3. Nitrogen analyses

Leaves harvested from wild and cultivated populations were freeze-dried and finely ground to pass a 20-mesh screen. 0.2 g leaf samples were digested at 370°C for 1 h in 100 ml tubes containing 4 ml of concentrated sulphuric acid, 2 ml of 30% hydrogen peroxide and 2.5 g catalyst, which comprised a powdered mixture of 15 g copper sulphate, 250 g potassium sulphate and stearic acid (Anon, 1972). Following digestion, 100 ml of distilled water was added to each sample and the hydrated samples filtered through Whatman No. 2 filter paper. Filtered samples were bottled and stored at -20°C prior to analysis. Nitrogen concentrations were determined in thawed samples using an Auto-Analyser (Anon, 1972) connected to a Sanplus Segmented Flow Analysis System (Skalar, Netherlands), and expressed as %N dry mass⁻¹.

2.4. Polyphenol analyses

0.5 g samples of finely ground and sieved (1.0 mm Endocott mesh) leaf material from each treatment were extracted in 5 ml acetone for 2 h in a shaker. Extracted samples were centrifuged for 5 min at 4000 rpm and the supernatant decanted. The extraction procedure was repeated three times on each leaf sample and the supernatants were combined to make up a volume of 15 ml. Concentration of total polyphenols were measured spectrophotometrically (Cecil Instruments, Cambridge, UK) at 760 nm in 10 times diluted 0.5 ml supernatant samples to which were added 2.5 ml of Folin-Ciocalteu phenol reagent (Waterman and Mole, 1994). The assay was calibrated against tannic acid in the 0.02–1.0 mg ml⁻¹ range. Polyphenol concentrations were expressed as mg tannic acid equivalents g⁻¹ (TAE, mg g⁻¹) leaf dry mass.

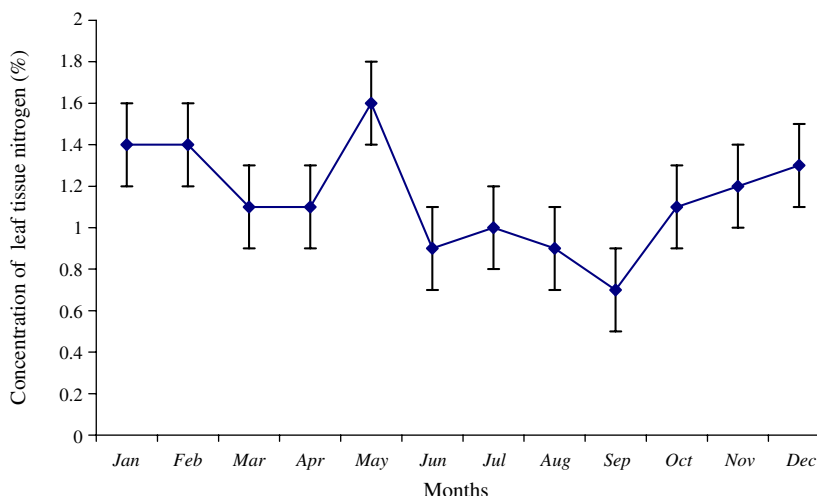


Fig. 1. Measured monthly leaf nitrogen concentrations ± standard errors in wild *A. phyllicoides*.

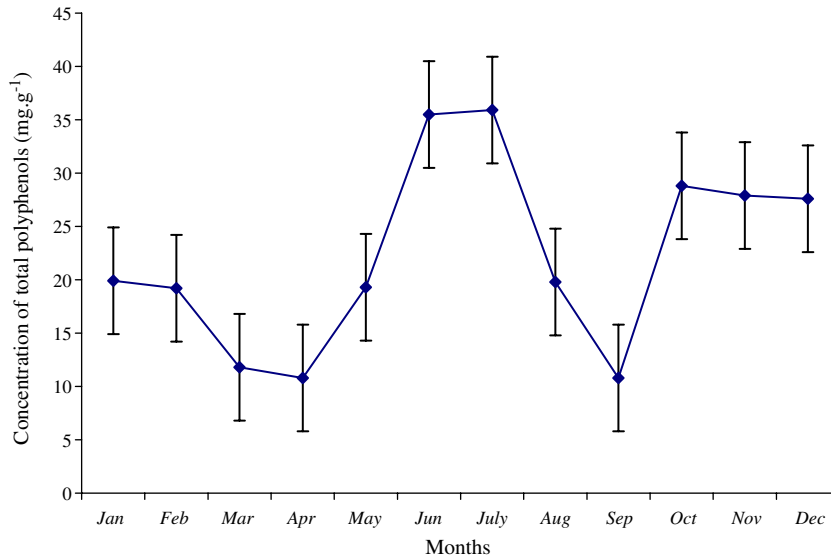


Fig. 2. Measured monthly leaf polyphenol concentrations \pm standard errors in wild *A. phylicoides*.

2.5. Statistical analyses

Differences in leaf nitrogen and polyphenol contents between seasons and nitrogenous fertilizer treatments were tested with a generalized linear model (GLM) (SAS ver. 8.0, SAS Institute Inc., 1999). Treatment sums of squares were partitioned into linear and quadratic polynomials for more efficient contrasts. Significantly different treatment means were separated using Duncan multiple range test (DMRT).

3. Results and discussion

3.1. Wild populations

Leaf nitrogen concentrations in wild *A. phylicoides* displayed seasonal differences (Fig. 1), with the lowest concentrations apparent in early spring (September) and the highest evident in early autumn (May). However, the overall seasonal difference was small ca. 1% and not statistically significant ($P \geq 0.05$). Similarly, leaf polyphenol concentrations also displayed seasonal differences (Fig. 2) with the lowest concentrations also apparent in early Spring (September) and the highest evident in midwinter (July) with the overall seasonal difference of ca 25.1 mg g⁻¹ statistically significant ($P \leq 0.05$). These findings concur with those of Owour (1992) who also reported that the highest concentrations of total polyphenols (24.1 mg g⁻¹) in black tea seedlings growing in eastern highlands of Kenya occurred during midwinter in July. These optimal polyphenol concentrations measured in plants during reduced solar radiation intensities in winter months contradict reports of correlations between the solar ultraviolet-B radiation intensity and foliar accumulation of secondary metabolites in plants (Rozema et al., 1997). They may alternatively reflect a response to increased water deficits (Hamilton et al., 2001) during the dry winter months in the predominantly summer rainfall areas.

3.2. Cultivated populations

Application of nitrogenous fertilizers resulted in significantly ($P \leq 0.001$) increased leaf nitrogen concentrations, with optimal levels measured in plants supplemented with nitrogenous fertilizers at applications of 300 kg N ha⁻¹. However, there was a significant ($P \leq 0.001$) interaction between level of nitrogenous fertilizer application and season on leaf nitrogen content. The highest leaf nitrogen contents were evident during winter and spring and the lowest concentrations apparent during summer, these seasonal differences more pronounced with increased level of nitrogenous fertilizer application. The optimal leaf nitrogen content of 3.8% measured in *A. phylicoides* grown

Table 1
Effects of nitrogenous fertilizer supplements on leaf N contents of *A. phylicoides* in different seasons

Treatment Applied N (kg ha ⁻¹)	Concentration of total leaf tissue N (%)			
	Autumn	Winter	Spring	Summer
0	1.7c	1.4d	1.6d	1.4b
100	2.1bc	2.4c	3.2c	1.9b
200	2.6ab	3.1b	3.5bc	2.5a
300	3.2a	3.8ab	3.8ab	2.6a
400	3.1a	3.7a	3.7ab	1.6b
500	2.8a	3.6ab	3.6ab	1.6b
Significance	<i>Q</i> **	<i>Q</i> **	<i>Q</i> **	<i>Q</i> **
Sources of variation	<i>df</i>	MS	<i>F</i>	Pr < <i>F</i>
Treatment (T)	5	12.2	45.3	0.0001
Season (S)	3	15.6	57.4	0.0001
T x S	24	1.2	4.3	0.0001
Error	152	0.3	—	—
Total	191	—	—	—

Linear (*L*) or quadratic (*Q*) effects significant at $P \leq 0.05$ (*), 0.01 (**) or non-significant (NS).

Values in each column with any letter in common not significantly different at $P \leq 0.05$.

Table 2
Effects of nitrogenous fertilizer supplements on leaf polyphenol contents of *A. phylicoides* in different seasons

Treatment	Concentration of total polyphenols (mg g ⁻¹)			
	Autumn	Winter	Spring	Summer
Applied N (kg ha ⁻¹)				
0	16.0b	28.0b	18.4b	16.6b
100	37.2a	46.2a	39.6a	38.9a
200	37.9a	47.8a	41.7a	42.1a
300	38.0a	51.1a	43.5a	48.9a
400	37.8a	51.0a	42.9a	47.7a
500	37.7a	50.1a	42.8a	47.3a
Significance	<i>Q</i> **	<i>Q</i> **	<i>Q</i> **	<i>Q</i> **
Sources of variation	<i>df</i>	MS	<i>F</i>	Pr< <i>F</i>
Treatment (T)	5	1994.8	13.78	0.0001
Season (S)	3	1520.8	10.5	0.0001
T x S	24	151.3	1.04	NS
Error	152	0.1	–	–
Total	191	–	–	–

Linear (*L*) or quadratic (*Q*) effects significant at $P \leq 0.05$ (*), 0.01 (**) or non-significant (NS). Values in each column with any letter in common not significantly different at $P \leq 0.05$.

under nitrogenous fertilizer supplement of 300kg N ha⁻¹ compared favorably with an optimum foliar concentration 3.4% reported in black tea leaves grown under a similar nitrogenous fertilizer supplement (Wanyoko, 1983). Conversely, the minimal leaf nitrogen content of 2.2% measured in *A. phylicoides*

grown in the absence of a nitrogenous fertilizer supplement was substantially greater than the value of 0.8% reported by Owour and Odhiambo (1994) in black tea seedlings (Table 1).

Addition of nitrogenous fertilizer supplements resulted in significantly ($P \leq 0.001$) increased concentrations of total polyphenols in *A. phylicoides* leaves in all seasons (Table 2) with the most prominent increases evident with nitrogenous applications in the range 0 to 100kg N ha⁻¹ (Table 2). Unlike foliar N content, there was no significant ($P \geq 0.05$) interaction between level of nitrogenous fertilizer application and season on leaf polyphenol content (Table 2). However, there were significant ($P \leq 0.05$) linear relationships between leaf nitrogen and polyphenol content, regardless of season (Fig. 3). These observed positive relationships between leaf polyphenol content, level of nitrogenous fertilizer application and leaf nitrogen content contradicted the carbon/nutrient balance hypothesis which advocates that under conditions of low nutrient status and high water stress growth rates are low resulting in excess photosynthetic carbon being channeled into secondary phenolic compounds (Haukioja et al., 1998; Hamilton et al., 2001). Indeed, negative correlations between concentrations of carbon based secondary compounds and low nutrient availability have been reported in tissues of quaking aspen (*Populus tremuloides* Michx.) and aspen tortrix (*Choristoneura conflictana* (Walker) by Bryant et al. (1987) and Tuomi et al. (1984) and similar findings were also reported by (Muzika 1993) and Kainulanaine et al. (1996). In contrast,

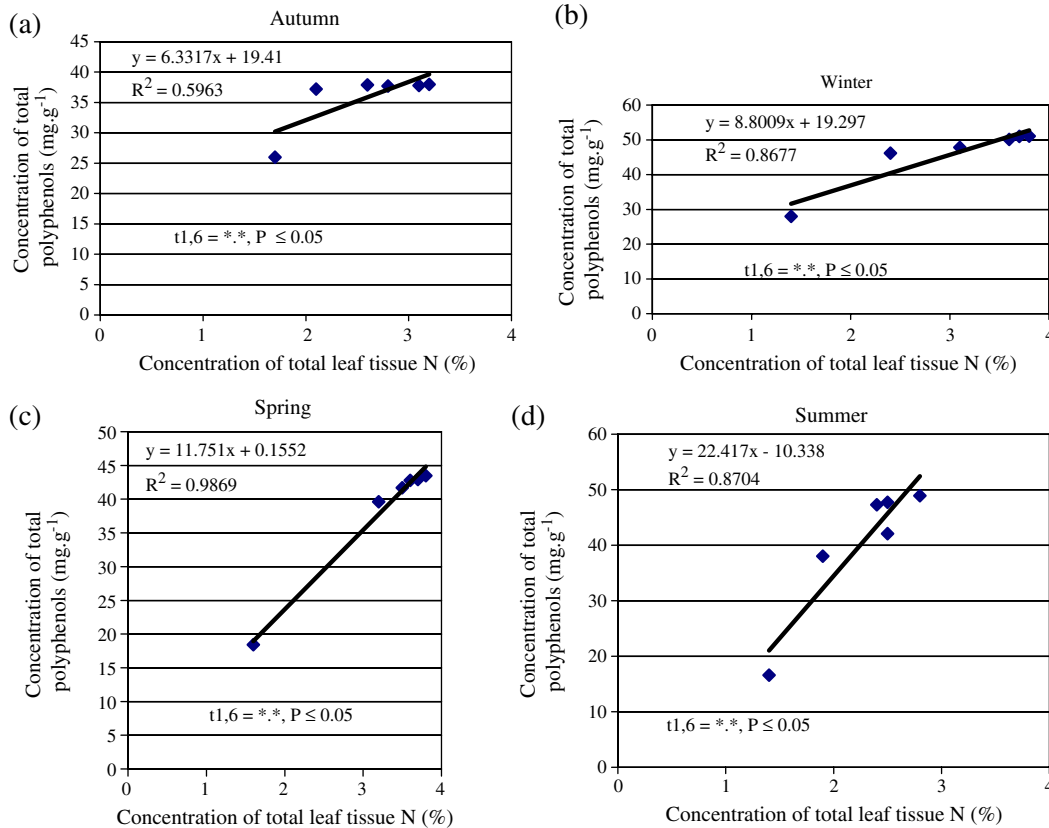


Fig. 3. Least squares regressions leaf nitrogen against leaf polyphenol content of cultivated *A. phylicoides* in different seasons.

Venkatesan et al. (2004) and Owour et al. (2000) found that nitrogenous fertilizer applications of 450 kg N ha^{-1} improved plant yields and leaf polyphenol and amino acid contents of green tea leaves. Similarly, nitrogenous fertilizer applications in the range 200 to 270 kg N ha^{-1} were reported to increase yield and concentration of polyphenols in black tea leaves (Rooster et al., 1985). These discrepancies may be partly related to the high growth rate of *A. phylicoides* (Roberts, 1990), and also partly to processing techniques (Fernando and Roberts, 1984) such as plucking (Owour et al., 2000), which have been reported to modify foliar polyphenol concentrations. Despite these discrepancies, the findings of this study conclude that the most suitable conditions for cultivating bush tea to obtain plants with optimal leaf polyphenol content are those of reduced light intensity during winter and in media supplemented with nitrogenous fertilizer applications below 300 kg N ha^{-1} further elevating polyphenol concentrations in leaves of cultivated plants grown under restricted lighting.

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