Effects of Nitrogen, Phosphorus, and Potassium Nutrition on Total Polyphenol Content of Bush Tea (*Athrixia phylicoides* L.) Leaves in Shaded Nursery Environment

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Abstract. Bush tea (*Athrixia phylicoides* L.) contains high concentrations of polyphenols that are the primary indicator of antioxidant potential in herbal teas. The objective of this study was to determine the seasonal effect of nitrogen (N), phosphorus (P), and potassium (K) nutrition on total polyphenol content in bush tea leaves. Treatments consisted of 0, 100, 200, 300, 400 or 500 kg·ha⁻¹ of N, P, or K in a randomized complete block design under 50% shade nets. Three (N, P, and K) parallel trials were conducted per season (autumn, winter, spring, and summer). Total polyphenols were determined using Folin-Ciocalteu reagents and analyzed in a spectrophotometer. The results of this study demonstrated that, regardless of season, application of nitrogenous, phosphorus, and potassium fertilizers increased quadratically the total polyphenols in bush tea, with most of the increase occurring between 0 and 300 kg·ha⁻¹ N, 300 kg·ha⁻¹ P, and 200 kg·ha⁻¹ K. Linear relationships between percentage leaf tissue N, P, and K with total polyphenols in bush tea were also observed. Therefore, for improved total polyphenol content in bush tea leaves, 300 kg·ha⁻¹ N, 300 kg·ha⁻¹ P, and 200 kg·ha⁻¹ K is recommended.

Herbal teas have high concentrations of total polyphenols (Owour et al., 2000; Venkatesan et al., 2004). Polyphenols are known to possess a wide range of beneficial biochemical and physiological properties (Hirasawa et al., 2002). The major polyphenol antioxidant reported in green tea is epigallocatechin-3-gallate (EGCG), which reduces the amount of free radicals and inflammatory prostaglandins in skin cells (Katiyar and Mukhtar, 1996). Bush tea contains 5-hydroxy-6,7,8,3',4,5'-hexamethoxyflavon-3-ol as a major flavonoid (Mashimbye et al., 2006).

Agronomic practices, such as plucking of leaves (Owour et al., 2000) and mineral nutrition (Owour, 1989; Owour et al., 1990; Owour and Odhiambo, 1994), have increased the concentration of total polyphenols in green tea. Among agronomic practices,

application of N, P, and K fertilizers was reported to have a pronounced effect on leaf total polyphenol content (Owour et al., 1991, 2000). N, P, and K application was also found to improve the accumulation of carbohydrates for plant growth (Wanyoko, 1983) and to increase photosynthetic rates (Haukioja et al., 1998). This resulted in the biosynthesis of carbon-based secondary metabolites, such as flavonoids, phenolic acids, and tannins, known as total polyphenols, which are antioxidant in nature (Haukioja et al., 1998). Nutrient-deficient plants often have lower growth rates and higher concentrations of carbon-based (non-nitrogen-containing) secondary compounds (CBSCs) than do plants with access to ample nutrients (Bryant et al., 1983; Coley et al., 1985). This negative correlation between concentrations of CBSCs and plant growth rate, or levels of nutrients in plant tissues, is assumed to indicate a trade-off between plant growth and the production of defensive compounds (Bryant et al., 1987).

Agronomic practices, such as the effects of mineral nutrition on total polyphenols in bush tea leaves, are not well established. Plant materials are harvested only from the wild for medical and herbal tea purposes. Total polyphenols in tea leaves are the main potential indicators for medicinal potential

due to their antioxidant activities (Hirasawa et al., 2002). Data that describe the response of total polyphenols in bush tea leaves to N, P, and K nutrition are lacking. Mudau et al. (2006) found that concentrations of total polyphenols in leaves of wild bush tea plants were lowest in March and April (autumn) and September (spring) and highest in June and July (winter). Therefore, the objectives of the study were to investigate the effects of N, P, and K fertilizer rates on polyphenol content in bush tea.

Materials and Methods

Experimental site and plant materials.

The study was carried out in Morgenzon, a commercial nursery in Louis Trichardt (Polokwane, South Africa) (23°N50'E, 30°S17'E; alt 610 m; a relatively cool subtropical climate with summer rainfall and cold, dry winter). On 13 Nov. 2002, plant materials were collected from Venda at Muhuyu village (South Africa, Limpopo Province) and 1500 apical cuttings were dipped in Seradix No. 2 hormone (0.3% IBA) (Bayer, Pretoria, South Africa) to encourage root formation and established in seed trays on a mist bed. The mist bed was supplemented with 24 h a day misting and fogging systems, which work automatically based on the humidity of the greenhouse. The mist bed was 5 m long, 1.5 m wide, and 1 m high and was supplied with an automated misting system operating through misting nozzles. The greenhouse temperatures were recorded by a Series 3020T Datalogger (Electronic Control Design, Mulino, Ore.). The measured mean minimum and maximum temperatures in the mist bed were 12.6 and 29.6 °C (autumn), 9 and 27.8 °C (winter), 13 and 34.2 °C (spring), and 17 and 34.7 °C (summer). The sprouted cuttings were grown with photoperiod extended to 16 h by 1000-W, high-pressure sodium lamps 16-h by 100-W, high-pressure sodium lamps (250 μmol·m⁻²·s⁻¹PPF) for 1 month.

Rooted cuttings on sand culture were transplanted into 1 L bags and placed in a hardening chamber maintained at a temperature of 20 °C. The transplants were grown with natural photoperiod extended to 16-h by 100-W, high-pressure sodium lamps (250 μmol·m⁻²·s⁻¹PPF) for 3 months. After 3 months, plants were transplanted into 20 L bags. The medium was a 1 pine bark:2 sand:1 Styrofoam bead mix (v/v), with AquaGro wetting agent (Aquatrols, Cherry Hill, N.J.) at 0.2 kg·m⁻³. The initial media chemical properties were determined using a procedure described by Hanlon et al., (1994). The EC was 0.9 dS·m⁻¹ and pH was 4.7. The composted pine bark contained 1.2 mg·kg⁻¹ NO₃-N, 0.1 mg·kg⁻¹ P, and 1.3 mg·kg⁻¹ K.

Experimental design and treatment details. Three (N, P, and K) parallel trials were conducted under 50% shade nets with one at each season (autumn, winter, spring and summer). Treatments consisted of 0, 100, 200, 300, 400, or 500 kg·ha⁻¹ N, P, or K, equivalent to 0, 2, 4, 6, 8, or 10 g per 20 L bag,

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respectively, in a randomized complete block design with six treatments replicated 8 times. Meteorological data on temperature ($^{\circ}\text{C}$), rainfall (mm), relative humidity (%), and evaporation (mm) were supplied by Agrometeorological Division at Morgenzon, a commercial nursery (Louis Trichardt, Limpopo Province, South Africa) (Table 1).

Fertilizer sources used were limestone ammonium nitrate (LAN, N = 28%) (for N trial), single superphosphate (P = 10.5%) (for P trial), and potassium chloride (K = 50%) (for K trial) applied as post plant 1 week after planting in the form of granules.

All plants received 1% MgSO_4 (Mg = 20%, S = 26%), Micrel ZnO (Zn = 78.6%), Micrel Fe 130 (Fe = 13%), Micrel soluble sodium borate (B = 20.5%) monoammonium phosphate (MAP, N = 12%, P = 27%) (except for P trial), and urea (N = 46%) (except for N trial), and potassium chloride (except for K trial) [Ocean Agriculture (Pty) Ltd, Muldersdrift, South Africa] twice per week as foliar sprays to supplement the rest of the elements necessary for the production of good-quality tea. At the end of each season (90 d after transplanting, DAT), all plants were harvested and leaves were washed with distilled water and freeze-dried for percentage N, P, and K analysis and assay of total polyphenols.

Leaf tissue N concentrations. Leaves harvested from wild and cultivated populations were freeze-dried and finely ground to pass a 20-mesh screen. Leaf samples of 0.2 g were digested at 370°C for 1 h in 100-mL tubes containing 4 mL of concentrated sulfuric acid, 2 mL of 30% hydrogen peroxide, and 2.5 g of catalyst. The catalyst composed a powdered mixture of 15 g of copper sulfate, 250 g of potassium sulfate, and stearic acid (Anon., 1972). Following digestion, 100 mL of distilled water was added to each sample, and the hydrated samples were filtered through Whatman No. 2 filter paper. Filtered samples were bottled and stored at -20°C before analysis. Nitrogen concentrations were determined in thawed samples using Auto-Analyser (Anon., 1972) on a rapid-flow analyzer (series 300; Alchem, Wilsonville, Ore.).

Leaf tissue phosphorus concentrations. Finely ground bush tea leaves of 2 g were transferred into crucibles and then ashed in a muffle furnace at 500°C for 4 h. The contents were allowed to cool, and 10 mL of deionized water and hydrochloric acid [1:1 (v/v)] were added and dried in a steam bath (Adrian, 1973). The contents of the crucibles were transferred to 100-mL volumetric flasks, which were then filled up to the mark with deionized water and filtered through Whatman No. 42 filter paper. From these solutions, 5 mL was pipetted into 100-mL glass beakers and 10 mL of concentrated HNO_3 was added. The mixture in the beakers was subjected to lower heat, and 10 mL of HNO_3 was added to dissolve the residues. The contents of the beakers were transferred into 100 mL volumetric flasks, and 10 mL of ammonium vanadate and 10 mL of ammonium molybdate solutions were added. The flasks were filled up to the mark with deion-

Table 1. Average seasonal variation in temperature, rainfall, relative humidity, and evaporation during growth of bush tea under 50% shade nets in 2003–2004.

Season	Avg temperature ($^{\circ}\text{C}$)	Avg rainfall (mm)	Avg relative humidity (%)	Avg evaporation (mm)
Autumn	28	300	51	68
Winter	24	100	44	45
Spring	34	400	86	65
Summer	38	500	77	75

ized water. The solutions were allowed to develop color for half an hour, and the absorbance at wavelength of 430 nm was read using a Bruker spectrophotometer (Spectronic Instruments Company, Leeds, United Kingdom).

Leaf tissue potassium concentrations. Finely ground bush tea leaves of 2 g were ashed in crucibles in a muffle furnace at 500°C for 4 h. To the ashed material were added 10 mL deionized water and hydrochloric acid [1:1 (v/v)], and the resulting solution was then dried in a steam bath (Adrian, 1973). The contents of the crucibles were transferred into 100-mL volumetric flasks and were filled up to the mark with deionized water and filtered through Whatman No. 42 filter paper. The nutrient element K was quantified using an AA flame spectrophotometer [Varian Technology (Pty) Ltd, Mulgrave, Australia].

Preparation of leaf extracts of total polyphenols. About 15 g of finely ground leaf material was sieved (≤ 1.0 mm; Endecotts test sieves; Endecotts Ltd, London, England) for 5 min. From the sieved material, 0.5 g was mixed in 5 mL of 75% acetone for 2 h in a shaker (Nanotech 5553/630, Johannesburg, South Africa) and then centri-

fuged for 5 min at 4000 rpm. The supernatant was carefully decanted, and the extraction procedure was repeated three times on residues. Three supernatants were combined and made-up to a volume of 15 mL of filtrate extracts. The residues were then discarded.

Fig. 1. Seasonal response of leaf total polyphenol content in bush tea to N nutrition. Total polyphenol content (autumn, $y = 0.787 + 1.8915x + 2.214x^2$, $r^2 = 0.9239$; winter, $y = 1.4686 + 1.4993x + 3.314x^2$, $r^2 = 0.9343$; spring, $y = 12.1221 + 2.3029x + 4.124x^2$, $r^2 = 0.9098$; and summer, $y = 0.9007 + 2.7053x + 3.145x^2$, $r^2 = 0.9239$) in bush tea. **Significant quadratic (Q) effect at 1% level of significance.

Fig. 2. Correlation and regression between total polyphenols and leaf tissue N in bush tea with respect to season.

Fig. 3. Leaf tissue N content (autumn, $y = 0.777 + 1.8815x + 2.014x^2$, $r^2 = 0.8239$; winter, $y = 1.3686 + 1.4993x + 2.314x^2$, $r^2 = 0.9303$; spring, $y = 11.1221 + 2.0029x + 3.124x^2$, $r^2 = 0.8098$; and summer, $y = 0.8007 + 1.7053x + 2.145x^2$, $r^2 = 0.8239$) in bush tea. **Significant quadratic (Q) effect at 1% level of significance.

Total leaf polyphenol concentrations. Total polyphenol concentrations were determined using the Folin-Ciocalteu (Waterman and Mole, 1994) method. In this method, 0.5 mL of the filtrate extracts was added to 50-mL volumetric flasks and filled up to 50 mL with deionized water. The contents were swirled to mix, and 0.5 mL of the solutions were pipetted and mixed into test tubes containing 2.5 mL of Folin-Ciocalteu phenol reagent (Fluka Ltd, Johannesburg, South Africa). Twenty (20) g of sodium carbonate was dissolved in 100 mL of distilled water, and 5 mL of sodium carbonate solution was added to the mixture in the test tubes. The mixture was shaken thoroughly, by inverting it several times, and allowed to stand for 2 h for completion of the reaction, when a blue color was formed. Measurements were done at 760 nm using a spectrophotometer (Du 530 Cecil Instruments, Cambridge, UK). The standards (preparations of 0.05 g tannic acid) were dissolved in the extracting solvent (75% acetone) up to 50 mL. The standard serial dilutions of 1, 0.8, 0.6, 0.4, 0.2, 0, 0.08, 0.06, and 0.02 mg·mL⁻¹ were prepared. The optical densities were converted into concentrations from a standard curve using 1 to 0.02 mg·mL⁻¹ tannic acid with phenol reagent and sodium carbonate in a similar manner. The standard curve obtained had an r^2 value of 0.987, passing through the origin.

Statistical analyses. Data were subjected to analysis of variance (ANOVA) using the GLM (general linear model) procedure of SAS, version 8.0. (SAS Institute, 1999). In all trials, treatment sums of squares were partitioned into linear and quadratic polynomial contrasts for total polyphenols and total leaf tissue nitrogen, phosphorus, and potassium.

Results and Discussion

Nitrogen trial. Results in Fig. 1 showed that all treatments increased ($P \leq 0.001$) total polyphenol content of bush tea leaves quadratically, regardless of season. Most of the total polyphenol response to N occurred between 0 and 300 kg·ha⁻¹ N. Similar results in biomass production studies were also reported by Mudau et al. (2005).

Fig. 4. Seasonal response of leaf total polyphenol content in bush tea to P nutrition. Total polyphenol content (autumn, $y = 0.2182 + 0.2438x + 2.334x^2$, $r^2 = 0.9397$; winter, $y = 0.2682 + 0.5489x + 4.214x^2$, $r^2 = 0.9471$; spring, $y = 0.24 + 0.3933x + 2.121x^2$, $r^2 = 0.8455$; and summer, $y = 0.2364 + 0.2241x + 4.104x^2$, $r^2 = 0.9761$) in bush tea. **Significant quadratic (Q) effect at 1% level of significance.

There were significant linear relationships between leaf tissue N and total polyphenol content, regardless of season (Fig. 2). This suggests that there was a strong trade-off in nutrients channeled toward the production of total phenolics. However, at higher N levels, Owour (1989) found that total polyphenols in black tea decreased with increasing nitrogenous fertilizer from 450 to 600 kg·ha⁻¹ N.

The carbon/nutrient balance (CNB) hypothesis emphasized that only when plants are restricted to mineral nutrient availability,

e.g., nitrogen was decreased, can carbon-based secondary compounds (CBSCs) accumulate in plant tissues (Hamilton et al., 2001; Haukioja et al., 1998). CNB emphasizes that low nutrient availability produce cheap or cost-free carbon to allocate to CBSCs (Bryant et al., 1983), which in practice could result in significant yield and productivity.

Indeed, negative correlations between the concentrations of CBSCs and low nutrient availability have been reported by Bryant et al. (1987) and Tuomi et al. (1984) in tissue of aspen tortrix (*Populus tremuloides* Michx.) and Alaska paper birch (*Choristoneura conflictana* Walker). Similar correlations were also reported by Muzika and Pregitzer (1993) and Kainulainen et al. (1996). Other factors—such as temperature, moisture stress, shading, and elevated CO₂—have been reported to induce the accumulation of total phenolics in plants, whereas no consistent changes were observed in terpenoids (Peñuelas et al., 1997; Peñuelas and Estiarte, 1998). Haukioja et al. (1998) reported that the inconsistency of the results of total phenolics were largely due to lower leaf nitrogen content, presumably due to increase in carbohydrate concentration when plants were stressed.

The results in Fig. 1, therefore, suggest that nitrogen treatments applied under 50% shade nets considerably increased the concentrations of total polyphenols in bush tea leaves, regardless of season. Roberts (1990) reported that bush tea had vigorous shoots, thus accumulation of carbohydrates reserves may have been channeled toward the

Fig. 5. Correlation and regression between total polyphenol and leaf tissue P in bush tea with respect to season.

Fig. 6. Leaf tissue P content (autumn, $y = 0.1182 + 0.2038x + 2.134x^2$, $r^2 = 0.9197$; winter, $y = 0.1682 + 0.4489x + 3.214x^2$, $r^2 = 0.8471$; spring, $y = 0.04 + 0.1933x + 2.101x^2$, $r^2 = 0.8400$; and summer, $y = 0.2364 + 0.1241x + 2.104x^2$, $r^2 = 0.9563$) in bush tea. **Significant quadratic (Q) effect at 1% level of significance.

production of total polyphenols, especially when plants were exposed to lower temperatures (24 °C) during winter and higher temperatures (38 °C) during summer (Table 1). Similar results were reported by Malec and Vigo (1988) and Sud and Baru (2000).

Venkatesan et al. (2004) and Owour et al. (2000) reported that the application of 450 kg·ha⁻¹ nitrogen improved green tea yield, polyphenols, and amino acid content compared with no nitrogen applied. Similarly, in South African tea industry, N application rates ranging from 200 to 270 kg·ha⁻¹ increased yield and concentration of total polyphenols in black tea compared with zero added N level (Rooster et al., 1985). Therefore, our results also suggest that the CNB hypothesis in bush tea is not plausible as it is generally reported. However, the hypothesis still needs further investigation on agronomic practices, such as mineral nutrition.

Leaf tissue nitrogen increased quadratically with increasing N, ranging from 21 to 31 g·kg⁻¹ (autumn), 24 to 38 g·kg⁻¹ (winter), 32 to 38 g·kg⁻¹ (spring), and 19 to 26 g·kg⁻¹ (summer) (Fig. 3). It is not clear why N concentration changed with season, but it could be related to differential seasonal changes in growth as reported by Mudau et al. (2005). Wanyoko (1983) reported that leaf N in a normal harvestable tea leaf (*Camellia sinensis* L.) was 30 to 34 g·kg⁻¹ during spring.

Phosphorus trial. Results in Fig. 4 showed that all treatments increased quadratically ($P \leq 0.001$) the total polyphenol content in bush tea leaves, regardless of season. Most of the total polyphenol response to P occurred between 0 and 300 kg·ha⁻¹. Similar results in growth and production of bush tea were also reported by Mudau et al. (2005).

Linear relationships between leaf tissue P and total polyphenol content were observed, regardless of season (Fig. 5), thus suggesting that there was a strong trade-off in nutrients channeled toward the production of total phenolics. This concurs with the findings reported by Haukioja et al. (1998).

The specific total polyphenol derivatives such as theaflavins (TFs) and thearubigins (TRs) in black tea have been established as

Fig. 7. Seasonal response of leaf total polyphenol content in bush tea to K nutrition. Total polyphenol content (autumn, $y = 6.879 + 2.5139x + 2.142x^2$, $r^2 = 0.8454$; winter, $y = 0.8876 + 2.3934x + 1.302x^2$, $r^2 = 0.8564$; spring, $y = 1.945 + 2.9104x + 3.145x^2$, $r^2 = 0.989$; and summer, $y = 0.279 + 2.3196x + 2.412x^2$, $r^2 = 0.895$) in bush tea. **Significant quadratic (Q) effect at 1% level of significance.

important nonvolatile green tea constituents. TFs contribute to the brightness and briskness, and TRs (Liang et al., 2003; Owour and Obanda, 1998) contribute to the depth of color, mouthfeel, and body of green tea (Kato and Shibamoto, 2001). Owour et al. (1991) reported that, in green tea, TF and TR (which were derived from polyphenol derivatives and caffeine) vary with time of the year and with application of 150 kg·ha⁻¹ P. Owour et al. (1998) reported that the levels of TR and flavor index (FI) were generally high when P was applied at 250 kg·ha⁻¹.

Fig. 8. Correlation and regression between total polyphenols and leaf tissue K in bush tea with respect to season.

Fig. 9. Leaf tissue K content (autumn, $y = 1.4555 + 2.5039x + 3.142x^2$, $r^2 = 0.9454$; winter, $y = 0.8876 + 2.2934x + 2.302x^2$, $r^2 = 0.9423$; spring, $y = 2.945 + 1.9104x + 2.145x^2$, $r^2 = 0.999$; and summer, $y = 0.0179 + 1.3196x + 2.312x^2$, $r^2 = 0.895$) in bush tea. **Significant quadratic (Q) effect at 1% level of significance.

Leaf tissue phosphorus was increased quadratically, ranging from 2 to 3 g·kg⁻¹ (autumn), 6 to 7 g·kg⁻¹ (winter), 2 to 5 g·kg⁻¹ (spring), and 3 to 5 g·kg⁻¹ (summer) (Fig. 6). Mudau et al. (2005) reported differential seasonal changes in growth of bush tea due to P application, and this could have resulted in differing leaf P concentrations with season. Wanyoko (1983) reported that leaf P in a normal harvestable tea leaf (*C. sinensis* L.) was 5 to 8 g·kg⁻¹ during spring.

Potassium trial. Results in Fig. 7 also showed that all the treatments ($P \leq 0.001$) increased quadratically ($P \leq 0.001$) the total polyphenol content in bush tea leaves,

regardless of season. Most of the total polyphenol response to K occurred between 0 and 200 kg-ha⁻¹.

Linear relationships were observed between leaf tissue K and polyphenol content, regardless of season (Fig. 8), thus suggesting a strong trade-off in nutrients channeled toward the production of total phenolics in bush tea leaves. This concurs with the findings reported by Haukioja et al. (1998) and Venkatesan and Ganapathy (2004).

In growth and production studies of bush tea, the application of K for maximum biomass production occurred between 0 and 200 kg-ha⁻¹ K (Mudau et al., 2005). Ruan et al. (1999) reported that total polyphenols significantly increased with K applications at a maximum level of 150 kg-ha⁻¹ during spring and autumn in black tea. In other herbal teas, such as oolong tea and green tea, total polyphenols and other aromatic compounds such as (Z)-3-hexenyl hexanoate, farnesene, and nerolidol were considerably increased with 300 kg-ha⁻¹ K applied as potassium sulfate (Ruan et al., 1998).

The percentage of leaf tissue potassium increased quadratically, ranging from 36 to 48 g·kg⁻¹ (autumn), 23 to 38 g·kg⁻¹ (winter), 22 to 23 g·kg⁻¹ (spring), and 17 to 22 g·kg⁻¹ (summer) (Fig. 9). Mudau et al. (2005) reported differential seasonal changes in growth of bush tea due to K application, and this could have resulted in differing leaf K concentrations with season. Wanyoko (1983) reported that leaf K in a normal harvestable tea leaf (*C. sinensis* L.) was 15 to 18 g·kg⁻¹ during spring.

Mudau et al. (2006) found that total polyphenol content in leaves of bush tea harvested from the wild ranged from ≈10 mg·g⁻¹ in autumn to ≈35 mg·g⁻¹ in winter, with the overall seasonal difference of ≈25 mg·g⁻¹ being statistically significant. With application of N, P, or K to cultivated bush tea, leaf total polyphenols increased from ≈38 mg·g⁻¹ in autumn to ≈49 mg·g⁻¹ in winter, a difference of only 11 mg·g⁻¹. N, P, and K nutrition also increased the level of total polyphenols in cultivated plants above the highest level in wild populations (35 mg·g⁻¹) and simultaneously decreased the seasonal differences in total polyphenols. This study, therefore, demonstrated that N, P, and K nutrition increased total polyphenols in bush tea leaves and reduced the apparent seasonal differences in total polyphenol concentrations. Harvesting of cultivated bush tea could thus be performed throughout the year due to increased total polyphenols with fertilization.

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