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| Item Type     | Article   |
| Authors       | Mabizelaa, G.S.;Muller, M.;De Beer, D.;Van der Rijst, M.;Slabbert, M.M.;Joubert, E.                               |
| DOI           | <a href="http://dx.doi.org/10.1016/j.sajb.2020.06.010">http://dx.doi.org/10.1016/j.sajb.2020.06.010</a>           |
| Publisher     | Elsevier B.V.   |
| Rights        | Attribution-NonCommercial-ShareAlike 4.0 International  |
| Download date | 2026-05-12 21:19:05   |
| Item License  | <a href="http://creativecommons.org/licenses/by-nc-sa/4.0/">http://creativecommons.org/licenses/by-nc-sa/4.0/</a> |
| Link to Item  | <a href="https://hdl.handle.net/20.500.14519/1482">https://hdl.handle.net/20.500.14519/1482</a>                   |



## Effect of genotype and harvest season on quality characteristics of *Cyclopia subternata*: Phenolic content and sensory profile

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### ARTICLE INFO

#### Article History:

Received 11 October 2019

Revised 6 April 2020

Accepted 9 June 2020

Available online 10 July 2020

Edited by AR Ndhkala

#### Keywords:

Honeybush

Benzophenones

Dihydrochalcones

Flavanones

Flavones

Xanthones

### ABSTRACT

Cultivation of selected *Cyclopia* species, including *C. subternata*, is the only viable option to ensure that the current growth trajectory of the honeybush industry is maintained. Gaps in honeybush production knowledge still exist, including the optimum harvest season for production of good quality honeybush, as defined by the phenolic content of the leaves, which is related to bioactivity of extracts, as well as the sensory profile of the herbal tea. The aim of this study was to determine the optimal harvest season for achieving the maximum accumulation of phenolic compounds in the leaves of six genotypes (SGD2, SGD3, SGD6, STB1, SHL2, SKB3) under evaluation by the honeybush breeding programme of the Agricultural Research Council. The major phenolic compounds were quantified by HPLC-DAD. Plants were harvested in summer, autumn, winter and spring. The plant material was also processed to determine whether genotype and harvest season affect the sensory profile of honeybush tea as determined by descriptive sensory analysis. Both genotype and harvest season affected the phenolic content of the leaves and the sensory profile of the herbal tea. Overall, the summer harvest delivered the better product. SGD3 emerged as the genotype with the highest total phenolic content of the leaves ( $p < 0.05$ ), predominantly due to higher accumulation of the benzophenone, 3- $\beta$ -D-glucopyranosyl-4-O- $\beta$ -D-glucopyranosyliriflophenone, and xanthones, mangiferin and isomangiferin. However, herbal tea from SGD3 also had a higher intensity of the negative aroma attribute, 'hay/dried grass', although not significantly different from SGD2, SHL2 and STB1 ( $p \geq 0.05$ ). Considering the positive aroma attributes, STB1 had either the lowest or lower intensities ( $p < 0.05$ ) of 'fynbos-floral', 'apricot jam', 'fruity-sweet' and 'fynbos-sweet' than the other genotypes. By identifying summer as optimum harvest time, a foundation is laid for screening of more genotypes to expand the genetic pool and to determine the effect of cultivation practices on quality.

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### 1. Introduction

In recent years, some *Cyclopia* species (family Fabaceae) proved to be high value fynbos plants. Their global use as a herbal tea, known as honeybush, has escalated since the first cultivation trials of a number of *Cyclopia* species were undertaken in the 1990s in an effort to develop a viable and sustainable honeybush industry (Joubert et al., 2011). Cultivation of selected *Cyclopia* species, including *C. subternata*, a re-seeder, is the mainstay of a growing commercial honeybush agricultural and agro-processing industry and the only viable

option to safeguard the growth trajectory of the industry. Recent studies focussed on aspects important in terms of the cultivation of *C. subternata* such as phenology of genotypes (Motsa et al., 2017), seed germination (Koen et al., 2017), rooting of cuttings (Mabizela et al., 2017) and weed management (Rhoda et al., 2020). This *Cyclopia* species is also currently under development by the honeybush breeding programme of the Agricultural Research Council of South Africa (Robertson et al., 2018).

Endemic to the Fynbos Floristic region, *Cyclopia* species are adapted to nutrient-poor soils and Mediterranean-type climate, the latter dictating to a large extent the time of harvesting. The major product produced by the honeybush industry is the 'fermented' herbal tea, produced by subjecting the plant material to a high temperature oxidation process. This oxidation step is essential for the

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formation of the brown to dark-brown leaf and infusion colour and sought-after sensory profile of honeybush tea, described as 'floral', 'sweet-associated', 'fruity', 'plant-like' and 'woody' with a slightly sweet taste and astringent mouthfeel (Theron et al., 2014).

Much research effort has been put into the potential health benefits of extracts prepared from *Cyclopia* species and their phenolic composition as recently reviewed (Joubert et al., 2019). Phenolic constituents of plants have become important from a value-adding perspective due to their beneficial health promoting properties, which overshadow to a large extent their negative impact on food. Their contribution to flavour, bitterness, astringency and colour of food and beverage products (Ares et al., 2009; Li et al., 2013) serves as a further incentive for investigating factors affecting the concentration of these secondary metabolites in plants. Environmental factors (amount of sunlight, ambient temperature and rainfall), agrarian factors, genotype and geographical location can potentially alter the production of phenolic compounds and thus their accumulation in plants (Treutter, 2010; Ahmed et al., 2014). Seasonal variation plays a significant role in the biosynthesis of polyphenols (Treutter, 2010) so that the harvest season is expected to affect their concentration. Investigation of the effect of harvest date on the phenolic content of *Cyclopia* plant material is limited to studies on *C. genistoides*, focussing on a few selected compounds, i.e. the xanthenes, mangiferin and isomangiferin, the benzophenone, 3- $\beta$ -D-glucopyranosyliriflophenone, and the flavanone, hesperidin (Joubert et al., 2003, 2014; North et al., 2017).

Phenolic compounds of *Cyclopia* currently of particular interest are those demonstrating anti-diabetic properties. Mangiferin and scolymside were shown to be equally effective in increasing glucose uptake by muscle cells (Schulze et al., 2016), while intestinal  $\alpha$ -glucosidase inhibitory activity of mangiferin, isomangiferin, 3- $\beta$ -D-glucopyranosyliriflophenone and 3- $\beta$ -D-glucopyranosyl-4-O- $\beta$ -D-glucopyranosyl-iriflophenone (Beelders et al., 2014; Bosman et al., 2017) holds further promise in prevention of diabetes. Inhibition of carbohydrate-hydrolysing enzymes is one of the strategies to decrease postprandial hyperglycaemia after a carbohydrate-rich meal (Derosa and Maffioli, 2012).

Consumers noted 'health enhancement', 'good taste' and 'refreshing' as some of the dominant motivators for drinking honeybush tea (Vermeulen, 2015), confirming that evaluation of both the phenolic composition and sensory profile of honeybush tea is justified when assessing the effect of various factors on the quality of *Cyclopia* species. These 'quality parameters' form second tier selection criteria of the honeybush breeding programme, with horticultural traits and biomass production forming first tier selection criteria (Bester et al., 2016).

Knowledge relating to optimum honeybush harvesting practices that will ensure a good quality product is extremely limited. The only study to date on the harvesting of *C. subternata* (North et al., 2017) found that the time of harvest could affect recovery of the plants, biomass yield and quality. However, in this instance, quality was defined in terms of the ratio of fine to coarse plant material obtained after mechanical shredding, with leaves and stems predominantly representing the fine and coarse fractions, respectively. A low ratio of fine to coarse material will result in a 'weak' infusion and will thus impact negatively on the sensory characteristics of honeybush tea, given that the soluble matter of the stems, as well as the phenolic content of the soluble matter, is substantially lower than that of the leaves (De Beer et al., 2012).

The aim of the present study was therefore to determine whether harvest season affects the phenolic composition and sensory profile of *C. subternata*. Six *C. subternata* genotypes, initially selected based on phenotypic characteristics, were harvested to delineate the effect of genotypic variation on the concentration of the individual phenolic constituents in the leaves, and to determine the optimal harvest season for achieving the maximum accumulation of major phenolic compounds in the leaves. In addition, given the major use of *Cyclopia* as herbal tea, the plant material was also subjected to high

temperature oxidation to determine whether genotype and harvest season affect the sensory profile of a cup of honeybush tea.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were of analytical grade and sourced from Sigma Aldrich (St Louis, MO, USA) or Merck (Darmstadt, Germany), unless otherwise specified. HPLC gradient grade acetonitrile and methanol were purchased from Merck. Sources of authentic phenolic standards for HPLC analysis of the leaves are provided in Robertson et al. (2018). Deionised and HPLC grade water were prepared using Elix and Milli-Q Academic water purification systems (Merck, Darmstadt, Germany), respectively.

### 2.2. Field site

The study site was the research farm situated at Elsenburg, Western Cape, South Africa ( $-33^{\circ} 58'221''S$ ,  $18^{\circ}83'048''E$ ; altitude 171 m) with a Mediterranean climate, i.e. cold winters with rainfall and warm, dry summers. The season varies as follows: summer (December - February), autumn (March - May), winter (June - August) and spring (September - November). The average daily min. and max. temperatures, relative humidity and rainfall were calculated for the 3-month period preceding the harvest date, supplied by the AgroMet (ARC-ISCW) weather station at Elsenburg, Western Cape (Table 1). The total rainfall for 12 months, spanning the period 17 October 2015 to 15 October 2016 was 569.85 mm.

### 2.3. Harvesting and preparation of plant material for analysis

Established clonal plants of each genotype (SGD2, SGD3, SGD6, STB1, SHL2 and SKB3), from a 6-year old plantation, were harvested by cutting the shoots ca 30 cm from the ground as per recommended practice. Harvesting took place in summer (15 Jan), autumn (15 Apr), winter (15 Jul) and spring (15 Oct) (Table 1). Seed pods and flowers were present on the shoots harvested in October. Approximately 1 kg of shoots was harvested from each clonal plant. At each harvest date, three clonal plants were harvested per genotype. Different clonal plants were harvested at each date (in total 12 plants per clone).

For analysis, only the leaves of *C. subternata* plants were used to better elucidate differences between genotypes and the effect of harvest season without adding variation in the leaf-to-stem ratio as confounding factor. Briefly, the plant material was handled as follows: Three shoots per clone per harvest season were randomly selected from the harvested plant material. The top part (ca 20 cm) of each shoot was removed and dried intact at 30 °C for 12 h under cross-flow air circulation (3 m/s) in a drying tunnel (Continental Fan Works c.c., Parow, South Africa). The leaves, which easily separated from the stems, were pooled per genotype and further dried under vacuum at 40 °C for 16 h in a vacuum oven. Each pooled leaf sample was milled to a very fine powder (Retsch MM301 ball mill, Retsch GmbH, Haan,

**Table 1**

Average daily values of climatic parameters and total rainfall for each 3-month period preceding the harvest date.

| Date period               | Tn (°C) | Tx (°C) | RHn (%) | RHx (%) | Rain (mm) |
|---------------------------|---------|---------|---------|---------|-----------|
| 17 Oct 2015 – 15 Jan 2016 | 14.6    | 28.6    | 28.6    | 84.6    | 22.84     |
| 16 Jan – 15 Apr 2016      | 12.2    | 32.9    | 24.8    | 89.1    | 47.23     |
| 16 Apr – 15 Jul 2016      | 7.6     | 20.9    | 52.8    | 94.1    | 308.28    |
| 16 Jul – 15 Oct 2016      | 6.6     | 20.5    | 44.6    | 94.2    | 191.5     |

**Abbreviations:** Tn, average daily minimum temperature; Tx, average daily maximum temperature; RHn, average daily minimum relative humidity; RHx, average daily maximum relative humidity.

Germany), sealed in a screw-cap glass vial and stored at 4 °C until analysis for quantification of individual phenolic compounds.

The remaining 'fresh' shoots of each clone were pooled to obtain enough plant material for processing as herbal tea according to a standard protocol (Le Roux et al., 2008; Theron et al., 2014). Briefly, the shoots were mechanically cut into small pieces (ca 3 mm length), moistened with deionised water (0.25 kg/kg plant material) and 'fermented' (oxidised) at 90 °C for 16 h as per recommended optimum temperature/time regime (Erasmus et al., 2017). After 'fermentation' the plant material was dried to a moisture content <10% at 40 °C for 6 h in the drying tunnel and the 'tea bag' fraction (<1.68 mm; >0.42 mm) obtained by mechanical sieving. The sieved fraction was stored in sealed glass jars at ambient temperature until sensory analysis.

#### 2.4. Determination of the phenolic content of dried leaves and herbal tea infusions

The milled, dried leaves were extracted using a slightly modified procedure to that described by Joubert et al. (2014). Briefly, duplicate samples (ca 40 mg) were weighed into 24 mL glass vials, 3 mL of a 33% (v/v) acetonitrile–water mixture was added to each vial and heated at 100 °C for 20 min in a temperature-controlled heating block, followed by sonication for 10 min. The samples were then cooled to room temperature and 1 mL of a 2% (m/v) ascorbic acid solution was added to prevent oxidation of the phenolic compounds during analysis. Samples were filtered through 33 mm Millex-HV 0.45 µm syringe filter devices (Merck) and aliquots of the filtrates (500 µL) diluted 1:1 (v/v) with deionised water (500 µL) before storage at –20 °C.

The extracts and the infusions prepared for sensory analysis were analysed in duplicate to quantify individual phenolic compounds, using a validated HPLC–diode array detection method previously developed for *C. subternata* (De Beer et al., 2012). Separation was achieved on a Gemini-NX C<sub>18</sub> column (150 × 4.6 mm, 3 µm; Phenomenex, Santa Clara, CA, USA), employing gradient separation (solvent A: 2% acetic acid and solvent B: acetonitrile) at 30 °C and a flow rate of 1 mL/min: 0–2 min (8% A), 2–27 min (8%–38% A), 27–28 min (38%–50% A), 28–29 min (50% A), 29–30 min (50%–8% A), 30–40 min (8% A). Calibration series of the available authentic phenolic standards were prepared for quantification. When no authentic standard was available, content values were expressed as equivalents of a chemically related compound. UV–Vis spectra were recorded from 200 to 450 nm for all samples. Xanthenes and flavones were quantified at 320 nm and flavanones, benzophenones and dihydrochalcones at 288 nm. The quantified compounds are the following: xanthenes (mangiferin, isomangiferin), benzophenones [3-β-D-glucopyranosylriflophenone (IMG), 3-β-D-glucopyranosyl-4-O-β-D-glucopyranosylriflophenone (IDG)], flavanones (eriocitrin, hesperidin), flavones (scolymoside, vicianin-2) and dihydrochalcones [3',5'-di-β-D-glucopyranosylphloretin (PDG), 3-hydroxyphloretin-di-C-hexoside (HPDG)].

#### 2.5. Descriptive sensory analysis of herbal tea

An infusion of the 'tea bag fraction' was prepared at 'cup-of-tea' equivalent strength according to the protocol used by Erasmus et al. (2017). Briefly, this entailed infusing 12.5 g of the sieved, dried plant material for 5 min in 1000 g freshly-boiled distilled water. The infusion was strained directly into a pre-heated 1-litre stainless steel vacuum flask where after ca 70 mL aliquots of the infusions were poured into white porcelain mugs pre-heated to 70 °C in an oven. Each mug was covered with a plastic lid to prevent the loss of volatiles and placed without delay in a water bath at 65 °C to ensure standardisation of infusion temperature during sensory analysis.

Twelve female panellists, all with extensive experience of descriptive sensory analysis (DSA) of a wide range of food and beverage products, including honeybush tea, participated in the study. They completed an official consent form before commencing with DSA of the samples. DSA was performed at the Sensory Research Laboratory of the Department of Food Science, Stellenbosch University, as described by Robertson et al. (2018).

The panellists were trained according to the consensus method (Lawless and Heymann, 2010) to familiarise them with the samples including scoring of the sensory attributes according to intensity and to generate a list of terms describing the sensory characteristics of the respective samples. Descriptors that best described the aroma (perceived orthonasally), flavour (perceived retronasally), taste and mouthfeel of the samples (Supplementary material, Table A.1) were generated during an open discussion led by an experienced panel leader. Following training, samples were analysed by the panel and the attributes scored on unstructured line scales ranging from 0 to 100, where 0 represented 'not present' and 100 represented 'prominent'. Scores were captured electronically, using Compusense<sup>®</sup> software (Compusense, Guelph, Canada). Samples were labelled with random, 3-digit codes to ensure blind tasting. Panellists evaluated four samples per session in completely random order during three replicate sessions per day over a 2-week period. Panellists were given a 10 min break between replicate sessions to reduce panel fatigue. All evaluations were conducted in a light-and-temperature controlled laboratory (21 °C) equipped with separate booths.

#### 2.6. Statistical analysis of data

##### 2.6.1. Phenolic content of dried leaves

Analysis of variance (ANOVA), with harvest season and genotype as factors, was performed using the General Linear Model procedure of SAS<sup>®</sup> software version 9.4 (SAS Institute Inc., Cary, NC, USA). The Shapiro-Wilk test was used to test for deviation from normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5% significance level to compare treatment means (Ott, 1998).

Agglomerative Hierarchical Clustering was performed using Ward's method, grouping genotypes and harvesting seasons based on dissimilarities and presenting hierarchy of partition in a dendrogram, which shows the progressive grouping of the data. Heat maps were produced to provide a visual summary of the phenolic content of the dried leaves. It was done by shading values below the lower 95% bound on the mean in red with red text and above the upper 95% bound on the mean in green with green text, for each compound. Values between the lower and upper 95% bounds on the mean were unshaded with black text for each compound.

##### 2.6.2. Descriptive sensory analysis data

Panel performance was monitored using Panelcheck software (Version 1.4.0, Nofima Mat, As, Norway). The DSA data were pre-processed to test for panel reliability by applying a model that includes panellist, replicate and sample effects and interactions (Næs et al., 2010) using SAS software (Version 9.4; SAS Institute Inc., Cary, USA). The residuals were tested for deviation from normality using the Shapiro-Wilk test. Outliers were removed when the standardised residual for an observation deviated with more than three standard deviations from the model value. Following confirmation of panel reliability and normality, statistical analyses on the DSA data were conducted on means over triplicate infusions and judges. Principal component analysis (PCA) of the sensory data, using the correlation matrix, was performed with XLStat (Version 18.06, Addinsoft, Paris, France) to visualise and elucidate the association between the samples and their attributes (Næs et al., 2010).

### 3. Results

#### 3.1. Effect of genotype and harvest season on phenolic composition of *C. subternata* leaves

Ward's Agglomerative Hierarchical Clustering produced two main groups, with all harvest samples of SGD3, the summer, winter and spring harvest samples of SGD2, as well as the summer harvest sample of SGD6 forming one cluster and the remaining samples forming the second cluster (data not shown). The autumn sample of SGD2 therefore clustered with the SHL2, SKB3 and STB1 samples.

For further exploration of the data, a heat map (Fig. 1) was used to show the effect of harvest season and genotypes on the accumulation of individual compounds in the leaves. Data sorted per season are

available as Supplementary material (Fig. A.1). The levels of the individual phenolic compounds in the leaves varied substantially, depending on genotype × harvest time. However, at a glance, certain features of the heat map stand out, i.e., irrespective of harvest season, SGD3 consistently accumulated higher levels of IDG and vicenin-2, while the mangiferin and isomangiferin content of its leaves from all harvests, except winter, was higher than the upper bound of the 95% confidence interval. Leaves of SGD2, harvested in winter had a higher mangiferin content than that of SGD3 (3.87 vs 2.44 mg/100 g DM), whereas similar values were obtained for isomangiferin (0.72 vs 0.79 mg/100 g DM). Another feature of SGD3 leaves is that their scolyoside, eriocitrin and PDG content, irrespective harvest season, was lower than the 95% lower bound. The HPDG content of SGD3 in winter and spring was also lower than the 95% lower bound, but for

| Genotype                   | Season | MG    | ISOMG | IDG   | ERIO  | VIC2  | SCOL  | HPDG  | PDG   |
|----------------------------|--------|-------|-------|-------|-------|-------|-------|-------|-------|
| SGD2                       | SU     | 6.083 | 1.011 | 1.153 | 0.660 | 0.306 | 0.628 | 0.174 | 0.464 |
| SGD2                       | A      | 0.638 | 0.211 | 0.236 | 0.596 | 0.079 | 4.638 | 0.290 | 0.522 |
| SGD2                       | W      | 3.869 | 0.716 | 0.918 | 0.593 | 0.256 | 0.674 | 0.099 | 0.341 |
| SGD2                       | SP     | 3.740 | 0.707 | 0.794 | 0.484 | 0.231 | 0.680 | 0.110 | 0.265 |
| SGD3                       | SU     | 6.194 | 1.639 | 7.683 | 0.382 | 0.559 | 1.097 | 0.197 | 0.358 |
| SGD3                       | A      | 4.115 | 1.191 | 6.201 | 0.415 | 0.434 | 1.385 | 0.203 | 0.333 |
| SGD3                       | W      | 2.441 | 0.791 | 4.632 | 0.323 | 0.306 | 1.002 | 0.138 | 0.210 |
| SGD3                       | SP     | 6.618 | 1.785 | 6.182 | 0.237 | 0.443 | 0.740 | 0.157 | 0.320 |
| SGD6                       | SU     | 4.974 | 1.000 | 3.025 | 0.464 | 0.235 | 1.303 | 0.135 | 0.493 |
| SGD6                       | A      | 1.294 | 0.442 | 1.416 | 0.610 | 0.166 | 3.552 | 0.275 | 0.778 |
| SGD6                       | W      | 2.035 | 0.450 | 2.020 | 0.476 | 0.158 | 2.740 | 0.137 | 0.330 |
| SGD6                       | SP     | 2.490 | 0.572 | 1.755 | 0.296 | 0.155 | 0.946 | 0.084 | 0.226 |
| SHL2                       | SU     | 1.191 | 0.383 | 1.219 | 0.692 | 0.215 | 4.389 | 0.184 | 0.911 |
| SHL2                       | A      | 0.790 | 0.269 | 0.982 | 0.651 | 0.147 | 4.287 | 0.231 | 0.566 |
| SHL2                       | W      | 2.370 | 0.559 | 1.939 | 0.468 | 0.175 | 1.889 | 0.117 | 0.347 |
| SHL2                       | SP     | 0.827 | 0.307 | 1.298 | 0.594 | 0.174 | 4.897 | 0.180 | 0.502 |
| SKB3                       | SU     | 0.899 | 0.283 | 0.321 | 0.632 | 0.103 | 3.772 | 0.291 | 0.664 |
| SKB3                       | A      | 1.039 | 0.340 | 1.587 | 0.679 | 0.199 | 5.199 | 0.211 | 0.673 |
| SKB3                       | W      | 0.701 | 0.245 | 0.278 | 0.483 | 0.087 | 4.332 | 0.223 | 0.385 |
| SKB3                       | SP     | 0.583 | 0.210 | 0.252 | 0.491 | 0.081 | 4.185 | 0.197 | 0.271 |
| STB1                       | SU     | 3.295 | 0.799 | 1.626 | 0.610 | 0.234 | 2.430 | 0.303 | 0.790 |
| STB1                       | A      | 0.648 | 0.227 | 0.247 | 0.524 | 0.081 | 4.071 | 0.280 | 0.414 |
| STB1                       | W      | 0.879 | 0.313 | 1.552 | 0.540 | 0.120 | 2.698 | 0.224 | 0.700 |
| STB1                       | SP     | 1.298 | 0.472 | 1.355 | 0.527 | 0.145 | 2.746 | 0.274 | 0.513 |
| Mean                       |        | 2.459 | 0.622 | 2.028 | 0.518 | 0.212 | 2.678 | 0.196 | 0.474 |
| Standard deviation (n-1)   |        | 1.955 | 0.436 | 2.056 | 0.123 | 0.124 | 1.589 | 0.066 | 0.194 |
| Standard error of the mean |        | 0.399 | 0.089 | 0.420 | 0.025 | 0.025 | 0.324 | 0.013 | 0.040 |
| Lower bound on mean (95%)  |        | 1.633 | 0.437 | 1.160 | 0.466 | 0.160 | 2.007 | 0.168 | 0.392 |
| Upper bound on mean (95%)  |        | 3.284 | 0.806 | 2.896 | 0.570 | 0.264 | 3.349 | 0.224 | 0.556 |

**Fig. 1.** Heat map of the phenolic content of dried leaves from different genotypes and harvesting seasons, grouped per genotype. Samples with values below the lower 95% bound on the mean are shaded in red with red text and above the upper 95% bound on the mean are shaded in green with green text, for each compound. Values without shading in black text are between the lower and upper 95% bounds on the mean for each compound. *Abbreviations:* ERIO, eriocitrin; HPDG, 3-hydroxyphloretin-di-C-hexoside; IDG, 3-β-D-glucopyranosyl-4-O-β-D-glucopyranosyliriflophenone; ISOMG, isomangiferin; MG, mangiferin; PDG, 3',5'-di-β-D-glucopyranosylphloretin; SCOL, scolyoside; VIC2, vicenin-2.

the summer and autumn harvests, the HPDG content fell within the 95% confidence interval.

The second cluster of genotypes showed less defining patterns (Fig. 1). Interesting features were that for SHL2, SKB3 and STB1, most of the content values for eriocitrin, scolymoside, HPDG and PDG were higher than the upper 95% bound or within the confidence interval. SKB3 stands out as its leaves show exactly the opposite trend than SGD3 for mangiferin, isomangiferin and IDG and scolymoside. SKB3, and to a lesser extent SDG2, produced mostly less IDG than the other genotypes (mean per genotype: 0.61 and 0.78 g/100 g DM, respectively). However, whereas SKB3 had the highest IDG content in autumn (1.59 g/100 g DM), SDG2 produced the least in autumn (0.24 g/100 g DM). The genotype STB1 emerged as having the highest content of the dihydrochalcones, HPDG and PDG (0.27 and 0.60 mg/100 g DM).

Large genotypic variation in individual phenolic content occurred (Fig. 1). For instance in summer, mangiferin varied from 0.90 – 6.08 g/100 g DM, IDG from 0.32 – 7.68 g/100 g DM and scolymoside from 0.63 – 4.39 g/100 g DM, whereas eriocitrin, HPDG and PDG showed an approximate two-fold variation.

It is relevant to probe patterns for members of the same compound class. Correlation coefficients and their p-values for the compounds are provided as Supplementary material (Tables A.2 and A.3, respectively). The two xanthones, mangiferin and isomangiferin, produced more or less the same pattern ( $r = 0.943$ ;  $p = 0.000$ ), with deviations relating to the extent of variation from the mean centred value. Vicenin-2 and scolymoside, both flavones, did not react the same, but showed opposite trends ( $r = -0.642$ ;  $p = 0.001$ ). The flavanone, eriocitrin, was present in the highest levels, either in summer (SGD2, SHL2, STB1) or autumn (SGD3, SGD6, SKB3). A moderate correlation was observed between eriocitrin and PDG ( $r = 0.740$ ;  $p < 0.0001$ ). Both dihydrochalcones, PDG and HPDG, were mostly similarly affected ( $r = 0.609$ ;  $p = 0.002$ ) with summer (0.61 and 0.21 g/100 g DM, respectively) and autumn (0.55 and 0.25 g/100 g DM, respectively) delivering higher leaf concentrations than winter (0.39 and 0.16 g/100 g DM, respectively) and spring (0.35 and 0.17 g/100 g DM, respectively).

Next, stacked bars were used to provide a broader overview of the impact of harvest and genotype on the phenolic composition of *C. subternata* in terms of phenolic sub-classes (xanthone, benzophenone, flavanone, flavone and dihydrochalcone). Data are presented for genotypes (Fig. 2a) and for harvest seasons (Fig. 2b). The total phenolic content (sum of quantified compounds) of SGD3 was significantly higher ( $p < 0.05$ ) than that of the other genotypes (not significantly different;  $p \geq 0.05$ ). When the phenolic classes are considered, further differentiation between genotypes emerges. SGD3 had a much higher mean benzophenone content ( $p < 0.05$ ) than the other genotypes (6.17 vs 2.05 g/100 g DM or less). The

benzophenone content reflects that of IDG only, as IMG and MMG could not be quantified due to only trace quantities being present. The xanthone content of SGD3 was also higher than that of four genotypes (SGD6, SHL2, STB1 and SKB3). Overall, mangiferin was present in higher levels in the leaves than isomangiferin (means over genotypes: 2.46 vs 0.62 g/100 g DM). Of the latter genotypes, SKB3 in particular, had the lowest xanthone and benzophenone content (means over harvest season: 0.81 and 0.61 g/100 g DM, respectively), although not significantly different ( $p \geq 0.05$ ) from SHL2 and STB1. These three genotypes stand out for having a high flavone content relative to the other phenolic classes, including xanthones and benzophenones. Scolyoside predominantly contributed to the flavone content with the overall mean scolyoside content (genotype  $\times$  harvest season) ca 10 times more than the vicenin-2 content (2.68 and 0.21 g/100 g DM, respectively). It is clear that *C. subternata* genotypes accumulated compounds belonging to the xanthone, benzophenone and flavone classes in higher levels than the flavanone and dihydrochalcone classes.

Considering the effect of harvest season (Fig. 2b), a summer harvest ( $p < 0.05$ ) compared to winter and spring harvests would deliver leaves with the higher total phenolic content. However, the total phenolic content of autumn-harvested leaves was not significantly different from those harvested in summer, winter and spring ( $p \geq 0.05$ ). The small differences in the total phenolic content of leaves harvested in summer and autumn were mainly due to a higher xanthone/lower flavone content in summer, and a lower xanthone/higher flavone content in autumn ( $p < 0.05$ ). Accumulation of higher xanthone levels in STB1, SGD2, SGD3 and SGD6 in summer and scolyoside in STB1, SGD2, SKB3 and SGD6 in autumn (Fig. 1) explains this result. The mean scolyoside content of the genotypes was 3.85 g/100 g DM in autumn compared to 2.29 g/100 DM for the other seasons (mean over genotype for summer, winter and spring harvests). The benzophenone (IDG) content of the leaves (mean = 2.03 g/100 g DM) was not significantly ( $p \geq 0.05$ ) affected by harvest season. Harvesting in summer and autumn produced leaves with a slightly higher flavanone and dihydrochalcone content than leaves harvested in winter and spring ( $p < 0.05$ ), due to accumulation of eriocitrin, PDG and HPDG in most genotypes (Fig. 1).

### 3.2. Sensory profile and phenolic content of *C. subternata* herbal tea

The intensity scores for the aroma and flavour attributes followed similar trends, however, in most cases the flavour attributes were perceived at lower intensities than the comparable aroma attributes. Data for the flavour attributes were therefore not included in the PCA bi-plot (Fig. 3). The data for aroma attributes, sweet taste and astringency with treatment means  $\geq 20$  were used to illustrate the ANOVA results (Figs. 4 and 5).

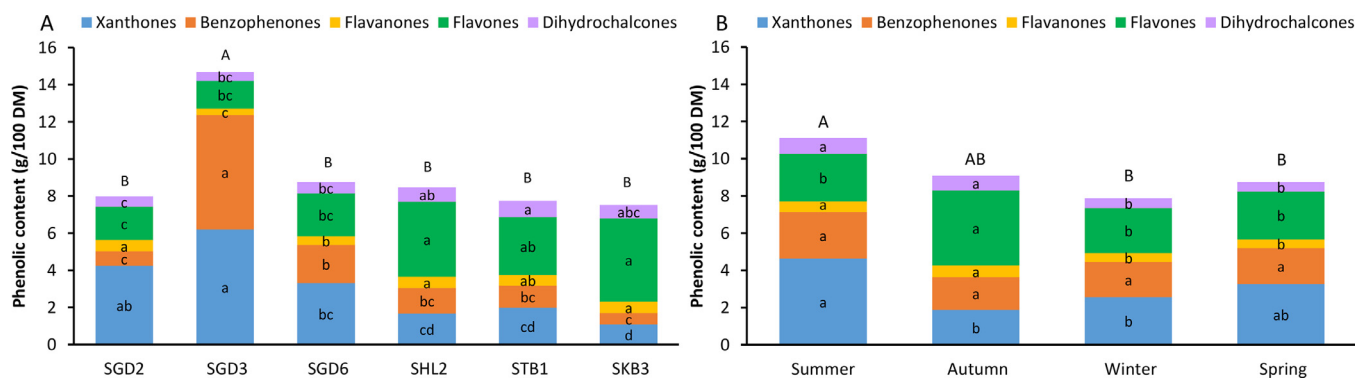


Fig. 2. Stacked bar graphs showing the effect of (a) genotype (SGD2, SGD3, SGD6, SHL2, SKB3 and STB1) and (b) effect of harvest season (summer, autumn, winter and spring) on the phenolic content of *C. subternata* leaves (g/100 g dry matter, DM) in terms of major phenolic classes/sub-classes. Statistical differences between samples are indicated with different letters (uppercase letters compare total phenolic content; lower case letters compare samples per phenolic class/sub-class) ( $p < 0.05$ ).

### 3.2.1. PCA of the sensory profile of *C. subternata* herbal tea

The first two components (F1 and F2) of the PCA explained only 57.91% of the variance (Fig. 3), yet some trends could be observed. The positive and negative aroma attributes were positioned on opposite sides of F1 indicating that they are negatively correlated. The positive aroma attributes and sweet taste associated mostly with plant material harvested in winter and summer, and to a lesser extent in spring, while the autumn harvest associated mostly with the negative attributes.

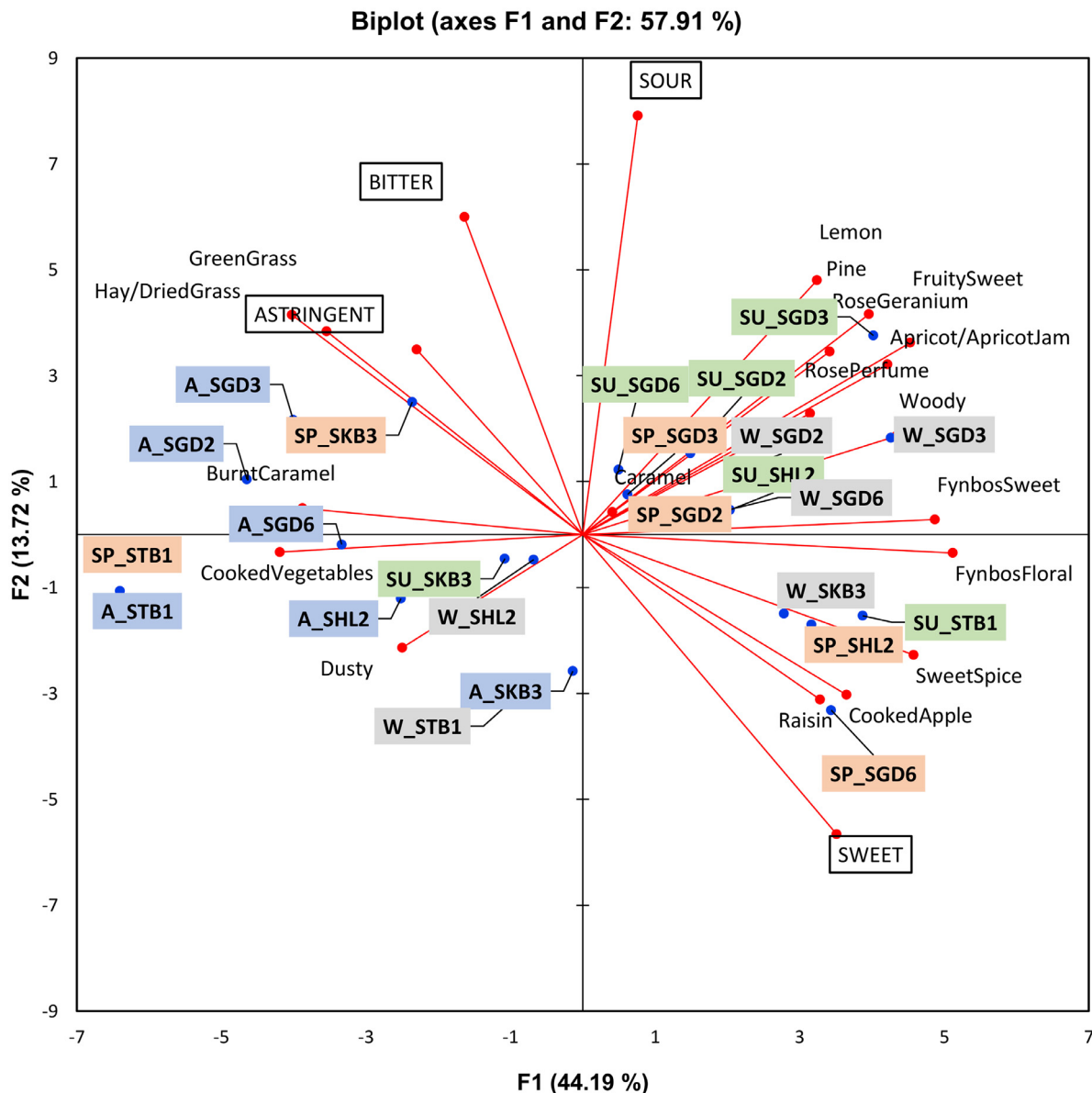
### 3.2.2. Effect of genotype on the sensory profile of *C. subternata* herbal tea

The effect of genotypic variation on the sensory attributes of *C. subternata* is depicted in Fig. 4. The most prominent positive aroma attributes were 'fynbos-floral', 'apricot jam', 'woody', 'fruity-sweet' and 'fynbos-sweet' (Fig. 4). The different genotypes produced herbal teas with very similar aroma profiles, however, notable was STB1, with lower or the lowest intensities ( $p < 0.05$ ), depending on the aroma attribute in question. Genotypes SGD2, SGD3, SGD6, SHL2 and SKB3 did not differ significantly ( $p \geq 0.05$ ) with respect to 'fynbos-

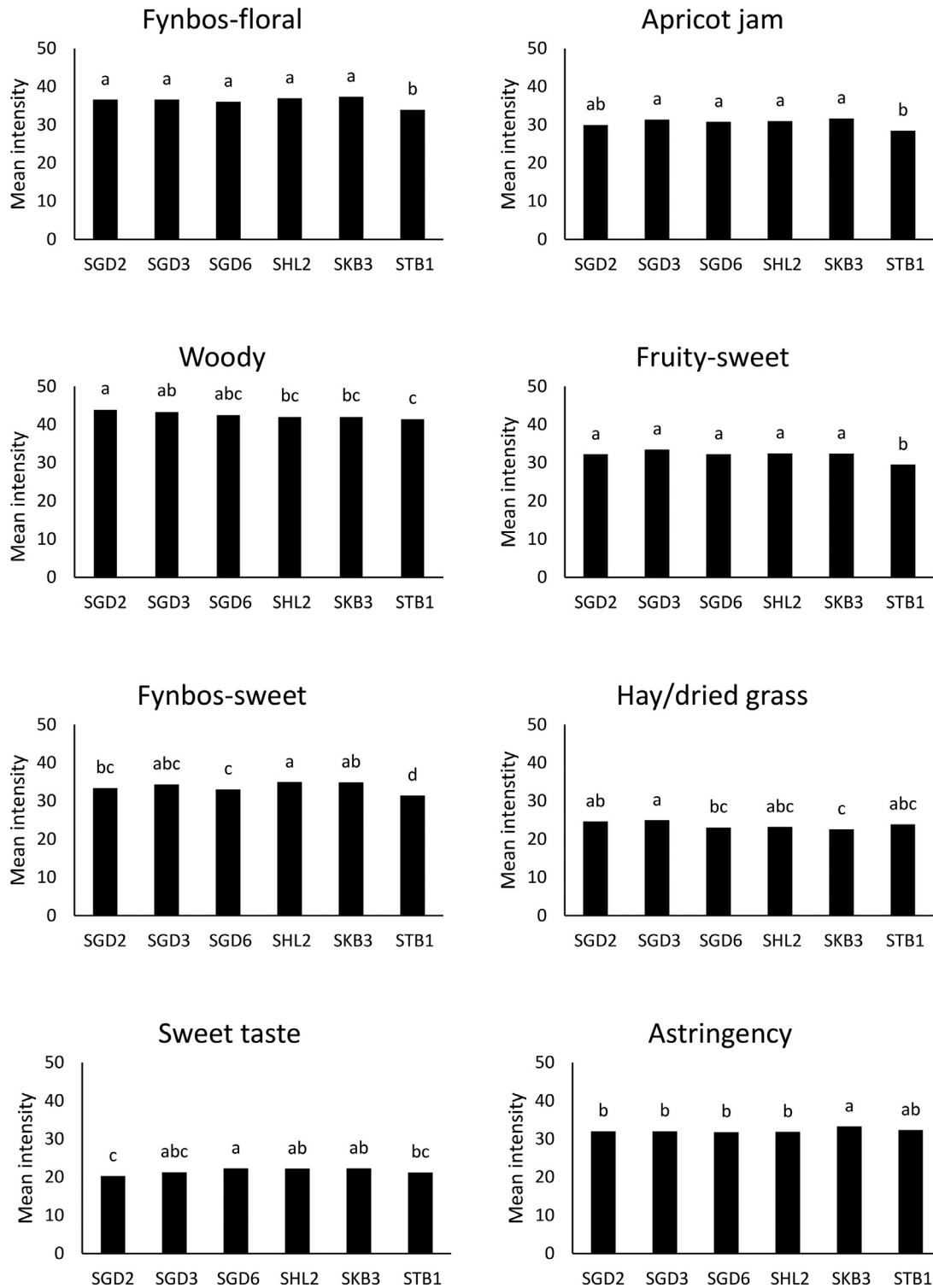
floral', 'fruity-sweet' and 'apricot jam'. Considering 'woody', STB1 had a lower intensity ( $p < 0.05$ ) than SGD2 and SGD3, although it did not differ significantly ( $p \geq 0.05$ ) from SGD6, SHL2 and SKB3. For the negative aroma attribute, 'hay/dried grass', SGD3 had the highest intensity ( $p < 0.05$ ), although not significantly different ( $p \geq 0.05$ ) from SGD2, SHL2 and STB1. SGD6 had the sweetest taste, although not significantly different ( $p \geq 0.05$ ) from SGD3, SHL2, SKB3. SGD2 tasted the least sweet ( $p < 0.05$ ), but not significantly different ( $p \geq 0.05$ ) from STB1. Astringency was more prominent in SKB3 and except for STB1, differed significantly ( $p < 0.05$ ) from the other genotypes.

### 3.2.3. Seasonal effect on the sensory profile of *C. subternata* herbal tea

The effect of harvest season is depicted in Fig. 5. The positive aroma attributes of the herbal teas, 'fynbos-floral', 'apricot jam', 'fynbos-sweet' and 'fruity-sweet', show a similar seasonal effect, i.e. no significant differences ( $p \geq 0.05$ ) were noted for summer and winter, whereas harvesting in autumn resulted in herbal teas with significantly lower ( $p < 0.05$ ) intensities. The 'woody' note of the herbal teas



**Fig. 3.** Principal component analysis bi-plot showing association between sensory attributes (loadings) and samples (scores). Samples are genotype × harvest seasons with SGD2, SGD3, SGD6, SHL2, SKB3 and STB1 indicating genotypes and SU, A, W and SP indicating summer, autumn, winter and spring, respectively.

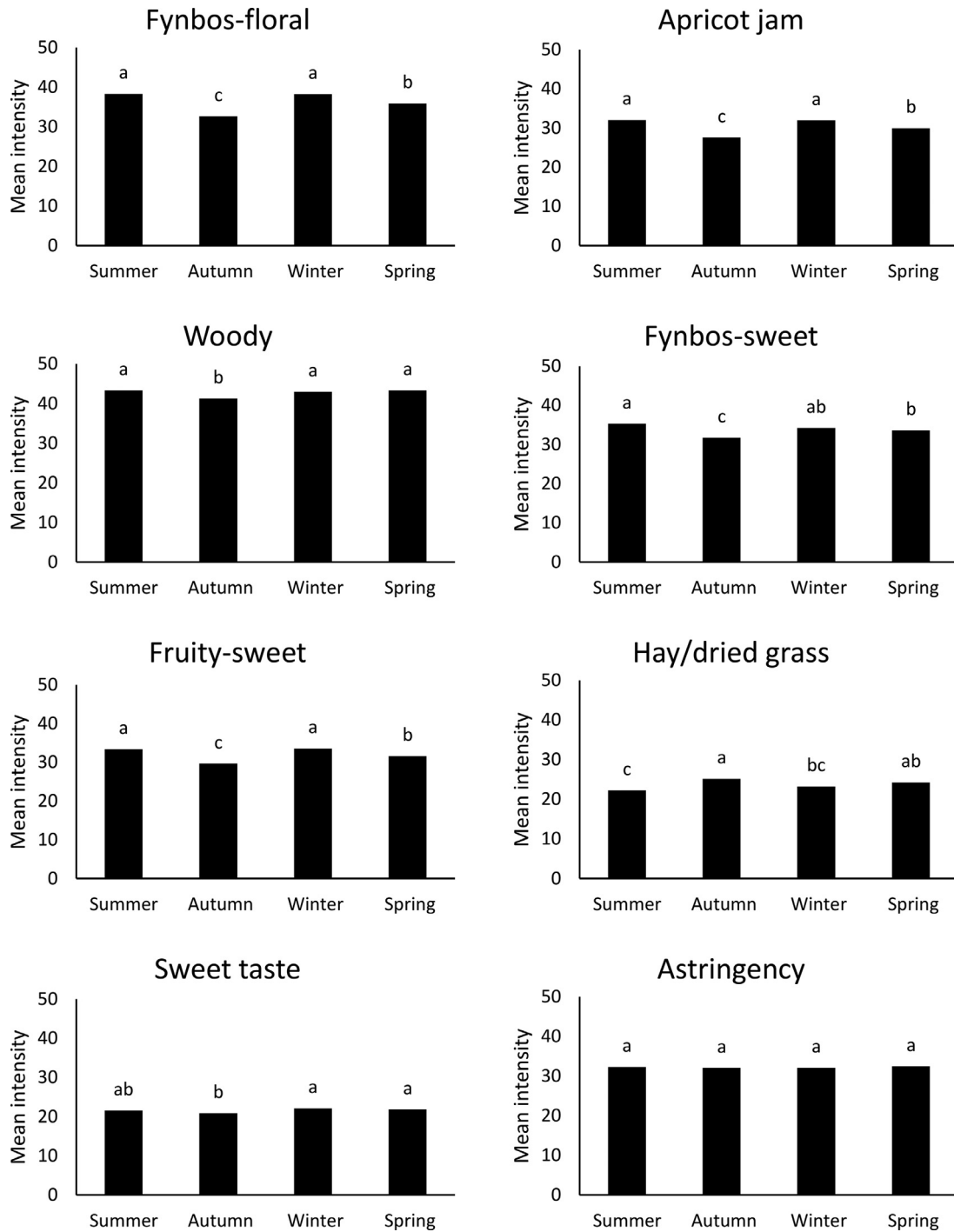


**Fig. 4.** Bar graphs showing the effect of genotype (SGD2, SGD3, SGD6, SHL2, SKB3 and STB1) on mean intensity of selected aroma attributes, sweet taste and astringency of *C. subternata* herbal tea. Statistical differences between genotypes are indicated with different letters ( $p < 0.05$ ).

did not differ significantly ( $p \geq 0.05$ ) between summer, winter and spring harvests, but harvesting in autumn reduced its intensity significantly ( $p < 0.05$ ) compared to the other harvest seasons. The negative aroma attribute, 'hay/dried grass', was at its highest intensity ( $p < 0.05$ ) in autumn, although not significantly different ( $p \geq 0.05$ ) from the spring harvest. Furthermore, the autumn harvest also produced herbal teas slightly less ( $p < 0.05$ ) sweet tasting than the winter and spring harvests. Astringency was not affected by harvest season ( $p \geq 0.05$ ).

#### 3.2.4. Phenolic content of *C. subternata* herbal tea

Table 2 summarises the soluble solids and individual phenolic content of the infusions, subjected to sensory analysis. Data represent means over harvest season for each genotype. The most prominent compounds in the infusions were IDG and scolymoside with mean values of 43.19 and 17.67 mg/L, respectively, followed by hesperidin, eriocitrin and IMG (9.36, 7.47 and 6.33 mg/L). The xanthenes, dihydrochalcones and vicenin-2 were present in the infusions at concentrations  $<5$  mg/L. SGD3 and SKB3 had the



**Fig. 5.** Bar graphs showing the effect of harvest season on mean intensity of selected aroma attributes, sweet taste and astringency of *C. subternata* herbal tea. Statistical differences between harvests are indicated with different letters ( $p < 0.05$ ).

**Table 2**

Mean soluble solids (g/L) and phenolic content (mg/L) of herbal tea infusions produced from different *Cyclopia subternata* genotypes.

| Genotype | SS   | Mangiferin | Isomangiferin | IDG    | IMG  | Eriocitrin | Hesperidin | Vicenin-2 | Scolymoside | HPDG | PDG  |
|----------|------|------------|---------------|--------|------|------------|------------|-----------|-------------|------|------|
| SGD2     | 2.13 | 3.56       | 3.00          | 24.82  | 7.04 | 9.72       | 10.65      | 3.69      | 14.39       | 3.29 | 3.91 |
| SGD3     | 2.48 | 6.05       | 6.21          | 131.44 | 6.08 | 6.90       | 11.61      | 7.09      | 10.14       | 4.17 | 4.95 |
| SGD6     | 2.08 | 3.90       | 3.10          | 43.56  | 4.83 | 5.71       | 7.68       | 2.51      | 8.73        | 2.93 | 4.04 |
| SHL2     | 1.94 | 1.60       | 1.68          | 24.77  | 9.29 | 8.27       | 8.10       | 2.96      | 26.69       | 3.55 | 4.47 |
| STB1     | 1.98 | 1.96       | 2.19          | 27.37  | 6.34 | 7.48       | 9.09       | 2.31      | 20.27       | 4.88 | 7.09 |
| SKB3     | 1.78 | 0.96       | 1.13          | 7.18   | 4.40 | 6.73       | 9.01       | 1.52      | 25.80       | 5.02 | 2.71 |
| Mean     | 2.06 | 3.01       | 2.89          | 43.19  | 6.33 | 7.47       | 9.36       | 3.35      | 17.67       | 3.97 | 4.53 |

**Abbreviations:** HPDG, 3-hydroxyphloretin-di-C-hexoside; IDG, 3- $\beta$ -D-glucopyranosyl-4-O- $\beta$ -D-glucopyranosyliriflophenone; IMG, 3- $\beta$ -D-glucopyranosyliriflophenone; PDG, 3',5'-di- $\beta$ -D-glucopyranosylphloretin.

highest and lowest IDG content, i.e. 131.44 mg/L and 7.18 mg/L, respectively. The scolymside content of SHL2, STB1 and SKB3 infusions was much higher (ca twice as high) than those of the other genotypes. The soluble solids content of infusion averaged 2.06 g/L.

#### 4. Discussion

This study was carried out to investigate the effect of harvest season on the phenolic profile of several *C. subternata* genotypes, originally selected for cultivation and breeding trials, based on phenotypic traits. Processing of the plant material, which entailed 'fermentation', was necessary to evaluate the sensory profile of the 'fermented' herbal tea as, ultimately, it will be a major factor guiding the honeybush breeding programme and harvesting practices.

##### 4.1. Effect of genotype and harvest season on the phenolic composition of *C. subternata* leaves

A previous study on *C. subternata* demonstrated substantial intra-species variation in the phenolic content of hot water extracts prepared from the leaves of a large number of genotypes (De Beer et al., 2012). The six genotypes investigated in the present study clustered broadly into two groups, with SGD3 and SGD2 predominantly forming the first cluster and the other genotypes (SGD6, SHL2, SKB3 and STB1) forming the second cluster. Based on mean values for harvest season, SGD3 and SGD2 contained the highest levels of mangiferin, which together with IDG and scolymside, were the most abundant phenolic compounds in *C. subternata* leaves. The latter flavone was, however, present in the lowest concentration in SGD3, while its IDG content was the highest. Further study would be necessary to determine whether these and other differences in composition between the two genotypes translate into a noticeable difference in bioactivity and merit the selection of one above the other in the honeybush breeding programme. (Dis)similarity of *Cyclopia subternata* extracts to an active reference extract, based on their HPLC fingerprints, did not translate into lower or higher bioactivity (Schulze et al., 2016). Mangiferin, isomangiferin, IDG, IMG, scolymside and PDG were the major compounds responsible for the variation between the HPLC fingerprints. A similar argument as for SGD3 and SGD2 is valid for the other genotypes when choosing one from the second cluster for the breeding programme.

An understanding of the response of the biosynthesis of secondary plant metabolites to fluctuations in environmental factors, such as temperature, rainfall, precipitation and solar radiation intensity conditions (Cezarotto et al., 2017; Hernández and Van Breusegem, 2010; Isah, 2019; Petrusa et al., 2013; Yang et al., 2018) lays the groundwork for identifying the optimal harvest time of plant material when production of polyphenol-rich extracts is the aim. Seasons present a combined environmental effect on biosynthesis so that specific factors cannot be identified as being responsible for the up- or down-regulation of biosynthetic pathways. Honeybush is harvested once a year, normally during the period spanning spring to autumn, but for this study a winter harvest was included.

The present study showed that the total phenolic content of the leaves peaked in summer and autumn (Fig. 2b), which was attributed to high levels of specific compounds that accumulated during these harvest seasons. A high xanthone/low flavone content in summer was more or less off-set by a low xanthone/high flavone content in autumn. These classes of compounds are biosynthesised via the shikimate pathway, with chalcone synthase catalysing the formation of naringenin chalcone and benzophenone synthase catalysing the formation of a benzophenone skeleton, a central step in xanthone biosynthesis. Xanthones are regioselectively cyclised from benzophenone derivatives. Coumaroyl-CoA and benzoyl-CoA act as precursors for biosynthesis of flavonoids and benzophenones, respectively

(Beerhues and Liu, 2009; El-Awaad et al., 2016; Ibdah et al., 2018; Liu et al., 2003). IDG, the only representative of the benzophenone class that could be quantified, was not significantly affected by season (Fig. 2b). To date the corresponding xanthone of IDG has not yet been identified in *Cyclopia*. The monoglucosyl of iriflophenone was present in trace quantities, but as for IDG, its corresponding xanthone has not yet been found in *Cyclopia*. Cyclisation of the monoglucosyl of iriflophenone and addition of a hydroxyl group would produce mangiferin/isomangiferin. For more insight, the effect of environment stress factors on enzymes in the biosynthetic pathways should be studied.

Accumulation of phenolic compounds during the dry months of the Mediterranean climate may reflect drought response (Boscaiu et al., 2010). For compounds not affected by harvest season it could be hypothesised that their rate of accumulation was near the saturation level, as there is a limit to the extent to which phenolic compounds can accumulate in a biological material under particular conditions (Bita and Gerats, 2013; Kacjan Maršić et al., 2011; Rivero et al., 2001).

Generally, high and low temperatures may positively or negatively impact the biosynthesis of polyphenols (Bita and Gerats, 2013; Han et al., 2018; Verma and Shukla, 2015). High temperatures induced the production of soluble polyphenols in tomato by increasing phenylalanine ammonia-lyase activity. Furthermore, low temperatures can slow plant growth, causing reduction in the photosynthesis rate which may lead to a decrease in the biosynthesis of secondary metabolites (Koç et al., 2010). Increased UV irradiation in summer may also up-regulate enzyme activity in the biosynthetic pathway (Santos-Sánchez et al., 2019). In the case of *C. subternata*, the high temperatures of summer and the low temperatures of winter may thus be a contributing cause for the difference in phenolic content of the leaves, harvested in summer and winter, respectively, confirming previous results for optimum xanthone production in *C. genistoides* leaves (Joubert et al., 2014). The latter study found the same trend for the monoglucosyl benzophenone, IMG, as for the xanthones, however the IDG content of the leaves was not quantified.

Evident from the heat map (Fig. 1) was that the response of the genotypes to seasonal effects was not the same, in particular SKB3, harvested in summer, stands out. In-depth investigation of differences between gene and protein expression in the genotypes is needed to gain insight.

##### 4.2. Effect of genotype and harvest seasons on the sensory profile of *C. subternata* herbal tea

Sensory analysis of the infusions of *C. subternata* herbal tea showed that harvest season has a notable impact on its sensory profile. This could be related to changes in accumulation of precursors of volatile compounds. The various pathways responsible for the biosynthesis of carotenoids, fatty acids, terpenes and phenylpropanoid/benzenoids, as well as glycoside hydrolysis, relevant to aroma development of *Camellia sinensis* teas (Zheng et al., 2016), may also be relevant for *Cyclopia*. Linalool and geraniol, both important to the aroma of *C. subternata* (Le Roux et al., 2012), are present in plants as glycosidically bound volatiles which are released upon hydrolysis (Song et al., 2018). (*E*)- $\beta$ -damascene and (*E*)- $\beta$ -ionone, important aroma-impact volatiles of *C. subternata*, derive from carotenoids. Other major aroma impact compounds were (*E*)- $\beta$ -damascenone and (7*E*)-megalstigma-5,7,9-trien-4-one (Le Roux et al., 2012). Terpenoids comprise the largest number of volatile organic compounds of *C. subternata* (Le Roux et al., 2012), but no information is available on the effect of seasonal variation on their evolution or that of their precursors. A study by Matich et al. (2010) on the enantiomeric composition of linalool and linalool oxide, produced by flowers of kiwifruit species, indicated that enantiomeric ratios of linalool differed between genotypes and year-

to-year. Sugawara (2000) reported that enantiomeric stereospecificity of linalool evokes a different aroma perception.

From a sensory perspective of the herbal tea as final product, raw plant material harvested in autumn not only produced herbal teas with lower intensities of the major positive aroma attributes than the summer and winter harvests, but the autumn harvest also produced a herbal tea with the highest intensity of the negative aroma attribute, 'hay/dried grass' (Fig. 3), clearly making a case for limiting harvesting to summer or even winter. However, other considerations such as survival of the plants and a practical issue such as drying of the processed plant material when using open-air drying would also come into play.

Considering genotypic variation, STB1 emerges as a genotype largely differing from the others in that the characteristic aroma attributes of *C. subternata*, i.e. 'fynbos-floral', 'apricot jam', 'woody' 'fruity-sweet' and 'fynbos-sweet', were less intense in its herbal tea infusion (Fig. 4). Small differences between genotypes were found in the sweet taste intensity, in particular SGD2 and SGD6, with the least and sweetest taste, respectively.

#### 4.3. Phenolic content of *C. subternata* herbal tea

The phenolic content of the infusions needs consideration when discussing taste and astringency, thus adding another dimension to the relative importance of these secondary metabolites. Infusions of SKB3, which was the most astringent, had the highest concentration of scolymoside and HPDG, yet the lowest concentration of xanthenes, benzophenones, PDG and vicenin-2. At this stage it is not known to what extent the individual compounds contribute to astringency as the astringency of a single phenolic compound may be modulated when combined with other phenolic compounds (Ferrer-Gallego et al., 2014), relevant for a complex mixture such as honeybush tea. To date, studies on the sensory impact of *Cyclopia* polyphenols, in particular xanthenes and benzophenones, are limited to bitter taste (Alexander et al., 2019, Alexander et al., 2019). The bitter intensity of the *C. subternata* infusions was barely perceptible, not surprising given that the mangiferin content of infusions was less than 11 mg/L (maximum concentration noted for SDG3 harvested in summer), a concentration typical of *C. subternata* infusions (Schulze et al., 2015) that elicits practically no perceivable bitter intensity (on a 100-point scale) (Alexander et al., 2019). As for astringency, the effect of the interplay between compounds on bitterness (Alexander et al., 2019) cannot be ruled out. However, bitterness of *C. subternata* herbal tea has not been a factor to date.

It should be noted that consideration of the phenolic content of the unfermented leaves is important for production of polyphenol-rich extracts, but it cannot be directly extrapolated to the phenolic content of the infusions. Apart from the presence of stems in the 'tea bag' fraction, the high temperature oxidation process causes degradation of compounds at different rates, changing the relative ratios between compounds, which could increase or decrease the relative prominence of a compound in the infusion. For example, IDG is very stable under fermentation conditions, and mangiferin degrades faster than its regio-isomer, isomangiferin (Beelders et al., 2015).

## 5. Conclusions

From this study, it is clear that the harvest season and genotype affected the phenolic content of the leaves and the sensory profile of 'fermented' *C. subternata* herbal tea. Summer and autumn may be regarded as the optimum harvest season to maximise the phenolic content of the leaves, however, a shortcoming of autumn harvesting is that the plant material produced a herbal tea less intense in the positive aroma attributes, while having a higher intensity of the negative aroma note, 'hay/dried grass', compared to the other harvests. SGD3 emerged as the genotype with the highest total leaf phenolic content, largely due

to accumulation of 3- $\beta$ -D-glucopyranosyl-4-O- $\beta$ -D-glucopyranosyliriflophenone, mangiferin and isomangiferin. Genotypic differences in phenolic content indicate that it would be possible to select a genotype (s) for the honeybush breeding programme, depending on the target polyphenolic compound as determined by intended use/bioactivity. An untargeted metabolomics approach in future research could further elucidate differences between genotypes and the effect of harvest season on the phenolic profile of this *Cyclopia* species.

## Declaration of Competing Interest

None.

## Acknowledgements

The authors wish to acknowledge funding by the Department of Science & Innovation (previously, Department of Science & Technology) (DST/CON 0023/2015 to CB), the National Research Foundation (grant 107805 to EJ) and the ARC Professional Development Programme for a PhD bursary to GM. Nico Walters and George Dico of the Plant Bioactives Group assisted with HPLC analysis of samples and processing of plant material, respectively.

## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2020.06.010.

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