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Chemical Variation in Essential Oil Profiles Detected Using Headspace Solid-Phase Microextraction Gas Chromatography Spectrometry in Response to Potassium, Nitrogen, and Water Available to Micropropagated Plants of *Salvia stenophylla* (Burch. *ex* Benth.)

Hannibal T. Musarurwa · Lerí Koegelenberg · Nokwanda P. Makunga

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Abstract The effects of nitrogen, potassium, water stress, and phytohormones were studied using a *Salvia stenophylla* (Burch. *ex* Benth.) microplant system. In vitro regeneration was monitored and then followed with volatile secondary metabolite profiling through headspace solid-phase microextraction gas chromatography. Plantlet growth was most prolific on half-strength Murashige and Skoog medium without phytohormones. An increase in macronutrients supplied to the microplants enhanced accumulation of the commercially important (−)-α-bisabolol, while no significant changes to the relative abundance of β-bisabolene, α-muurolene, α-patchoulene, and D-limonene (among others) became apparent. Water-stressed plants, treated with sorbitol and polyethylene glycol, had a lowered rooting capacity in vitro. Overall, as a plant production system, micropropagation did not have deleterious effects on the biochemistry of *S. stenophylla* as no significant differences in metabolic profiles existed between conventional garden plants and in vitro propagules, regardless of phytohormone treatment. We also show that nutrient manipulation can be used efficiently as a strategy for positively altering secondary metabolism. This will ultimately benefit the domestication of this commercially important medicinal herb.

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H. T. Musarurwa · L. Koegelenberg · N. P. Makunga
Department of Botany and Zoology, Stellenbosch University, Matieland, South Africa

N. P. Makunga (✉)
Private Bag X1, Matieland, Stellenbosch, Western Cape 7602, South Africa
e-mail: makunga@sun.ac.za

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Introduction

Plant secondary metabolism is biochemically and genetically complex. It is a function of spatial, temporal, and phenological factors, with seasonal changes and species growth patterns impacting both metabolite synthesis and subsequent accumulation. Therefore, metabolite heterogeneity within species is often reliant on biotic and abiotic factors. *Salvia stenophylla* Burch. *ex* Benth. (Lamiaceae) (Fig. 1a), or blue mountain sage, is one of 26 indigenous South African species (Kamatou and others 2005; Germishuizen 2006). This small, bushy perennial herb (average height = 40 cm), with flowers varying from blue to purple and which bloom from August to February (Jequier and others 1980), has a wide geographical distribution in South Africa (Germishuizen 2006).

Similar to other sages, *S. stenophylla* produces a diverse range of volatile compounds of commercial significance (Kamatou and others 2008) consisting primarily of monoterpenes and sesquiterpenes along with their oxygenated derivatives, which could be alcohols, aldehydes, esters, ethers, ketones, phenols, or oxides (Longaray Delamare and others 2007), rendering the oil aromatic. Apart from (−)-α-bisabolol, an important component of dermaceutical and cosmeceutical products due to its anti-inflammatory, anti-irritant, antimicrobial, and cicatrizant activity (Kamatou and others 2010), major compounds of industrial importance include manool, limonene, δ-3-carene, α- and β-pinene, camphor, and camphene, among many others. These biochemicals play a major role in the quality of

Fig. 1 In vitro propagation of *Salvia stenophylla*. **a** An example of a wild plant grown outdoors as a potted garden plant. **b** Continuous stock plant culture grown on agar-solidified MS medium (1%; w/v) with 2.9 μM IAA and 4.4 μM BA. **c** Plantlets grown for experimental use in test tubes rooted easily in culture. **d** Plantlets grown on medium with reduced nitrogen levels (½N medium) became chlorotic with increased production of callus. **e** Plantlets grown on medium with increased nitrogen levels (2N medium) maintained prolific shoot regeneration and rhizogenesis. **f** Roots produced on the 2N medium were strong, thick, and fibrous. **g** An example of rooted plants growing on ½N medium with roots that were thin and fragile. **h** Plants grown on the 2K medium produced well-formed in vitro flowers. **i** Inclusion of sorbitol reduced plantlet productivity, increasing the incidence of callus at shoot bases. **j** In vitro-propagated *Salvia stenophylla* plants growing in the greenhouse

essential oils and are thus monitored for quality assurance in commercial preparations. The essential oil of *S. stenophylla* shows chemical polymorphism, a phenomenon that has been linked to changing ecological microclimates (Viljoen and others 2006) as populations of these plants are found in a diverse range of habitats (ranging from the Eastern Cape, KwaZulu-Natal, and the Highveld of Gauteng Province).

Field cultivation has been applied broadly to aromatic plants for essential oil production worldwide [for example, *Pelargonium graveolens* L'Heritier (Saxena 2008), *Lippia javanica* (Burm. f.) Spreng, *Anthemis nobilis* L., and *Matricaria recutita* L. (Nikolova and others 1997)], ensuring adequate supply to meet commercial demands. Even though in its infancy, domestication of aromatic and medicinal plants (both exotic and indigenous species) is becoming increasingly important for poverty alleviation schemes (Makunga and others 2008; Horak and others 2009) in South Africa as the essential oils industry is part of a wider government initiative to stimulate rural socio-economic development. Currently, both cultivated and wild-crafted plant material is distilled for the production of "African indigenous" essential oils to serve local and export markets, but *S. stenophylla* cultivation is limited to a few agricultural commercial producers that are mainly resident in the Western Cape Province. In view of this, a better understanding of the nutritional and other cultivation requirements would assist with the domestication of key aromatic indigenous crops such as *S. stenophylla*.

Like most medicinal and aromatic plants, genetic and abiotic factors such as water availability, temperature, photoperiod, and herbivory affect the essential oil production of *S. stenophylla* populations (Viljoen and others 2006). The need for the cultivation of medicinal plants in an economy with a fast-growing phyto-pharmaceutical industry cannot be overemphasized as access to many aromatic plants in South Africa is largely through wild harvesting, increasing concerns regarding the loss of biodiversity. Tissue culture is also a well-used means of conservation (Kamatou and others 2008; Makunga and Van Staden 2008) for those medicinal plants that are accessible only through wild harvesting. In vitro culture also allows for rapid plant propagation (Nalawade and others 2003). The generation of disease-free, clonal propagules is highly attractive for commercial use (Kintzios 2000) as the production of clonal plants minimizes qualitative and quantitative chemical variation, ensuring a reliable source of bioactive ingredients. Apart from the studies that focused on the in vitro propagation of *S. chamelaeagnea* (Huang and Van Staden 2002), *S. africana-lutea* (Makunga and Van Staden 2008), and *S. stenophylla* (Musarurwa and others 2010), investigations have centered mostly on the phytochemical characterization and pharmacological efficacy of local endemic *Salvia* species which are used as traditional medicines (Kamatou and others 2005, 2008, 2010; Viljoen and others 2006).

Here, the in vitro system was ideal for generating plants that are genetically similar, facilitating the aim of studying the effect of changing nitrogen, potassium, water supply, and plant growth regulator (PGR) combinations on the volatile oil profiles of *S stenophylla*. Such manipulations cause great shifts in secondary metabolic flux leading to significant changes in the chemical constituents of essential oils (Rout and others 2000). We chose to vary the concentrations of nitrogen and potassium supplied to the plants as they greatly influence plant growth and vigor (Van Iersel and others 1998; Kadota and others 2001). Volatile constituents were monitored easily through headspace solid-phase microextraction gas chromatography spectrometry (HS-SPME-GCMS). Plants derived from the in vitro system may serve as an ex situ stock for commercial agricultural cultivation.

Materials and Methods

General Procedures and In Vitro Plantlet Culture

A continuous microplant culture of *S. stenophylla* Burch. *ex* Benth. (Fig. 1b, c), generated from seedling explants in 2008 according to the protocol described by Musarurwa and others (2010), was used in this investigation. Eighteen-month-old microplants were maintained in culture vessels (110 × 55 mm) on a basal medium (30 ml) of Murashige and Skoog (1962) (MS), with 2.9 μM NAA, 4.4 μM BA, 0.1 g l^{-1} myo-inositol, 30 g l^{-1} sucrose, and 10 g l^{-1} agar (pH 5.8; 1 M NaOH) (unless otherwise stated). All media were autoclaved at 121°C and 103 kPa for 20 min. In vitro plantlets were grown under cool white fluorescent lights (L75 W/20 × Osram, USA; code F96T12), supplying light for 16 h (50 μmol m^{-2} s^{-1}) at shelf-level in the growth room (25 ± 2°C). Nodal segments (2–5 cm), each containing an axillary bud pair that had the capacity for producing adventitious shoots, were subcultured to generate plantlet stocks every 6 weeks. Plants were acclimatized similarly to the methods described by Musarurwa and others (2010). Throughout the study, a set of ex vitro plants derived from tissue culture was maintained as potted glasshouse plants. The plants were acclimated by reducing the relative humidity over a 2-week period from about 90 to 70%. Plants were watered regularly every 3 days by hand. The conditions in the glasshouse were maintained using a thermostat that regulated the temperature between 15°C (minimum) and 25°C (maximum). In Stellenbosch, the natural sunlight ranged daily between 540 and 810 μmol m^{-2} s^{-1} at midday during the time of acclimatization.

We also kept a stock of plants, donated by Mrs. Jenny Ferreira (Wellington, Western Cape, South Africa), under garden conditions. The plants were watered daily using a sprinkler system but otherwise they were exposed to normal environmental conditions prevailing in Stellenbosch during the study period from February 2009 until December 2010.

Effect of Nitrogen and Potassium Macronutrients

The control medium (referred to here as MS) had NH_4NO_3 at 25.76 M and KNO_3 at 18.79 M as the nitrogen source, and all other macro- and micronutrients were kept as stipulated by Murashige and Skoog (1962). For the nitrogen experiment, the concentration of NH_4NO_3 and KNO_3 was either doubled (2N), tripled (3N), or increased by a factor of one and a half (1½N), or two and a half (2½N); all other nutritional elements were kept similar to the control. The potassium source, KH_2PO, which was added to MS medium at a concentration of 1.25 M (Murashige and Skoog 1962), was varied in a similar manner as described above and treatments henceforth are denoted as 1½K, 2K, 2½K, and 3K. Furthermore, plants were also grown on a medium that had half the concentration of all MS salts and vitamins (½MS). For this treatment, the sucrose and myo-inositol were added at 15 and 0.05 g l^{-1}, respectively. Otherwise, for all other media, sucrose and myo-inositol were added at 30 and 0.1 g l^{-1}, respectively. The PGRs were omitted from all media, including the control. The experiment was conducted in glass vessels (110 × 55 mm) with one plantlet representing a replicate. Each treatment had at least ten replicates (unless otherwise stated), and three samples from each treatment (sampled at random) were used for metabolite profiling (Tables 2, 3). All experiments were conducted twice. Data were collected at the end of 6 weeks by recording the number of regenerated shoots, shoot length (cm), plantlet mass (g), and root number from each explant. The number of leaves from each regenerating shoot was also counted (Table 1), and in this particular instance, each regenerating shoot was regarded as a replicate for statistical purposes.

Effect of Osmotica

Two dehydrating compounds were added independently as osmotica [sorbitol and polyethylene glycol (PEG)] to the MS medium (Table 4). One group of plantlets was treated with 110 μM sorbitol, similar to the study of Kadota (2001), as plants are still able to maintain normal shoot proliferation at this concentration. In parallel, 5% (w/v) PEG-6000 (Merck, Germany) was added to the basal medium of the second group of plantlets. Plant growth regulator supplementation was achieved by adding a combination of NAA and BA at 2.9 and 4.4 (μM), respectively (Musarurwa and others 2010). The controls had no sorbitol or PEG added to the basal MS medium. *S. stenophylla* internodal sections (1–3 cm in length) were individually transferred to glass test tubes (7 cm × 2 cm) containing 10 ml growth medium (Fig. 1c). Plastic caps were used as a seal and secured with a Parafilm "M" strip (American National Can, USA). Each plant in a test tube was regarded as a replicate (for examples refer to Fig. 1c). The experiment was terminated after 4 weeks. The number of leaves per plantlet was counted at day 0 and day 30. The shoot mass, the callus mass, and the total biomass produced from each plantlet were recorded. The number of roots formed was also noted. Thereafter, these plantlets were immediately sent for GCMS analysis (Table 5).

Effect of Plant Growth Regulator Combinations

Because plant metabolite synthesis is also affected by the presence and concentration of PGRs, plantlets were grown on MS media with varying concentrations of cytokinins and auxins. The PGR combinations were 2.9 μM IAA and

Table 1 Effect of potassium and nitrogen concentration on in vitro growth and organogenesis of *S. stenophylla* on Murashige and Skoog medium

Nutrient	Nutrient level	Shoot number	Shoot weight (g)	Shoot length (cm)	Leaf number	Root number
Control	½MS	1.63 ± 0.25 abcd	0.21 ± 0.04 ab	**4.89 ± 0.59 b**	10.9 ± 1.71 a	**16.8 ± 2.65 b**
	MS	1.17 ± 0.28 ab	0.16 ± 0.05 ab	1.83 ± 0.68 a	7 ± 1.97 ab	7.50 ± 3.06 ab
Nitrogen	1½	1.00 ± 0.28 a	0.16 ± 0.04 ab	1.85 ± 0.68 a	7 ± 1.97 ab	2.67 ± 1.71 a
	2	1.78 ± 0.23 bcd	0.17 ± 0.03 ab	1.58 ± 0.55 a	8.11 ± 1.61 ab	0.67 ± 0.55 a
	2½	**2.22 ± 0.23 d**	0.35 ± 0.11 ab	1.68 ± 0.55 a	7.22 ± 1.61 ab	1.00 ± 0.57 a
	3	2.00 ± 0.28 cd	0.30 ± 0.04 ab	1.68 ± 0.68 a	6.00 ± 1.97 ab	0.00 ± 0.00 a
Potassium	1½	1.86 ± 0.26 bcd	**0.68 ± 0.34 b**	2.16 ± 0.63 a	**11.3 ± 1.83 a**	5.86 ± 2.99 ab
	2	1.14 ± 0.26 ab	0.17 ± 0.06 ab	1.53 ± 0.63 a	9.57 ± 1.83 ab	5.00 ± 3.92 ab
	2½	1.02 ± 0.22 a	0.09 ± 0.02 a	1.30 ± 0.68 a	4.83 ± 1.97 b	2.17 ± 2.17 ab
	3	1.40 ± 0.31 abc	0.20 ± 0.05 ab	2.34 ± 0.74 a	8.6 ± 2.16 ab	8.40 ± 5.88 ab

Letters that are different within the same column indicate statistical differences ($P \leq 0.05$). Values in boldface type are the highest recorded means for that variable

4.4 μM BA, 2.9 μM IAA and 8.9 μM BA, and 1.1 μM IAA and 8.9 μM BA (Table 6). Data were collected at the end of 6 weeks.

Routine HS-SPME-GC-MS Protocol

The essential oils were assessed through HS-SPME-GCMS, in a manner similar to the report of Musarurwa and others (2010) but oven ramping temperatures and injection ratios were modified. Briefly, directly after harvesting, all plant material (0.4 g) was placed inside 20-ml headspace vials. Each container was sealed with an aluminum-coated silicone rubber septum. The extraction of the volatiles was facilitated by heating samples for 15 min at 80°C. Released shoot volatiles were adsorbed by the SPME fiber [DVB/Carboxen/PDMS, StableFlex (Supelco)]. With the exception of the osmotica-treated material and those experiments that examined the effect of PGRs, a 1:20 split injection ratio was used to introduce compounds into the Waters GCT Premier instrument fitted with a HP5 column (30 m, 0.25 mm i.d., 0.25 μm film thickness). Otherwise, a 1:5 split injection ratio was used. An initial temperature of 40°C with a 5-min hold was used. Thereafter, the temperature was raised to 150°C at a rate of 5°C min^{-1} before the rate was further adjusted to 10°C min^{-1} until 280°C. Mass spectrometry transfer and the chemical standards (Sigma-Aldrich, Germany) used for analysis [namely, (−)-α-bisabolol, camphene, (1R)-(+)-camphor, (+)-3-carene, β-caryophyllene, (1R)-(+)-α-pinene, R-(+)-limonene] were kept the same as in the report by Musarurwa and others (2010). An additional chemical standard, azulene, also purchased from Sigma-Aldrich (Germany), was included in this set (refer to Supplementary Material for chromatograms) used in the present study. The NIST Mass Spectral Search Program Library ver. 2.0 d (2005; Standard Reference Data Program of the National Institute of Standards and Technology, USA), in conjunction with previous work of Viljoen and others (2006), was used for compound identification. The plantlets for each experiment were pooled together for analysis as we had determined that the differences between the chemical constituents of three individual tissue culture samples were negligible.

Data Collection and Statistical Analysis

Each treatment had at least ten replicates and three samples from each treatment (sampled at random) were used for metabolite profiling. All experiments were conducted twice. The means were compared using the analysis of variance (ANOVA; $P \leq 0.05$) followed by the HSD post-hoc analysis. For the data that were not normally distributed, Kruskal-Wallis post-hoc analysis was used to separate means. All percentage data were first arcsine-transformed prior to ANOVA. A principal component analysis (PCA) was also carried out to show differences between in vitro propagules and ex vitro-propagated plants and those exposed to varying potassium and nitrogen medium components. All statistical procedures were performed using Statistica ver. 9 (2007; Stata Corp., College Station, TX, USA).

Results and Discussion

Effect of Nitrogen and Potassium on Plantlet Growth

The impacts of mineral nutrition on both microplant productivity and volatile leaf constituents of *S. stenophylla* in vitro were determined. Most treatments were able to induce the development of two microplants per explant, with the exception of the 1½N and 2½K treatments. Plants growing on 1½K medium were able to maintain prolific shoot mass production (0.68 g) (Table 1) which was paralleled by adventitious leaflet regeneration. Although this was statistically insignificant, the MS medium strongly promoted rhizogenesis. Roots produced in culture were typically well developed and appeared to be thick and strong (Fig. 1c). The number of leaves produced per shoot was not correlated to increasing concentrations of nitrogen. However, explants on ½MS and 1½K media produced the highest number of leaves per individual regenerated shoot. Those plants grown on ½MS elongated best (4.89 cm), while all other treatments were statistically similar in terms of their ability to promote shoot extension. However, nutrient manipulation strongly influenced overall growth patterns and organogenesis. Increased levels of supplied potassium salts corresponded to a higher rooting frequency and the number of adventitious roots formed in culture.

It is evident from Table 1 that total shoot mass correlated with supplied nitrogen, but this effect was also accompanied by a drastic reduction of root formation. Plants in nutrient-limiting environments invest in mass proliferation of roots so as to increase the surface area for maximum nutrient absorption. The root network thus becomes much thicker with increased production of lateral root branches (Pellny and others 2008). Our data corroborate these well-established effects as reduction of nutrients (½MS) dramatically stimulated rooting. Although unrooted microplants are able to survive ex vitro transplantation, acclimatization is often better when roots are present. Furthermore, plants with roots associated with callus often have a lower hydraulic activity due to the disruption of the vasculature, potentially lowering their ex vitro acclimatization frequencies (Makunga and Van Staden 2008). The in vitro propagation system for *S. stenophylla* is generally highly prolific, with adventitious shoot regeneration from axillary nodes.

Stitt (1999) reported that high nitrate concentrations may inhibit root growth. This may explain the loss of the rooting potential. Nitrates are important in nucleic acid and protein production, acting as a stimulant for gene expression while playing a crucial role in resource allocation (Hermans and others 2006). Moderate nitrogen deficiency reduces shoot growth but it may have a stimulatory effect on root growth resulting in a smaller shoot:root ratio (Scheible and others 1997). Plantlets often became chlorotic on lower nitrogen medium (Fig. 1d), indicating inefficient production of chlorophyll pigments. Production of roots under high levels of nitrogen (2N) coupled with inefficient reallocation of carbohydrates due to lowered potassium resulted in strong, thick roots that were more fibrous (Fig. 1e, f), but those on lower nitrogen levels appeared more fragile, breaking off easily when plants were extracted from culture tubes (Fig. 1g).

Salvia stenophylla shoot regeneration was inversely proportional to changing potassium concentrations (Table 1), suggesting that a shortage of this mineral may favor shoot growth (Zhang and others 1999; Lahti and others 2005). A similar effect was noted by Benlloch-González and others (2010) using sunflower plants deprived of potassium. Potassium plays a crucial role in osmoregulation, photosynthesis, enzyme activation, and transport of assimilates in plants and, thus, it is possibly responsible for a biomass production shift. To improve their uptake in low-potassium environments (Li and others 2006), plants activate more potassium transporters to counter low levels of this mineral. All these factors affect in vitro plantlet regeneration and metabolite synthesis. Interestingly, doubling the potassium in the medium precipitated in vitro flowering (Fig. 1h), and those plantlets that flowered had floral parts that were well developed and typical of wild *S. stenophylla* plants in flower.

Effect of Nitrogen and Potassium on Essential Oil Components

A metabolomic approach was used in this study to differentiate the effects of changing the microenvironmental medium components. Compounds that had a NIST library match of 85–100% were regarded as likely hits (Tables 2, 3). The true identity of nine chemical compounds was confirmed through the use of standards and the molecular ion peaks, fragmentation patterns, and retention times. These concluded a positive match, providing us with greater confidence in the mass spectral analysis conducted for putative compound identification in this study. Over 100 volatile compounds were detected in propagated plants of *S. stenophylla*, and the profiles of the propagated plants were similar to those occurring in the wild (Fig. 2). These compounds accounted for 85–95% of the headspace volatiles (Tables 2, 3). As expected, the major compounds were monoterpenes and sesquiterpenes and overall profiles were similar to those previously reported by Viljoen and others (2006). Altering supplied levels of both macronutrients had little effect on many secondary volatiles with the exception of (−)-α-bisabolol, which responded positively to an increase in potassium and nitrogen levels (Tables 2, 3; Fig. 3). The MS- and ½MS-grown plants were the least complex in terms of chemical composition (Fig. 3). Furthermore, individual plantlets extracted from these media did not show significant essential oil intraspecific variation upon carrying out the PCA (Fig. 4), implying an almost uniform accumulation of metabolites reflected by 27 major constituents (Fig. 3). All replicates for ½MS and MS, denoted as A and B in Fig. 4, loaded on the same component. We regarded this as being good and beneficial when considering commercial production because uniformity can be used as a measure of quality.

Overall, changing the composition of potassium and nitrates did not significantly alter the major volatile chemical constituents produced by *S. stenophylla* plants in vitro as few to no effects were detectable regarding the essential oil quality. There was no direct relationship (or statistically valid trend) linking increasing concentrations of these nutrients with elevated accumulation of the major essential oil metabolites, with the exception of a few chemicals (Table 2). Only a few metabolites responded positively to higher amounts of tested nutrients. For instance, (−)-α-bisabolol (Fig. 2), which makes up the greatest fraction of headspace volatiles irrespective of treatment, was positively influenced by changing concentrations of supplied potassium and nitrogen (Table 2; Fig. 3). (−)-α-Bisabolol accumulation showed an interesting trend in response to higher potassium supply, with its relative abundance rising from a mean of 48.58% (½MS) to 61.01% when potassium was increased threefold in the growth medium (3K) (Table 2). Abundance of the monoterpene D-limonene was reduced when potassium was increased, but varying the nitrogen provided to microshoots had an insignificant effect on this particular metabolite. However, for those plants exposed to the 2½N medium, a decrease was also noted. Among these, cis-lanceol and verticiol are likely to be some of the metabolites that distinguish this treatment as a separate cluster (Fig. 4). The growth of plantlets on media with different nitrogen or potassium content did not affect the accumulation of α-muurolene (a sesquiterpene). Its relative abundance ranged from 0.56 to 0.92% and was not significantly different at the 95% confidence level (Table 3).

There is no congruent idea on the effect of genetic regulation and/or environmental impact on essential oil biochemistry in plants, so several research groups (Nikolova and others 1997; Emongor and Chweya 1992; Van den Heever and others 2008) have monitored the effects of

Table 2 Volatile secondary metabolite accumulation in response to changes in potassium concentrations in MS media

Compound	Retention time (min)	Kovats index	Relative abundance (%)					
			½MS	MS	1½K	2 K	2½K	3 K
α-Pinene	8.74	937	0.23 ± 0.08 ab	–	0.18 ± 0.06 ab	0.18 ± 0.01 ab	0.23 ± 0.15 ab	0.13 ± 0.02 b
Camphene	9.24	947	0.19 ± 0.05 a	–	–	0.10 ± 0.04 a	0.18 ± 0.14 a	0.10 ± 0.03 a
β-Pinene	11.00	965	4.61 ± 1.36 ab	–	3.57 ± 1.22 ab	3.86 ± 1.17 ab	3.11 ± 1.56 ab	2.61 ± 0.88 ab
α-Phellandrene	11.34	1000	0.97 ± 0.22 a	0.34 ± 0.04 bc	0.20 ± 0.02 b	0.90 ± 0.31 a	0.66 ± 0.18 abc	0.62 ± 0.16 ac
δ-3-Carene	11.52	1009	12.60 ± 0.05 a	–	9.42 ± 1.35 ab	12.18 ± 2.99 a	9.57 ± 2..52 ab	9.51 ± 2.09 ab
D-limonene	12.10	1018	5.12 ± 1.05 a	4.05 ± 1.08 ab	3.62 ± 0.64 ab	5.08 ± 1.49 a	3.63 ± 1.68 ab	3.53 ± 0.86 ab
D-sylvestrene	12.19	987	1.81 ± 0.40 ab	1.36 ± 0.35 ab	1.33 ± 0.30 ab	1.87 ± 0.53 ab	1.31 ± 0.46 ab	1.23 ± 0.30 ab
Terpinolene	14.15	1052	0.97 ± 0.19 bc	0.55 ± 0.13 ab	0.47 ± 0.01 ab	0.92 ± 0.31 bc	0.21 ± 0.11 b	0.68 ± 0.17 abc
Camphor	15.95	1144	0.29 ± 0.06 a	0.38 ± 0.21 ab	0.63 ± 0.22 ab	0.19 ± 0.05 a	0.14 ± 0.09 a	0.19 ± 0.10 a
Borneol	16.67	1156	1.25 ± 0.30 a	1.73 ± 1.38 ab	1.22 ± 0.22 a	1.22 ± 0.32 a	1.39 ± 1.28 ab	1.22 ± 0.28 a
β-Caryophyllene	23.62	1415	2.95 ± 0.22 a	1.39 ± 0.28 a	–	2.84 ± 0.47 a	3.13 ± 0.60 a	1.69 ± 0.34 a
trans-ß-Bergamotene	24.07	1674	2.98 ± 0.23 abc	2.47 ± 0.46 a	2.76 ± 0.46 ab	3.89 ± 0.58 c	3.05 ± 0.71 abc	2.48 ± 0.19 a
α-Caryophellene	24.47	1579	0.69 ± 0.17 b	–	1.31 ± 0.38 b	0.65 ± 0.11 a	0.74 ± 0.05 a	0.42 ± 0.08 a
Z-ß-farnesene	24.62	1438	2.46 ± 0.45 abc	1.51 ± 0.32 ad	1.86 ± 0.26 abd	2.44 ± 0.35 abc	1.72 ± 1.09 abd	1.24 ± 0.25 d
β-Bisabolene	25.87	1483	2.16 ± 0.44 a	1.73 ± 0.21 a	2.60 ± 0.09 a	2.28 ± .036 a	1.87 ± 0.56 a	1.62 ± 0.06 a
τ-Muurolene	25.98	1486	0.64 ± 0.12 a	0.70 ± 0.13 a	0.36 ± 0.09 a	0.56 ± 0.06 a	0.24 ± 0.10 bc	.059 ± 0.04 b
Cedrene	26.23	1398	0.60 ± 0.10 abcd	–	0.49 ± 0.63 acd	–	0.41 ± 0.15 a	0.44 ± 0.08 a
α-Patchoulene	26.43	1464	0.21 ± 0.05 a	–	0.25 ± 0.03 a	–	0.30 ± 0.09 a	0.27 ± 0.02 a
α-Muurolene	28.70	1440	0.82 ± 0.17 a	–	0.71 ± 0.10 a	0.55 ± 0.16 a	–	0.83 ± 0.14 a
Bisabolol oxide B	29.02	1707	0.21 ± 0.05 a	–	0.32 ± 0.04 a	0.31 ± 0.14 a	–	0.36 ± 0.14 a
(−)-α-Bisabolol	29.52	1625	47.58 ± 3.75 ac	51.87 ± 4.57 ab	50.25 ± 7.25 abd	56.98 ± 4.19 ac	55.04 ± 6.25 abd	61.01 ± 3.70 d
cis-Lanceol	30.61	1737	1.48 ± 0.32 ae	1.21 ± 0.24 e	2.48 ± 0.41 abcd	1.60 ± 0.53 abe	1.03 ± 0.39 e	2.51 ± 0.62 abcd
Verticiol	33.29	1290	2.57 ± 1.38 ab	2.04 ± 0.38 ad	2.85 ± 0.49 abc	2.85 ± 1.27 abc	1.35 ± 1.01 d	2.66 ± 0.48 ab
Trachylobane	33.56	1698	0.09 ± 0.01 b	–	–	–	0.25 ± 0.23 ab	0.28 ± 0.03 ab

Table 3 Volatile secondary metabolite accumulation in response to changes in nitrogen concentrations in MS media

Compound	Retention time (min)	Kovats index	Relative abundance (%)					
			½MS	MS	1½N	2 N	2½N	3 N
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Camphene	9.24	947	0.38 ± 0.03 b	0.24 ± 0.02 ab	0.16 ± 0.02 a	–	0.16 ± 0.04 a	0.35 ± 0.06 b
β-Pinene	11.00	965	2.23 ± 0.23 ab	3.73 ± 0.10 ab	3.06 ± 0.34 ab	5.41 ± 2.03 b	2.02 ± 0.55 a	3.59 ± 0.95 ab
α-Phellandrene	11.34	1000	0.77 ± 0.02 ac	0.87 ± 0.04 a	0.82 ± 0.12 a	0.81 ± 0.15 a	0.35 ± 0.02 bc	0.61 ± 0.13 abc
δ-3-Carene	11.52	1009	8.33 ± 0.34 ab	10.26 ± 0.09 ab	9.90 ± 0.87 ab	10.27 ± 1.56 ab	5.60 ± 0.24 b	10.05 ± 2.56 ab
D-limonene	12.10	1018	3.25 ± 0.16 ab	4.06 ± 0.09 ab	4.04 ± 0.43 ab	3.90 ± 0.77 ab	1.96 ± 0.12 b	3.55 ± 0.97ab
D-sylvestrene	12.19	987	1.19 ± 0.05 ab	1.42 ± 0.05 ab	1.42 ± 0.14 ab	154 ± 0.34 ab	0.85 ± 0.09 a	2.05 ± 0.67 b
Terpinolene	14.15	1052	–	1.07 ± 0.05 c	–	–	–	0.69 ± 0.14 abc
τ-Terpinene	15.88	1056	0.20 ± 0.01 a	0.36 ± 0.03 a	0.32 ± 0.08 a	–	–	0.25 ± 0.04 a
Camphor	15.95	1144	0.62 ± 0.06 ab	0.61 ± 0.12 ab	0.44 ± 0.09 ab	0.97 ± 0.65 b	0.20 ± 0.03 a	0.48 ± 0.05ab
Borneol	16.67	1156	3.13 ± 0.44 b	1.24 ± 0.24 a	0.62 ± 0.06 a	0.72 ± 0.28 a	1.24 ± 0.57 a	1.66 ± 0.37 ab
β-Caryophyllene	23.62	1415	10.59 ± 2.05 b	3.15 ± 0.24 a	3.08 ± 0.48 a	1.10 ± 0.14 a	2.03 ± 0.38 a	1.76 ± 0.21 a
trans-ß-Bergamotene	24.07	1674	2.62 ± 0.06 ab	4.02 ± 0.15 c	3.56 ± 0.51 bc	1.97 ± 0.47 a	2.36 ± 0.27 a	2.82 ± 0.28 ab
α-Caryophellene	24.47	1579	2.70 ± 0.50 c	0.82 ± 0.04 a	0.75 ± 0.01 a	–	0.53 ± 0.10 a	0.45 ± 0.06 a
Z-ß-farnesene	24.62	1438	2.61 ± 0.13 bc	2.42 ± 0.12 abc	2.98 ± 0.37 c	–	2.02 ± 0.38 abcd	2.07 ± 0.12 abcd
β-Bisabolene	25.87	1483	7.54 ± 0.54 b	2.25 ± 0.09 a	2.57 ± 0.17 a	1.77 ± 0.29 a	2.09 ± 0.08 a	2.01 ± 0.13 a
τ-Muurolene	25.98	1486	0.07 ± 0.02 a	0.63 ± 0.06 a	0.65 ± 0.01 a	0.50 ± 0.08 ac	0.24 ± 0.03 bc	0.53 ± 0.06 a
Cedrene	26.23	1398	0.77 ± 0.05 b	0.71 ± 0.04 bcd	0.77 ± 0.08 b	0.49 ± 0.11 ac	0.74 ± 0.14 bd	0.61 ± 0.02 abcd
α-Patchoulene	26.43	1464	0.24 ± 0.00 a	0.28 ± 0.02 a	0.23 ± 0.01 a	0.22 ± 0.03 a	0.25 ± 0.01 a	–
α-Muurolene	28.70	1440	–	0.57 ± 0.04 a	0.56 ± 0.05 a	–	0.92 ± 0.14 a	0.64 ± 0.06 a
Bisabolol oxide B	29.02	1707	–	–	–	–	–	–
(−)-α-Bisabolol	29.52	1625	40.12 ± 2.78 c	48.08 ± 0.53 ac	48.58 ± 1.91 ac	54.82 ± 3.67 abd	52.01 ± 0.77 bd	61.14 ± 0.10 ab
cis-Lanceol	30.61	1737	2.75 ± 0.43 bcd	2.20 ± 0.12 abcde	2.48 ± 0.29 abce	2.01 ± 0.42 abce	3.37 ± 0.14 d	2.85 ± 0.69 bc
Epi-manool	33.19	2085	0.83 ± 0.09 a	0.63 ± 0.12 a	1.59 ± 0.27 a	–	–	0.98 ± 0.10 a
Verticiol	33.29	1290	1.76 ± 0.45 ad	2.71 ± 0.55 ab	4.22 ± 1.21 bc	3.02 ± 0.77 abc	4.87 ± 0.97 c	3.80 ± 0.56 abc
Trachylobane	33.56	1698	0.22 ± 0.06 ab	0.29 ± 0.05 ab	0.45 ± 0.15 ab	0.27 ± 0.08 b	0.45 ± 0.05 a	0.33 ± 0.04 ab

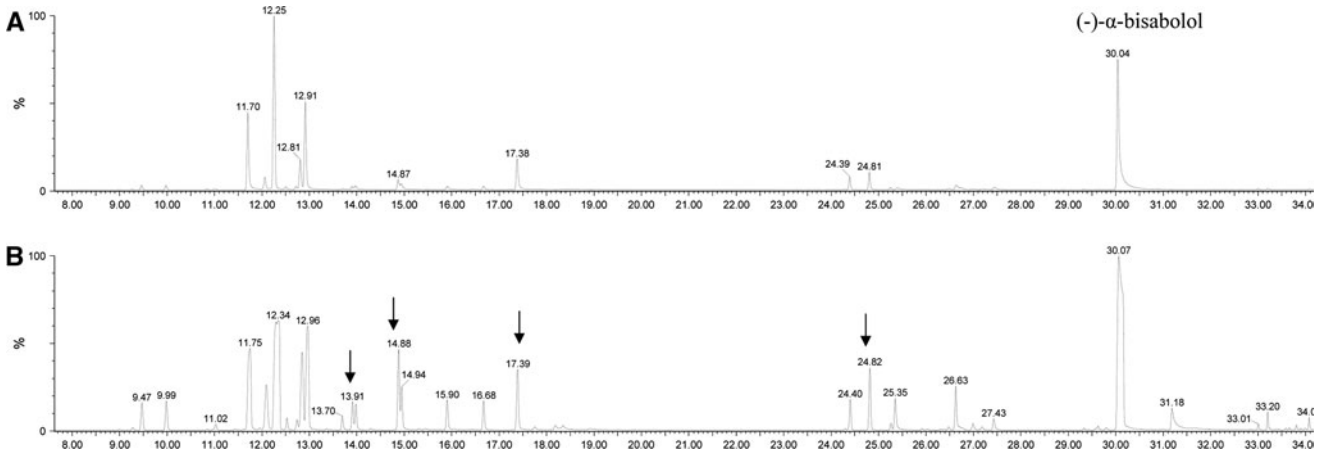

Fig. 2 HS-SPME-GCMS analysis of (**a**) nonpropagated plants maintained in the garden and (**b**) in vitro-derived microplants of *S. stenophylla*. Overall, metabolite profiles were similar, with tissue-cultured samples at times having higher levels of specific compounds (indicated by arrows) and (−)-α-bisabolol (eluting at 30 min) was one of the major constituents. Arrows indicate peaks that were more prominent in the tissue-cultured material

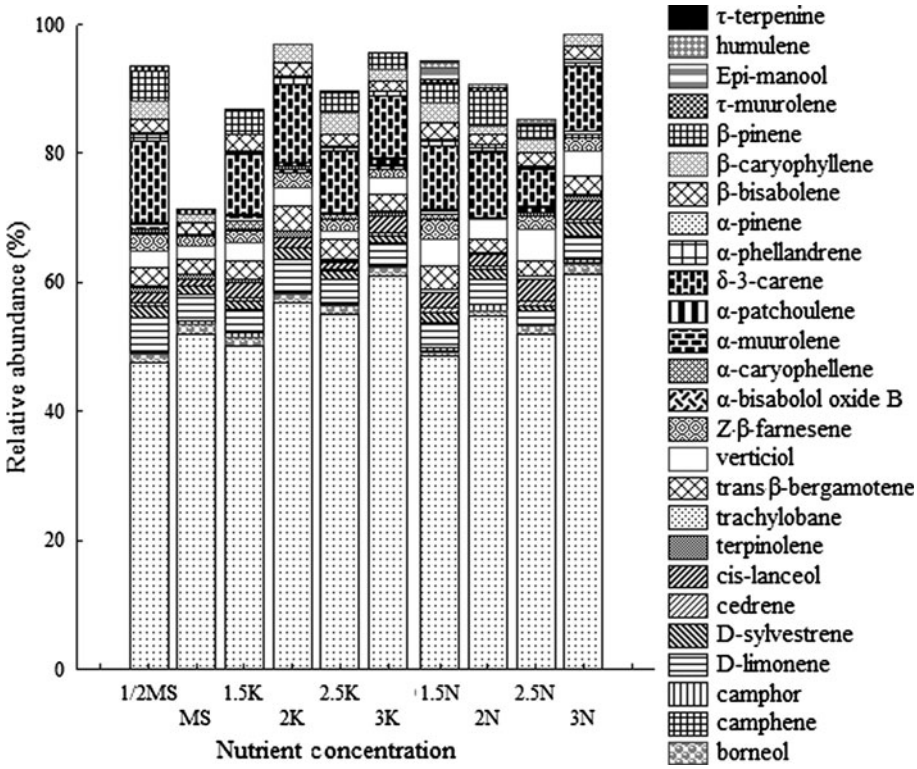

Fig. 3 Relative abundance (%) of *Salvia stenophylla* metabolites in response to different nutrient concentrations in vitro after a 6-week growth period on solid agar MS medium. Stacked columns represent major metabolites identified for each treatment using the NIST05 library

nitrogen and potassium nutrition on the essential oil yield in plants. Low essential oil heterogeneity in response to alterations in nutrient supply may indicate that genetic control of secondary metabolism has a larger role in controlling the synthesis of compounds rather than the environment in *S. stenophylla*, as was also noted by Ma and Gang (2008) in micropropagated *Phlegmariurus squarrosus*. Nikolova and others (1997) reported that essential oil synthesis in different lines of chamomile was not affected by different combinations of nitrogen, potassium, and phosphorus. Nitrogen is often associated with enhanced vegetative growth and differentiation, which in aromatic plants may possibly result in increased peltate glandular trichome development. Trichomes are vital for oil accumulation and storage, leading to elevated monoterpene production (Yamaura and others 1989). (−)-α-Bisabolol and bisabolol oxides B and A remained within their normal range. On the other hand, Emongor and Chweya (1992) noted higher (−)-α-bisabolol and chamazulene levels. The increased essential oil yield due to higher nitrogen supplementation led to lowered

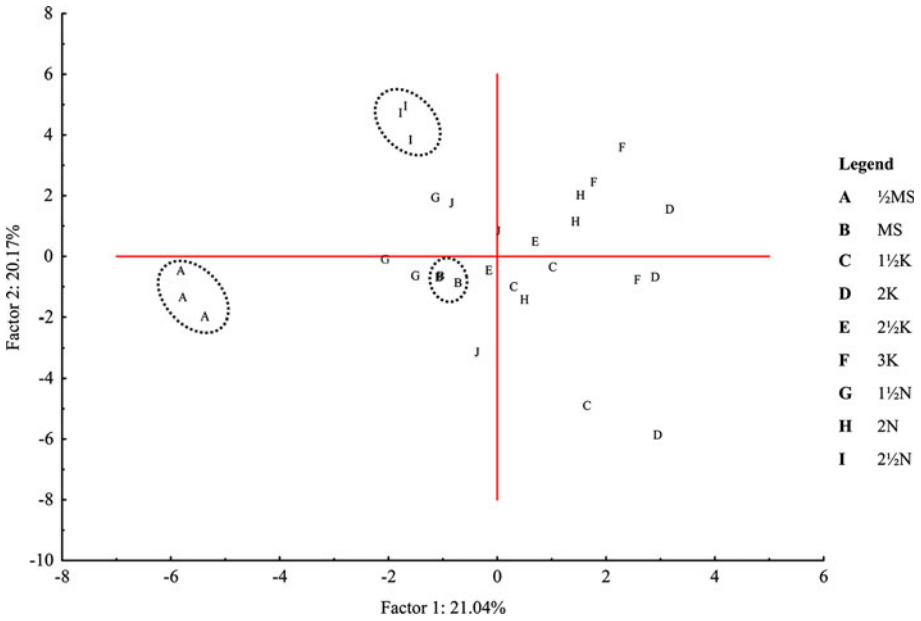

Fig. 4 A score plot obtained from PCA showing aggregation of HS-SPME-GCMS profiles based on ½MS, MS, nitrogen, and potassium treatments. Factor 1 captured 21.04% of the variance while factor 2 captured 20.17% of the variance

antifungal activity in *Tulbaghia violacea*, thus implying a decrease in the accumulation of secondary bioactives responsible for pharmacological action (Ma and Gang 2008). In this study we were unable to detect any azulene in the plant samples, even though analysis of the cocktail containing the pure chemical standards showed positive identification of this compound. Differences in the response of plants to minerals suggest that perceived metabolomic changes are species-specific.

Plants that grow in the Dordrecht region in South Africa have the highest (−)-α-bisabolol content of 46% (Viljoen and others 2006). The region is characterized by low rainfall, plus *S. stenophylla* and other broad-leafed plants occur along highly nutritious drainage lines. Through in vitro manipulation, we could supersede recorded levels of (−)-α-bisabolol in naturally occurring plants, making our strategy a powerful tool to increase production of this commercial metabolite. Similarly, nitrogen supplementation was correlated to the amount of (−)-α-bisabolol produced in vitro, with superior production recorded at 61.14% when plants were grown on medium with three times the normal levels of nitrogen (Table 3).

(−)-α-Bisabolol in wild *S. stenophylla* plants may range from 1.8 to 46.50% as the primary compound, depending on the locality of the plant population (Viljoen and others 2006). We therefore cannot emphasize enough the importance of being able to easily produce plants with a superior, more predictable (−)-α-bisabolol content. Such treatments could thus be directly adopted for agricultural production because (−)-α-bisabolol synthesis responds readily to microenvironmental alterations. This is of great benefit to the pharmaceutical and cosmeceutical industries as this chemical product is highly sought after. Although ½MS was beneficial for microplant production and organogenesis (Table 1), (−)-α-bisabolol production decreased (Table 2). Effects of this medium could be clearly discriminated from the other treatments, resulting in both fewer and lower metabolite concentrations. This was confirmed through its distinct grouping upon carrying out the PCA (Fig. 4).

Interestingly, Green and others (2009) recently concluded that potassium is required for the synthesis of a terpene synthase, known as α-farnesene synthase, isolated from apple. This enzyme is paramount in the formation of sesquiterpenes, and its response to potassium is similar to other terpene synthases from unrelated gymnosperms (Degenhardt and others 2009). Production of (−)-α-bisabolol, a sesquiterpene, is linked to prenyl transferases which increase the amount of available precursors that ultimately lead to greater α-farnesene synthase activity, and subsequent synthesis of the sesquiterpene intermediate, farnesene, is of vital importance in bisabolol production. It is thus possible that potassium functions in a similar fashion in *S. stenophylla*, that is, increasing farnesene production.

Some compounds occur as trace elements and are thus not easily detectable via HS-SPME-GCMS. Nonetheless, adjustment to the GCMS method helps with the detection of these minor chemical constituents. In this study, those compounds that generally accumulated at a proportion of 2% or less were regarded as trace chemicals and modifications to them were also obvious (Tables 2, 3) with changing available nutrients. Some compounds (bisabolol B oxide and terpinolene) were not always quantifiable in nitrogen-treated samples (Table 3), just as trachylobane was not readily detected in potassium treatments (Table 2), accumulating at undetectable levels which suggests production at extremely low to negligible concentrations.

Table 4 Effect of sorbitol and PEG as osmotica on in vitro growth and development of *S. stenophylla* plantlets

Treatment	No. of new leaves[a]	Leaf weight (g)	Callus weight (g)	Total biomass	Root formation
Sorbitol	4.70 ± 0.54 a	0.08 a	0.18 ± 0.03 a	0.25 a	0.00 ± 0.00 a
PEG	6.00 ± 0.71 a	0.07 a	0.18 ± 0.03 a	0.24 a	0.00 ± 0.00 a
Control	4.22 ± 1.22 a	0.05 a	0.34 ± 0.10 a	0.39 a	0.00 ± 0.00 a

Means within columns noted with different letters are significantly different according to Kruskal–Wallis ANOVA at $P \leq 0.05$

[a] The amount of new leaves was calculated as number of leaves on day 30—number of leaves on day 0 associated

Effect of Osmotica on Plantlet Growth

Inclusion of osmotic agents in the growth medium adversely influenced morphogenetic responses, and plantlets treated with 5% (w/v) PEG were unable to maintain normal in vitro organogenesis (leaf mass = 0.07 g) during the culture period. Instead, the frequency of callus accumulation became higher (18%) (Table 4). Intense callugenesis (Fig. 1i), which is undesirable as it increases the incidence of somaclonal variation, lowers the capacity for in vitro microplants to produce volatile compounds. Dedifferentiation negatively affects the development of glandular trichomes which are crucial as essential oil storage organs (Serrato-Valenti and others 1997; Rout and others 2000; Arikat and others 2004; Kamatou and others 2006). Plantlets subjected to the dehydrating treatments became chlorotic compared to controls. Generally, dehydration influences osmotic potential, lowering the capacity for water uptake and absorption of macro- and micronutrients from the surroundings. In turn, one of the effects of water deprivation is reduced plantlet growth and photosynthetic ability (Pasternak 1987). Apart from this, the probability for ion toxicity in plants becomes elevated (Levitt 1980) and this may lead to many genetic and biochemical changes related to the stress responses (Cassells and Curry 2001). The negative impacts of water stress are known to be complicated and plants in tissue culture respond to stress in many ways. Among other effects (such as lowered nutrient translocation across membranes and enzyme malfunction that may lead to poor functioning of biochemical pathways), the incidence of callugenesis and somaclonal variation increases (Cassells and Curry 2001), leading to unpredictable and suboptimal plant growth and vigor.

Effect of Osmotica on Essential Oil Components

Differences in the quantity of headspace volatiles were evident from one culture cycle to another. For instance, control plants used in the nutrient experiment had more (−)-α-bisabolol (48.08–51.87%) compared with those assayed to study the effects of water stress (19.95%). Age differences might explain this increase in (−)-α-bisabolol content, which was more than twofold. Even so, within a population of plants utilized for experimentation, little to no intraspecific clonal differences were detectable with our analytical method. (−)-α-Bisabolol was used as an internal marker (standard) and the injection ratio was adjusted accordingly for each experiment. Emission of (−)-α-bisabolol (27.5%), as a headspace volatile in PEG-supplemented medium, was more pronounced (Table 5) in comparison to controls and sorbitol-exposed microplants. Several publications provide supporting data. Positive upregulation of essential oil components produced by *S. officinalis* due to water deprivation (Bettaieb and others 2009) and salt stress (Taarit and others 2010) has been shown. In the latter situation, where NaCl was used to dehydrate plants, quantitative chemical differences were evident (Cassells and Curry 2001). Surprisingly, sorbitol treatments positively influenced production of α-guaiene, α-copaene, α-caryophyllene, muurolene, δ-guaiene, and guaiol, eluting between 28.6 and 30.1 min; these were either in trace quantities or undetectable in control and wild aerial parts (Table 6). (−)-α-Bisabolol and azulene,1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, 1S-(1à,4à,7à) (also known as α-guaiene) are commercially important in the cosmeceutical industry due to their anti-inflammatory and anti-irritant properties. When plants of *S. stenophylla* were subjected to water stress, α-guaiene became favored over (−)-α-bisabolol as it accumulated at higher levels than previously detected (Table 5). Although not statistically significant, water stress was detrimental to (−)-α-bisabolol accumulation. Both (−)-α-bisabolol and α-guaiene are sesquiterpenes which are synthesized via terpenoid metabolism (Ganzera and others 2006). Similar to (−)-α-bisabolol, which is used in the production of numerous products such as eye drops, body creams, sunscreens, and after-sun creams (Brown and Dattner 1998), guaiene derivatives are also useful as additives to skincare products. Furthermore, α-guaiene and related compounds are potent antiseptics.

Effect of Plant Growth Regulator Combinations

The ratio of PGRs is important as small changes in the concentrations of phytohormones have the ability to greatly

Table 5 Effect of phytohormone combinations (auxins and cytokinins) on major essential oil compounds of in vitro *Salvia stenophylla* plants analyzed using HS-SPME-GCMS

Major compounds	Retention time (min)	Kovats index	Tissue culture	Wild	BA:NAA 2.7:4.4 (μM)	BA:NAA 2.9:4.4 (μM)	IAA:BA 2.9:8.9 (μM)	IAA:BA 1.1:8.9 (μM)	IAA:BA 2.9:4.4 (μM)
α-Pinene	9.46	937	1.07	1.48	1.42	1.23	–	–	0.97
Camphene	9.97	947	–	–	1.12	–	–	–	–
β-Pinene	11.80	965	3.71	8.04	4.73	4.57	3.36	3.59	4.08
Myrcene	12.14	958	1.50	3.68	1.43	2.69	1.46	1.74	1.99
δ-3-Carene	12.38	1009	8.33	14.04	7.16	10.62	7.24	8.40	9.35
Sylvestrene	12.89	987	3.46	5.59	3.77	4.64	3.19	3.68	3.82
Limonene	13.03	1018	4.46	6.54	4.21	5.33	4.07	4.60	5.05
γ-Terpinene	13.91	998	–	1.06	–	–	–	–	–
Isoterpinolene	14.89	1023	1.91	2.74	1.74	2.51	2.00	2.24	2.45
Terpinolene	14.95	1052	1.03	1.13	1.02	1.27	1.19	4.61	1.44
α-Terpinene	15.88	998	–	–	–	–	–	1.00	–
Borneol	17.40	1156	–	–	1.75	1.17	–	1.39	1.16
α-Terpineol	18.16	1143	–	2.06	–	–	1.18	1.09	–
β-Caryophyllene	24.41	1415	9.31	–	6.50	3.60	5.84	4.60	2.14
trans β-Bergamotene	24.82	1674	3.37	3.51	3.96	3.29	1.60	3.43	3.91
Aromadendrene	24.89	1386	–	–	–	–	2.55	–	–
α-Caryophyllene	25.25	1579	–	–	2.02	1.01	–	1.58	–
Z-β-farnesene	24.34	1438	2.09	2.50	2.77	2.47	1.06	3.53	2.97
Varidiflorene	26.30	1419	–	–	–	–	1.36	–	–
β-Bisabolene	26.63	1483	4.66	2.75	4.19	3.34	4.10	6.79	2.86
β-Sesquiphellandrene	26.96	1446	–	–	1.30	–	–	1.22	1.12
α-Guaiene	28.54	1490	–	–	–	–	9.37	–	–
δ-Guaiene	29.54	1490	–	–	–	–	4.26	–	–
Guaiol	29.64	1614	–	–	1.09	–	1.92	–	–
(−)-α-Bisabolol	30.10	1625	18.46	21.07	19.95	20.48	11.65	19.13	21.60
cis-Lanceol	31.15	1737	5.12	2.42	4.00	4.64	3.69	5.50	4.56
Epi-manool	33.19	2085	1.17		–	1.34	–	1.08	1.65
Trachylobane	34.06	1698	–	–	–	–	–	–	1.07
Manool	34.52	2016	5.19	2.87	4.49	5.45	3.89	4.85	6.33

affect in vitro plantlet growth and development by affecting the multiplication, elongation, and rooting of plantlets (Emongor and Chweya 1992; Van den Heever and others 2008). Extensive discussion on the effect of PGRs on in vitro performance of *S. stenophylla* cultures has been conducted elsewhere (Musarurwa and others 2010). We focused on analyzing the metabolomic profiles of propagated plants and their responses to different PGR combinations. Plants grown on the 2.9:4.4 BA:IAA medium had harder and more serrated leaves, which were often chlorotic, hardly elongating (data not shown). Plants grown on the 2.9:4.4 IAA:BA medium produced (−)-α-bisabolol at similar levels to wild plants. Although explant elongation was minimal, with this PGR combination callus production could be circumvented. However, callus regeneration was more abundant on the 2.7:4.4 NAA:BA medium. Plants also failed to root. α-Guaiene (and other guaiene derivatives) plus α-caryophyllene became more prominent in plants maintained on IAA:BA (2.9:8.9; μM) (Table 5). Otherwise, chemical profiles were generally similar in propagated tissues irrespective of PGR treatment. Interestingly, *S. stenophylla* plants maintained their ability to flower in vitro, especially with a higher potassium supply. Flowers in vitro appeared to have fully formed floral parts that were morphologically similar to those found in nature (Fig. 1i). In vitro flowering occurred at the same time as ex vitro flowering in the greenhouse. Naturally, these plants flower from spring until the end of Southern Hemisphere summer (Jequier and others 1980). Micropropagated plants (Fig. 1j) were similar in their morphology to traditionally propagated *S. stenophylla* plants and there were no distinguishing metabolomic features derived from tissue

Table 6 Effect of PEG (5%, w/v) and sorbitol (2%, w/v) on major essential oil compounds of in vitro *Salvia stenophylla* plants analyzed using HS-SPME-GCMS

Major compounds	Retention time[a] (min)	Kovats index	Tissue culture	PEG 5% (w/v)	Sorbitol 2% (v/v)
α-Pinene	9.46	937	1.07	–	–
β-Pinene	1180	965	3.71	4.43	3.43
Myrcene	12.14	958	1.50	–	–
δ-3-Carene	12.38	1009	8.33	9.03	6.79
Sylvestrene	12.89	987	3.46	2.51	1.59
Limonene	13.03	1018	4.46	4.40	3.38
Isoterpinolene	14.89	1023	1.91	–	–
Terpinolene	14.95	1052	1.03	–	–
Camphor	16.65	1144	–	1.04	–
β-Caryophyllene	24.41	1415	9.31	7.10	7.33
trans-β-Bergamotene	24.82	1674	3.37	2.81	1.31
α-Caryophyllene	25.25	1579	–	1.77	2.04
Z-β-farnesene	25.34	1438	2.09	1.74	1.08
β-Bisabolene	26.63	1483	4.66	4.15	3.34
cis-α-Bisabolene	28.54	1518	1.04	–	–
α-Guaiene	29.30	1490	–	1.37	11.61
α-Copaene	28.80	1221	–	–	1.13
δ-Guaiene	29.54	1490	–	–	6.11
Guaiol	29.64	1614	–	–	1.57
(−)-α-Bisabolol	30.10	1625	18.46	27.50	15.59
cis-Lanceol	31.15	1737	5.12	3.55	3.95
Epi-manool	33.19	2085	1.17	–	–
Trachylobane	34.06	1698	–	1.02	–
Anthracene	34.23	1782	–	1.04	–
Manool	34.52	2016	5.19	4.63	4.60

culturing (Fig. 2), demonstrating that this propagation method can easily be adapted for massive agricultural multiplication of this commercially important medicinal crop. However, all tissue-cultured and glasshouse-acclimatized plants showed a slight difference in secondary compounds from the wild-growing plants, possibly due to different growth environments (Table 5).

In conclusion, micropropagation of *S. stenophylla* is not only an efficient system of producing plant material for agricultural cultivation and industrial essential oil processing, ensuring the production of quality essential oils, it is also a unique platform to optimize growing conditions and for studying metabolism within a highly controlled microenvironment. Such practices may easily be adopted for field production of *S. stenophylla* plants. Further greenhouse and field trials are required to maximize ex vitro performance of these plants.

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