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-Ciocalteau 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 K kg ha⁻¹ N is recommended.

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⁻¹ PPF) for 1 months. 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⁻¹ PPF) for 3 months. After 3 months, plants were transplanted into 20 L bags. The medium was a 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 0.99 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, 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.
respectively, in a randomized complete block design with six treatments replicated 8 times. Meteorological data on temperature (°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% MgSO₄ (Mg = 20%, S = 26%), Microl 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 thehydrated 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; Alpchem, 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 into 100-mL volumetric flasks and were filled up to the mark with deionized water and filtered through Whatmann 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 centrifuged 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.

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

<table>
<thead>
<tr>
<th>Season</th>
<th>Avg temperature (°C)</th>
<th>Avg rainfall (mm)</th>
<th>Avg relative humidity (%)</th>
<th>Avg evaporation (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autumn</td>
<td>28</td>
<td>300</td>
<td>51</td>
<td>68</td>
</tr>
<tr>
<td>Winter</td>
<td>24</td>
<td>100</td>
<td>44</td>
<td>45</td>
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<tr>
<td>Spring</td>
<td>34</td>
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<td>86</td>
<td>65</td>
</tr>
<tr>
<td>Summer</td>
<td>38</td>
<td>500</td>
<td>77</td>
<td>75</td>
</tr>
</tbody>
</table>

Fig. 1. Seasonal response of leaf total polyphenol content in bush tea to N nutrition. Total polyphenol content (autumn, $y = 0.787 + 1.891x + 2.214x^2$, $r^2 = 0.9239$; winter, $y = 1.4686 + 1.4993x + 3.134x^2$, $r^2 = 0.9343$; 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 ($Q^2$) 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.

$y = 0.6332x + 19.41$  
$R^2 = 0.5963$

$y = 2.2417x - 10.338$  
$R^2 = 0.5704$

$y = 0.8801x + 19.297$  
$R^2 = 0.667$

$y = 2.2417x - 10.338$  
$R^2 = 0.8704$
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 series dilutions of 1, 0.8, 0.6, 0.4, 0.2, 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 r² 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 ≤ 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 Mudad et al. (2005).

Fig. 3. Leaf tissue N content (autumn, y = 0.777 + 1.8815x + 2.014x², r² = 0.8239; winter, y = 1.3686 + 1.4993x + 2.314x², r² = 0.9303; spring, y = 11.1221 + 2.0029x + 3.124x², r² = 0.8007 + 1.7053x + 2.314x², r² = 0.8329) in bush tea. **Significant quadratic (Q) effect at 1% level of significance.

Fig. 4. Seasonal response of leaf total polyphenol content in bush tea to P nutrition. Total polyphenol content (autumn, y = 0.2182 + 0.2438x + 2.334x², r² = 0.9397; winter, y = 0.2682 + 0.5489x + 4.214x², r² = 0.9471; spring, y = 0.24 + 0.3933x + 2.121x², r² = 0.8455; and summer, y = 0.2364 + 0.2241x + 4.104x², r² = 0.9761) in bush tea. **Significant quadratic (Q) effect at 1% level of significance.

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

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 ≤ 0.001) increased quadratically (P ≤ 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 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 to 20 mg g⁻¹ in autumn to 35 to 49 mg g⁻¹ in winter, with the overall seasonal difference of 35 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.

### Literature Cited