Chemical variations, trichome structure and antifungal activities of essential oils of Helichrysum splendidum from South Africa

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a b s t r a c t

The chemical profiles of essential oils isolated from nine populations of Helichrysum splendidum were obtained by gas chromatography. Plants were harvested in both summer and winter seasons in two provinces of South Africa. Essential oils originating from Limpopo Province were characterized by high levels of β-phellandrene, 1,8-cineole, δ-cadinene, α-cadinol, τ-cadinol, and β-pinene. These could be clearly distinguished through multivariate analysis from those collected in Mpumalanga Province. The Mpumalanga oils contained germacrene D, spathulenol and bicyclogermacrene as major constituents. Seasonal variations in the volatile compositions and yields were observed. A prediction model obtained through orthogonal projection to latent structures discriminant analysis was used to compare the essential oil profiles to that of a commercial H. splendidum sample, in addition to those reported for Zimbabwean specimens. One of the specimens from Zimbabwe differed substantially from all the others, indicating the existence of more chemotypes. The structures of the oil-bearing trichomes, as observed on the leaf surfaces using scanning electron microscopy, were investigated and are described for the first time. Since this investigation forms part of an ongoing screening of plants to identify natural alternatives to synthetic fungicides, the essential oils were tested in vitro against several economically important pathogens of fruit. Essential oils from Mpumalanga specimens totally inhibited the growth of Alternaria alternata from avocado and Colletotrichum gloeosporioides isolated from mango at a concentration of 500 µL/L in a toxic medium assay. The presence of high concentrations of germacrene D, spathulenol and bicyclogermacrene probably contributed to the antifungal properties of these essential oils.

1. Introduction

The genus Helichrysum (Asteraceae) comprises about 600 species worldwide, with almost 250 species distributed throughout South Africa (Hillard, 1983). Helichrysum splendidum (Thunb.) Less., a perennial shrub, occurs throughout South Africa, as well as in Swaziland, Lesotho and Zimbabwe (Chagonda et al., 1999; Germishuizen et al., 2006). The plant is used as a fuel source and for the treatment of various conditions associated with rheumatism (Pooley, 2003). In Zimbabwe, the Shona people use the plant to combat colds, flu and pneumonia, and as a general antiseptic (Chagonda et al., 1999). Volatile compounds released from boiling the leaves are inhaled to promote sweating (Tramed Database, Index card 547 as cited by Swanepoel, 1997). The plant has a pleasant fragrance, explaining its popularity as a perfume amongst rural communities (Dlamini, 1981).

Since the chemical composition of an essential oil (EO) is inextricably linked to its pharmacological properties, it is vital that the composition is known prior to the development of antimicrobial agents. Two studies (Chagonda et al., 1999; Marongiu et al., 2006) have reported on the constituents of EOs from H. splendidum growing in Zimbabwe. Chagonda et al. (1999) identified α-pinene, β-pinene, α-phellandrene, α-terpinene, 1,8-cineole, bicyclogermacrene, δ-cadinene and cubebol as major constituents of the hydrodistillate. More recently, Marongiu et al. (2006) reported high concentrations of δ-cardinene and α-cadinol in the EO, together with β-phellandrene, α-cadinol, germacrene D, δ-cardinene, bicyclogermacrene, γ-cadinene and α-muurolol. These reports confirm the existence of different profiles for the same Helichrysum species from different localities. Variation in EO composition has been reported for a related species, Helichrysum cymosum (Bougatsos et al., 2004; Van Vuuren et al., 2006), but no comparative studies on the chemotypic variations of EOs from H. splendidum have been described. In this study, EOs were obtained in summer and winter from nine populations growing in two provinces of South Africa and analysed by gas chromatography (GC)-mass spectrometry (MS) and GC-flame ionization detection (FID). Chemometric models were constructed from the data to establish trends and clustering patterns within the dataset.

Volatile organic compounds are produced and stored in glandular trichomes, which can be used as reliable taxonomic characters for the

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identification of species and for resolving taxonomic conflicts (Hayat et al., 2009). Histochemical studies of surface appendages shed light on the location and type of secondary metabolites produced by the plant (Afolayan and Meyer, 1995). Although the glandular trichomes of a few Helichrysum species have been described (Afolayan and Meyer, 1995; Ascensão et al., 2001; Mathekga, 2001), such reports are sparse, considering the size of the genus Helichrysum worldwide. To contribute to the available knowledge, a preliminary investigation of the glandular structures of H. splendidum was conducted using scanning electron microscopy.

Effective natural mycobiocides are needed to protect the supply chains of fresh products against fungal decay, while minimizing environmental problems by eliminating synthetic fungicides (Du Plooy et al., 2009). High costs associated with the removal of hazardous chemical waste in packhouses may result in the irresponsible disposal of fungicide residues, with detrimental effects on the environment. The fate of crop protection products in soils is associated with a series of intricate processes, which sometimes result in the migration of residues into ground water (Taube et al., 2002). Our research team has been involved in the identification of plant extracts and particularly EOs as green alternatives to synthetic crop protection products. Towards this aim, the EOs isolated in this study were evaluated for their abilities to inhibit the growth of important fungal decay pathogens of fruit.

2. Materials and methods

2.1. Collection of plant material and isolation of essential oils

Aerial parts, including flowers, of H. splendidum (Genspec nos: 3240-397A to I) were collected in the Limpopo and Mpumalanga provinces of South Africa in summer (November 2010). Five specimens were harvested from each of nine populations (Table 1). Herbarium specimens were prepared and identified by staff of the South African National Biodiversity Institute, Pretoria. Voucher specimens were deposited at the Department of Chemistry, Tshwane University of Technology. The same sites were resampled in winter (May 2011).

Specimens from summer and winter seasons were air dried for three days at 23 °C ± 1 °C. Thereafter, the plant material (150–200 g) from each specimen, together with 800 to 1000 mL water, was placed in a Clevenger-type apparatus fitted with a 5 L roundball flask. The EO was isolated after hydrodistillation for 2 h, dried over anhydrous sodium sulfate (Merck, AR grade, Darmstadt, Germany) and stored in a refrigerator in a sealed amber vial at 4 °C prior to analysis. A commercial sample of H. splendidum EO of unknown origin, purchased from Mnandi (www.mnandioil.co.za), was analysed together with the isolated EOs.

2.2. Gas chromatography analyses of essential oils

Samples were diluted with dichloromethane and analysed using an Agilent 6890N gas chromatograph, coupled simultaneously to a flame ionization detector (FID) and a mass spectrometer (Model 5973). Using an autosampler, a 1.0 μL sample volume was introduced into the inlet, maintained at 250 °C, and split according to a 1:200 ratio. Separation was achieved on an HP-Innowax polyethylene glycol column (60 m × 250 μm i.d.; 0.25 μm film thickness). The oven temperature was held at 60 °C for the initial 10 min, then increased to 220 °C at a rate of 4 °C/min and held for 10 min, before increasing to 240 °C at a rate of 4 °C/min. Helium was used as carrier gas at a constant flow rate of 1.2 mL/min. The FID was maintained at 250 °C. Mass spectra were obtained, after ionization in electron impact mode at 70 eV, over the scanning range m/z 35 to 550. A transfer line temperature of 280 °C was maintained. The relative percentage peak areas of the individual components were obtained from the FID data, while the MS data were used for identification. Compound identities were verified by comparing their retention indices and mass spectra with those of authentic standards and compounds contained in the NIST®, Mass Finder® and Flavor® spectral libraries.

2.3. Chemometric analysis of data

Data obtained from GC analyses were exported to SIMCA-P 13.0 chemometrics software (Umetrics AB, Malmo, Sweden). Pareto scaling was applied, but no preprocessing of the data was done. Principal component analysis (PCA) models were constructed from individual and combined datasets and the resulting scores plots were visually inspected to determine the presence of possible outliers. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) were applied to distinguish the populations from the two provinces, as well as the EOs obtained from specimens harvested in different seasons. Loading plots generated from the model statistics indicated constituents contributing significantly to the differences observed between the EOs obtained from the various populations. Results from the analysis of the commercial oil and results published by Chagonda et al. (1999) and Marongiu et al. (2006) were incorporated into the data as a prediction set.

2.4. Scanning electron microscopy

A mixture consisting of equal parts glutaraldehyde (2.5%) and formaldehyde (2.5%), prepared in 0.15 M phosphate buffer (pH 7.4), was used for fixation of sections of fresh mature leaves. The material was subsequently washed in the phosphate buffer and post-fixed in 0.5% OsO_4. An ethanol dilution series (30, 50, 70, 90%, followed by 3 × 100%) was used for dehydration of the sections. After critical point drying (Biorad E3000, Polaron, West Sussex, United Kingdom), the samples were mounted on stubs, using double-sided carbon tape, and plasma-coated with gold (10 μm). The prepared sections were viewed using a JSM-601OLA InTouchScope (JEOL, Tokyo, Japan).

2.5. In vitro antifungal activities of essential oils

Essential oils, obtained from specimens harvested in November 2012 from Limpopo (Sites 1 to 4 pooled) and from Mpumalanga (Sites 5 to 8 pooled) were screened in a toxic medium assay at 1000 μL/L to evaluate their antifungal activities. Seven fruit decay pathogens, Alternaria alternata, Colletotrichum gloeosporioides, Fusarium oxysporum, Lasiodiplodia theobromae, Penicillium digitatum, Penicillium expansum and Penicillium italicum were purchased from the culture collection of the Plant Protection Research Institute (Roodeplaat, Pretoria, South Africa) of the Agricultural Research Council. The strains were preserved on malt extract agar (Oxoid, Johannesburg, RSA) at 24 °C. Spore suspensions of the Penicillium cultures were prepared by adding sterile ¼-strength Ringer's solution to fully colonized petri dishes. The concentration of the spore suspensions was then standardized to 10^5 spores/mL, using a haemocytometer.

Each of the two pooled EO samples was premixed with 200 μL of the emulsifier Triton X-100 (Ajax Laboratory Chemicals, Philadelphia, USA).

Table 1
Elevation and GPS coordinates for sites where Helichrysum splendidum specimens were harvested. Sites 1 to 4 (Limpopo Province); Sites 5 to 9 (Mpumalanga Province). Genspec nos: 3240-397 A to I were assigned by SANBI to each of the specimens.

Population	Location	Latitude, S	Longitude, E	Elevation, m ASL
Site 1	Haenertsberg	23°53′50.6	29°54′50.8	1449–1514
Site 2	Haenertsberg	23°56′33.9	29°53′23	1506–1861
Site 3	Haenertsberg	23°56′54.8	29°56′25.8	1421–1471
Site 4	Magoebaskloof	23°52′36.3	29°59′42.9	796–1424
Site 5	Ohrigstad	24°53′55.1	30°40′0.95	1467–1619
Site 6	Pilgrims Rest	24°52′32.0	30°42′10.2	1741–1758
Site 7	Pilgrims Rest	24°55′38.7	30°48′10.7	1653–1662
Site 8	Dullstroom	25°20′59.1	30°10′24.4	1954–1974
Site 9	Lydenburg	25°100′20	30°40′285	1782–1858

Sterilized potato dextrose agar (PDA) was then supplemented with either of the EOs (Limpopo or Mpumalanga) to yield a final concentration of 1000 μL/L. Controls containing agar medium and emulsifier only were prepared for each pathogen. Two fungicides, Kenopel® 200SL (1000 μL/L; Makhteshim-Agan, SA (Pty) Ltd., Brackenfell, South Africa) and ICA Thiabendazole® 500SC (1000 μL/L; ICA International Chemicals (Pty) Ltd., Stellenbosch, South Africa), were obtained from Citrus Research International, Nelspruit and prepared in PDA for comparison. The concentrations were selected based on the supplier recommendations. After solidification of the agar in 90 mm Petri plates (N = 10), the medium was either inoculated with a 5 mm agar plug of mycelia from a 7 day-old culture of the pathogens (A. alternata, C. gloeosporioides, F. oxysporum, L. theobromae) or with a 5 μL aliquot of the spore suspension of the Penicillium strains. The plates were incubated for 3–12 days at 25 °C, depending on the mycelial growth rate of each pathogen. The EOs were later re-tested in the same way, at a concentration of 500 μL/L, against the two most susceptible pathogens. Mycelial growth was measured (in mm) with a digital calliper (Absolute Digimatic-Mitutoyo Corp., Japan) and the percentage inhibition calculated using the control as reference, as described by Plaza et al. (2004).

3. Results and discussions

3.1. Essential oil analysis

The yields of EOs from H. splendidum varied, depending on the season of harvest. Substantially higher yields were obtained in summer (0.45–0.75%) than in winter (0.31–0.44%). Both season-dependent variation in EO yields (Kamatou et al., 2008) and variation as a result of geographical origin (Martinez-Nataren et al., 2012) have been reported for many aromatic species. However, findings from this study did not reveal variations in the oil yield that could be attributed to their origin.

Thirty compounds, representing between 84.53 and 95.86% of the total oil composition, were identified in the oil samples. Although the EOs from both provinces for the same season contained several corresponding compounds, marked differences in the relative amounts of the constituents present were evident. α-Muurolol (3.43−7.09%), germacrene-D (3.41−10.1%), δ-cadinene (9.32−16.9%), α-cadinene (2.27−7.58%), τ-cadinol (3.61−9.01%), and α-cadinol (5.84−16.8%) were present as major constituents of oils from Limpopo, while spathulenol (1.61−6.05%) and bicyclogermacrene (0.822−6.31%) were generally present in lower concentrations. In contrast, the Mpumalanga EOs were characterized by significantly higher levels of spathulenol (12.2−37.0%) and bicyclogermacrene (7.41−20.5%), with α-muurolol (0.349−2.85%), δ-cadinene (1.41−6.85%), τ-cadinol (0.427−3.44%), and α-cadinol (0.477−6.45%) at lower concentrations. Although germacrene-D was present as a major constituent of all of the isolated oils, higher amounts were present in the Mpumalanga oils (11.1−26.5%) than in the Limpopo oils (3.41−10.1%). Germacrene-D-4-ol occurred in all of the summer samples at levels above 1%, but was absent in all the oils from specimens harvested in winter. Higher concentrations of δ-cadinene were found in Limpopo oils (9.32−16.9%) than in Mpumalanga oils (1.41−6.85%), irrespective of the season. These results confirm that plants produce compounds in varying amounts, possibly as a result of the climatic differences of the growing sites (Medina-Holguı́n et al., 2007).

3.2. Chemometric analyses

The complex nature of EO profiles makes it impossible to establish relationships between large numbers of samples by direct observation. However, multivariate analysis allows a quantitative measurement of similarities and differences between samples through mathematical criteria, providing a visual overview of the spatial distribution of observations (Eriksson et al., 2006; Wiklund et al., 2008). An initial PCA model

was constructed from the GC profiles of specimens representing nine populations, harvested in both winter and summer (N = 84). The scores plot indicated that the specimens collected in winter from Site 9 were chemically distinct and therefore revealed by the model as outliers, due to their positions outside the Hotelling's T^2 ellipse. Removal of these five outliers from the dataset improved the separation obtained (Wold et al., 1987). The subsequent PCA model ($R^2 = 0.59$; $Q^2 = 0.42$; N = 79), indicated separation between samples originating from Limpopo (Sites 1 to 4) and those from Mpumalanga (Sites 5 to 9) from both seasons along the X-axis by the first model component (Fig. 1A). This separation suggests differences in the chemical profiles of EOs from different geographical sites. In addition, EOs from specimens collected in each province were separated by the second principal component along the Y-axis, indicating smaller differences in the chemical compositions according to season. Geographical and climatic conditions may have a pronounced influence on the production of secondary metabolites by plants (Namdeo et al., 2010; Faravani et al., 2011; Zouari et al., 2012).

Specimens harvested in summer and winter were subsequently assigned a class identifier of 1 and 2, respectively. Analysis by OPLS-DA ($R^2 = 0.87$; $Q^2 = 0.82$) indicates good separation by the first component along the X-axis between specimens, according to the season of harvest (Fig. 1B). The reliability of the model is reflected by R^2 and Q^2 values of above 0.5 and differing by less than 0.2 (Eriksson et al., 2006).

A PCA model (Fig. 2A) constructed from the GC profiles of specimens collected only in summer (N = 41), indicates clear separation of specimens according to the province where they were harvested. All of the specimens from Limpopo are clustered on the left side of the y-axis, while those from Mpumalanga are grouped on the right. Plants from Mpumalanga occurred at higher elevation (Table 1) than Limpopo specimens, possibly accounting partially for the differences in their chemical compositions. Inter-population variations can also be observed within EOs originating from Mpumalanga. Those from Sites 5 and 9 are separated by the second PC from EOs originating from Sites 6, 7 and 8 (all three sites in the vicinity of Pilgrim's Rest). This model is appropriate for discriminate analysis as reflected by the R^2 and Q^2 values of 0.64 and 0.47, respectively. Since PCA is an unsupervised cluster analysis method that does not rely on class information for discrimination of samples (Wold et al., 1987), the introduction of bias in the distinction between the GC profiles of specimens originating from the two provinces can be discounted.

For OPLS-DA analysis, populations were assigned a class identifier of 1 if they were harvested in Limpopo Province and an identifier of 2 if they originated from Mpumulanga. The scores plot (Fig. 2B) generated from the OPLS-DA model, constructed from the EO profiles of summer samples, indicates discrimination based on the province of origin. This model explains 98.3% of the data ($R^2 = 0.983$) and is characterized by an excellent predictive ability ($Q^2 = 0.945$). A seven-fold cross-validation analysis was applied to validate the model (Sandasi et al., 2011), wherein the Q^2 value reflects the average prediction results of the seven rounds. In addition, an external validation of the model was done by excluding nine random data points from both provinces (four from Limpopo and five from Mpumalanga) to form the prediction set. The remaining workset (N = 32) was used to construct a prediction model for the two provinces. All of the samples in the complementary set were correctly predicted according to the province of origin, as reflected by the miscalculation table. The small value obtained for Fisher's exact probability (4.1×10^{-12}) reflects a very slight possibility of obtaining such a classification by chance. Thereby the predictive ability of the OPLS-DA model to determine the origin of an unknown sample of H. splendidum oil as from Limpopo or Mpumulanga was established. Such OPLS-DA models offer better discrimination between classes than a PCA model and can be used to predict the class to which an unknown sample belongs (Wold et al., 1987). This is a supervised discriminant analysis method, which could introduce bias. However, the clear distinction obtained between groups on the PCA plot negates any form of bias in the OPLS-DA model.
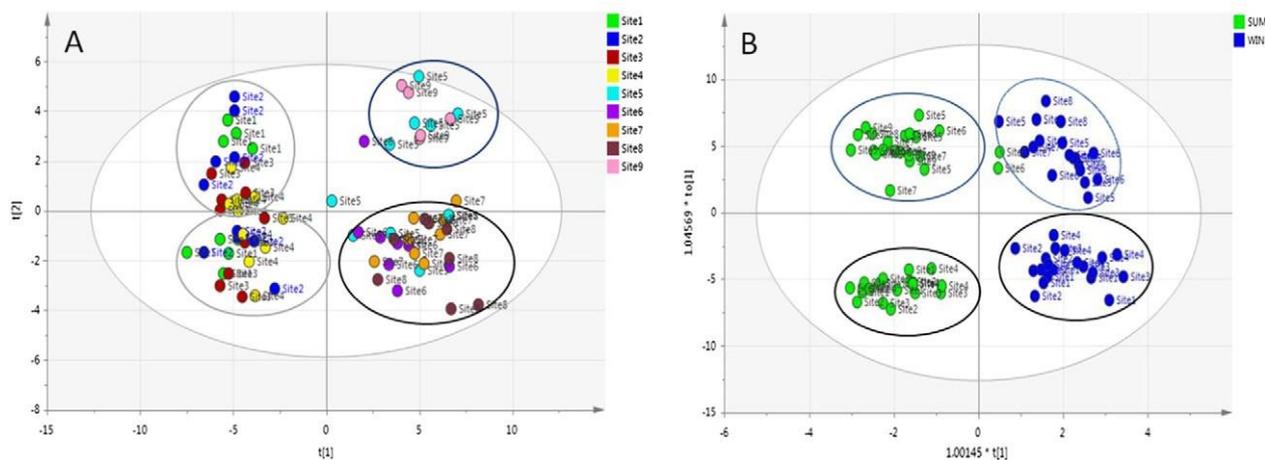
Fig. 1. Plots generated from chemometric analysis of the gas chromatography profiles of essential oils of Helichrysum splendidum harvested in both summer and winter (N = 79). A) PCA scores plot with circles on the left representing specimens from Limpopo Province (Sites 1 to 4) and those on the right indicating specimens from Mpumalanga Province (Sites 5 to 9). Circles above and below the parallel line distinguish oil profiles of summer (above) and winter (below) specimens. B) OPLS-DA scores plot indicating differences between oil profiles of specimens harvested in summer and winter from both provinces. Circles above the parallel line represent specimens from Sites 1 to 4 (Limpopo Province), while those below the line indicate specimens from Sites 5 to 9 (Mpumalanga Province).
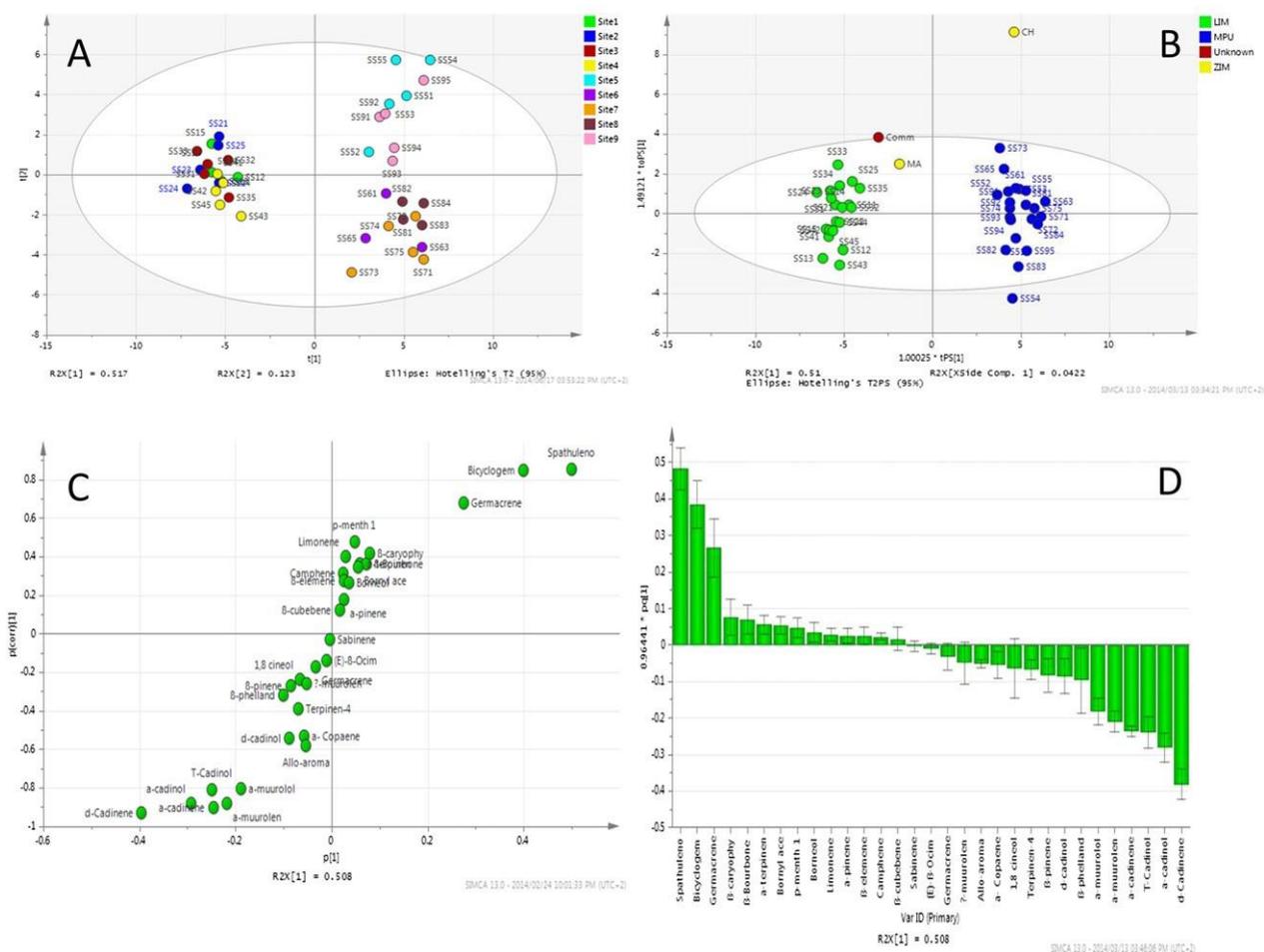

Fig. 2. Plots generated from chemometric analysis of gas chromatography profiles of essential oils of Helichrysum splendidum harvested in summer (N =41). A) PCA scores plot with specimens on the left originating from Limpopo Province (Sites 1 to 4) and those on the right from Mpumalanga Province (Sites 5 to 9). Some interpopulation differences are also evident. B) OPLS-DA scores plot indicating differences between oil profiles of specimens harvested from Limpopo Province (left) and those from Mpumalanga Province (right). Specimens labelled MA and CH are predicted by the model from the data reported by Marongiu et al. (2006) and Chagonda et al. (1999), respectively. The specimen labelled Comm is predicted from the data obtained following GC-FID analysis of a commercial essential oil sample. C) S-plot generated from the OPLS-DA model indicating marker compounds associated with the essential oils originating from Limpopo (lower left) and from Mpumalanga (upper right) provinces. D) Loadings plot with jack-knives indicating the degree of certainty associated with the role of specific compounds in distinguishing between the oils from Limpopo (below the line) and Mpumalanga (above the line) provinces.

To identify compounds associated with the differences observed in the EOs from the two provinces, an S-plot (Fig. 2C) and a loadings plot (Fig. 2D) were generated from the OPLS-DA model. The S-plot indicates that high levels of spathulenol, bicyclogermacrene and germacrene-D, at the extreme upper right (Wishart, 2008), characterize the Mpumalanga samples, while high levels of d-cadinene, α-cadinol. T-cadinol and α-cadinene, at the extreme left, are linked to the Limpopo samples. The loadings plot (Fig. 2D) is helpful in assessing the reliability of the role of these specific compounds. In this instance, the mentioned compounds can be considered reliable markers of sample origin as reflected by small jack-knives that do not cross the centre line (Eriksson et al., 2006). Chemometric analysis of samples collected in winter displayed similar trends to the summer specimens with separation based primarily on geographical differences, rather than seasonal differences (plots not provided). Model validation was carried out as described for the summer models.

The GC-FID data obtained for the commercial sample and those reported for EOs of H. splendidum from Zimbabwe (Chagonda et al., 1999; Marongiu et al., 2006) were added to the SIMCA worksheet. Subsequently, the origins of these three samples were predicted by the validated OPLS-DA model for summer data to establish similarities between the samples. Both the commercial sample of unknown origin and one of the Zimbabwe samples (according to the published data of Marongiu et al., 2006) were predicted as originating from Limpopo, since they were plotted within the 95% Hotelling's T^2 ellipse (Fig. 2B). The close proximity of Zimbabwe to Limpopo makes this prediction highly probable. Marongiu et al. (2006) also reported the presence of δ-cadinene (13.85%), α-cadinol (13.72%) and germacrene D (7.15%) as major constituents of the oil, but the levels of α-muurolol (7.54%) were higher than those found in this study. In contrast, it was found that the levels of β-phellandrene in EOs originating from Limpopo were significantly higher (7.11−18.7%) than those reported by Marongiu et al. (4.08%). Data supplied by Chagonda et al. (1999) indicates that the second EO from Zimbabwe differs substantially from the EOs isolated from our specimens, since it is revealed as a strong outlier on the scores prediction plot. It also differs from the other Zimbabwean sample, emphasizing that other typical chemical compositions probably exist for other localities. The oil sampled by Chagonda et al. (1999) contained 14.9% α-terpinene, which was present in lower levels (0.096−5.32%) in our oils. All oils isolated as part of this study contained spathulenol as one of major constituent, however low levels (0.4%) were reported by Chagonda et al. (1999). They reported the presence of α-phellandrene (5.5%), which was not identified in our samples.

3.3. Scanning electron microscopy

H. splendidum is characterized by lanceolate leaves that have light green adaxial and silver abaxial surfaces. The silver appearance is the result of a thick mat of extraordinarily long, filamentous non-glandular hairs that conceal the entire surface of the epidermis (Fig. 3A). In contrast, these appendages are far less abundant on the adaxial surfaces, allowing an unobscured view of glandular trichomes that are present on both sides of the leaf (Fig. 3B). The dense hirsute abaxial surface will impede sample preparation for light microscopy investigations.

Two to four parallel pairs of cells, culminating in two tapered head cells, comprise the body of the glandular trichomes (Fig. 3C). Fig. 3D illustrates the presence of a ruptured cuticular membrane that enveloped the head cells. A subcuticular space is evident and it can be reasonably assumed that this space was filled with exudates. Furthermore, the aromatic nature of the plant implies the secretion and storage of odiferous secondary metabolites. The base cells of the glandular trichomes appear to consist of a single cell of variable dimensions. These trichomes are similar to the glandular structures of Helichrysum stoechas described by Ascensão et al. (2001). However, the trichomes of Helichrysum aureonitens (Afolayan and Meyer, 1995) are noticeably

different in that this species seems to have a smaller secretory cavity, with the head cells less sheathed than those of H. splendidum.

The randomly arranged striate-textured hairs (Fig. 3E) are slightly twisted. Hairs are clearly segmented, indicating the presence of several transverse cell walls along the length of individual hairs (Fig. 3D). This uniseriate arrangement was also described by Afolayan and Meyer (1995), indicating morphological similarities within the genus. However, a major difference is evident in the basal arrangement of the hair origins. In the case of H. aureonitens, the columnar base appears slightly rounded, while the hairbase in H. splendidum is clearly spindle-shaped (Fig. 3E and F). A bulbous implant is visible on the base of each spindle. We agree with the deductions of Afolayan and Meyer that the hairy mass has evolved to serve as a mechanical barrier against predation.

3.4. Antifungal activities of the essential oils

The EOs from the two provinces were initially screened at a concentration of 1000 μL/L. Combrinck et al. (2011) indicated that many EOs are able to inhibit fungal growth at concentrations at or above 3000 μL/L. The concentration of 1000 μL/L was therefore selected to permit those pathogens most prone to inhibition by the EOs of H. splendidum, to be identified. Essential oils of H. splendidum at 1000 μL/L were ineffective towards most of the pathogens tested (Table 2). A. alternata and C. gloeosporioides were the most susceptible fungi, with both exhibiting total growth inhibition at 1000 μL/L for the Mpumalanga specimens. The EO of H. splendidum from Mpumalanga had the same efficacy at this concentration as the positive control, ICA-Thiabendazole. Displaying 84 and 51% inhibition against A. alternata and C. gloeosporioides, respectively, the Limpopo oil was significantly less active at the same concentration. At a concentration of 500 μL/L, oil from Mpumalanga also totally inhibited the growth of both pathogens (Table 2). However, lower activities were recorded for the oils from Limpopo Province. These two pathogens appear to be highly resistant to EOs in general. The study by Combrinck et al. (2011) revealed that of 18 EOs tested, only Cinnamonum zeylanicum and Thymus vulgaris were able to inhibit the fungal growth of A. alternata and C. gloeosporioides at 1000 μL/L. In addition, only Thymus vulgaris caused total mycelial growth inhibition of the two pathogens at 500 μL/L. Within this context, the oil originating from Mpumalanga can be considered as a good candidate for further investigations towards the development of ecofriendly crop protection products.

The observed differences in activity may be attributed to larger amounts of β-phellandrene, spathulenol, bicyclogermacrene, germacrene D and 1,8-cineole in the Mpumalanga specimens, as these constituents were previously found to be major compounds of oils that possess good antifungal activities (Moreno et al., 2009; Barra et al., 2010). Three of these compounds were identified by the S-plot (Fig. 2C) as markers indicating major differences between Mpumalanga and Limpopo oils. Although plants sourced from Limpopo were less active, they still displayed moderate activities that may be attributed to the presence of several compounds, β-pinene, β-phellandrene, spathulenol, δ-cadinene, α-cadinol, τ-cadinol and τ-muurolol, known to have antifungal properties (Chang et al., 2000, 2008; Chinou et al., 2004; Vukovic et al., 2007; Barra et al., 2010; Ho et al., 2011).

4. Conclusions

Through the use of multivariate analysis techniques, this study has demonstrated that the EOs of H. splendidum vary significantly and are dependent on the origin of the plant material. Inter-population variations and seasonal differences, although less pronounced, are also evident through cluster analysis. An OPLS-DA prediction model can be used to characterise EOs originating from the Limpopo and Mpumalanga provinces. This prediction model could be extended to include specimens from other areas to identify further chemotypes within the genus.
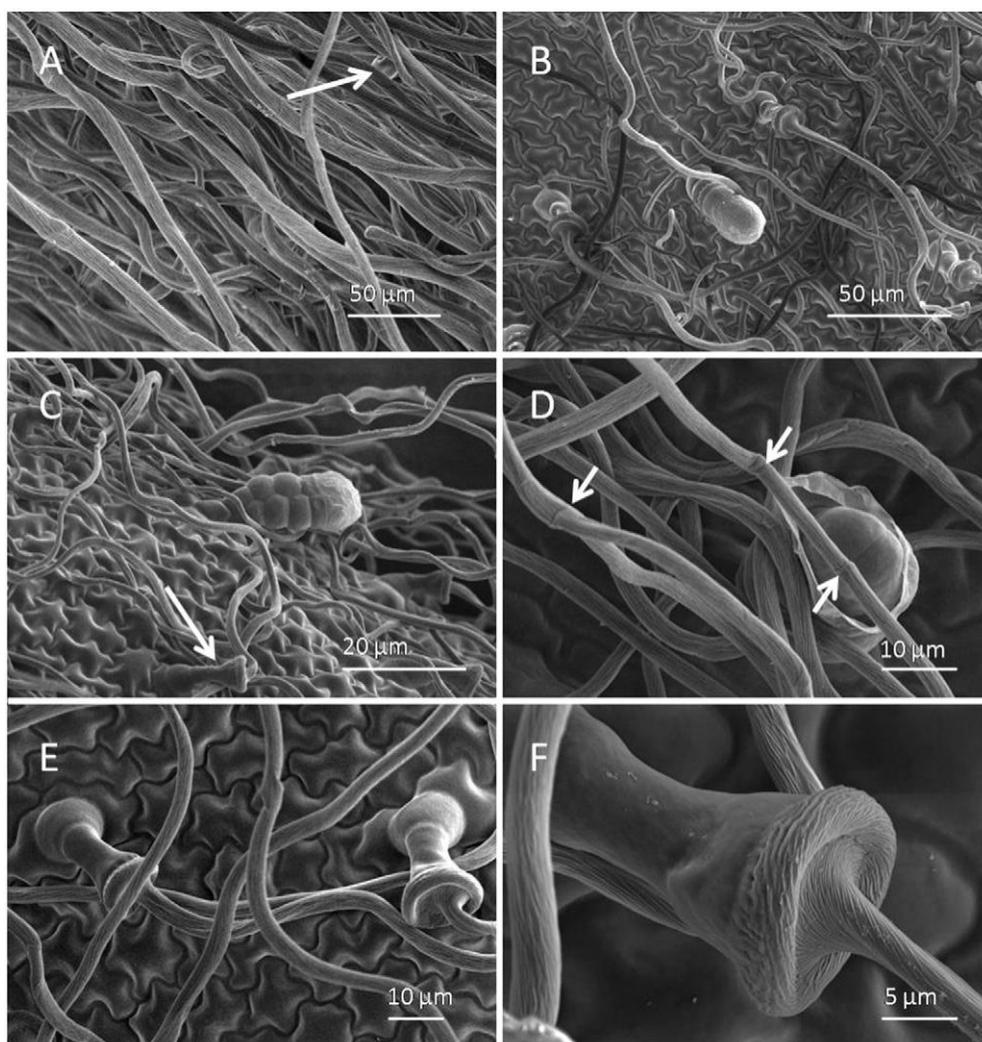
Fig. 3. Scanning electron micrographs of Helichrysum splendidum mature leaf surfaces. A) Abaxial view illustrating the abundant mat of filamentous hair obscuring a secretory gland (arrow); B) On the adaxial surfaces the hair is sparse, allowing an unobstructed view of both the secretory glands and the goblet-shaped hair base; C) Side-on view (adaxial surface) displaying glandular structure. Also visible is a hair base (arrow); D) Top-down view of a ruptured membranous envelope that served to cover the two head cells of a secretory gland. Arrows indicate the transverse cell walls of the filamentous hair; E) Detailed view of two individual hair bases; F) Base cell with striations of the cuticular wax clearly visible.

The morphology of the trichomes, which are storage sites for EOs, is described for the first time. These appendages display many similarities with the glandular structures of H. stoechas. Essential oil from Mpumalanga Province displayed excellent in vitro antifungal

Table 2
Percentage growth inhibition indicating the antifungal activities of Helichrysum splendidum essential oils from Mpumalanga and Limpopo provinces against a series of postharvest fungal pathogens (N = 10).

Pathogen	PPRI no:	Source	Province	% growth inhibition		Positive control (1000 µL/L)	
				1000 µL/L	500 µL/L	Imazacure®	Kenopel®
Alternaria alternata	5110	Avocado	Mpumalanga Limpopo	100	100	100	N/A
				84 ± 1.75	81 ± 2.08		
Collectotrichum gloeosporioides	6853	Mango	Mpumalanga Limpopo	100	100	100	N/A
				51 ± 4.78	47 ± 6.78		
Fusarium oxysporum	10189	Maize	Mpumalanga Limpopo	58 ± 3.65	53 ± 3.32	N/A	100
				37 ± 4.73	30 ± 1.87		
Lasiodiplodia theobromae	6759	Avocado	Mpumalanga Limpopo	62 ± 6.44	53 ± 4.21	100	N/A
				60 ± 2.79	50 ± 2.91		
Penicillium digitatum	5846	Citrus	Mpumalanga Limpopo	42 ± 3.80	35 ± 2.50	N/A	100
				60 ± 5.76	58 ± 6.42		
Penicillium expansum	5654	Apple	Mpumalanga Limpopo	35 ± 6.64	37 ± 5.57	N/A	100
				48 ± 5.64	45 ± 6.23		
Penicillium italicum	10380	Citrus	Mpumalanga Limpopo	65 ± 4.52	63 ± 3.45	N/A	100
				62 ± 6.24	60 ± 2.87		

[a] Plant Protection Research Institute culture collection number.

activities against A. alternata and C. gloeosporioides, isolated from avoca-do and mango, respectively. Results obtained in the study highlight the potential of exploiting H. splendidum EO to combat pathogens that are responsible for heavy losses of fruits harvests. Several strains of A. alternata and C. gloeosporioides have been reported to be resistant to-wards chemical fungicides (El-Goorani et al., 1984; Kumar et al., 2007; Maouni et al., 2007).

The biological activity of an essential oil is related to its chemical composition and to possible synergistic effects between the compo-nents. Marker compounds associated with EOs that have demonstrated good antifungal activities are of value in the quest for natural antifungal agents.

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