The Composition of the Floral Fragrance of *Polianthes Tuberosa* L Cut Flower Grown in Kenya

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Abstract

Tuberose sold on the domestic market fetches very low prices during the low export season. Value addition to the crop would make its production more sustainable. A study was carried out to identify the constituent chemical compounds of tuberose fragrance grown in high altitude regions (Meru, 2068m asl and Tigoni, 1850m asl) and low altitude regions (Sagana, 1214m asl and Juja, 1350m asl) in Kenya. The volatiles were trapped between 5.30pm to 9.00pm from fully-opened intact tuberose flowers using a portable volatile collection technique. The volatiles were analysed to identify the chemicals compounds present using gas chromatography and mass spectrometry. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of n- hydrocarbons, and by computer matching against commercial standards (NIST 98 and ADAMS). Twenty eight chemical compounds were repeatedly identified and categorized. The main fractions with large peak area were methyl benzoate 47% followed by 1. 8-cineole 29%. The two volatiles are important commercially; methyl benzoate has flavor and aroma qualities and 1,8-cineole is a commercial food additive with medicinal, perfumery and insecticidal properties. These two major compounds accounted for 69-84% of the total volatiles in tuberose.

Keywords: Agavaceae, plant volatiles, smallholder production, summer flower, 1, 8-cineole, Methyl benzoate.

Introduction

Tuberose is cultivated in many countries for commercial production as a cut flower, pot plant and for fragrance production (Anon, 2001). In Kenya it is planted in March during the rainy season and flowering coincides with the peak export period from September through to March. However flowers produced during the low export period of May to August do not command a competitive price (HCDA 2007; Muthoka and Muriithi 2008). In neighbouring Rwanda in where tuberose is also grown as a cut flower agribusiness development technical experts have recommended processing of non-export grade flowers for organic essential oil in order to command higher profits (Turner 2001).

Plant volatiles accumulate in all types of plant organs e.g. flowers, pollen and leaves (Maccioni et al. 2007). Floral volatiles have a role in pollinator attraction (Raguso 2009) and assumed to be its primary function. However, pollinator attraction and visitation is also linked to flower morphology and colour, though dynamic patterns in floral scent emission can be related to pollinator behaviour (Raguso 2004). Chemical compounds in the floral scent show close association with the pollinators attracted (Knudsen et al. 1993). The strong, sweet fragrances of moth-pollinated flowers are characterized by specific classes of volatile compounds commonly used in perfumery (Knudsen and Tollsten 1993). Tuberose extracts have been shown to have anti –fungal activity (Nidiry and Babu 2005).

Floral fragrance may be composed of one or more than 100 compounds varying from pico-grams to micrograms emitted per hour. The composition varies spatially different parts of the flower (Maccioni et al. 2007) and based on the circadian rhythm or external stimuli such as light or temperature. An increase in temperature leads to increase in emission (MacTavish et al. 2000; Picone et al. 2004) In other studies increase temperature and rainfall in the field increased emission of volatiles in apples (Vallat et al. 2005). The monoterpene pinene was three times higher at 30°C compared to 20°C in *Pinus halepensis* and *Quercus ilex* (Llusia and Penuelas 2000).

Tuberose volatiles are very unstable and show variable polarity, solubility, volatility, pH, and concentration. They are easily oxidized by contact with air or degraded by heat (Guenther, 1948). The push and pull collection device is a novel method for volatile collection where only the desired portion of the plant is enclosed in a chamber. Air is pushed into the chamber regulated by a flow meter and pulled through a collection trap by a vacuum pump regulated by another flow meter (Knudsen and Gershenzon 2006). The process has the advantage of not trapping non-volatile compounds, therefore yielding a fragrance that should be closer to the flower's true natural odor and is therefore more accurate than other sample extraction methods. Therefore, extraction of volatiles for the cosmetic, pharmaceutical and food industry would provide more income to the grower. The objectives of the study were analyse and identify the chemicals compounds present in the volatiles emitted in vivo by tuberose flowers grown in Kenya and also determine the effect of the environment on emission of tuberose flower volatiles.

MATERIALS AND METHODS

The study sites and plants

The plants used in the study were field grown and the study was carried out during the export period. The locations were Meru Tigoni, Sagana and Juja as described in table .1.

Experimental set up

The treatment was the experimental site with tuberose plants grown in four sites of different altitudes (table 1). The plants were grown by commercial flower farmers in Sagana and Meru, while Tigoni and Juja they were grown using procedures according to the tuberose growing guidelines (Anon, 2001). The bulbs were planted at a spacing of 20cm by 20 cm. Fertilizer NPK 17:17:17 was applied at planting and CAN (26%) was used for top-dressing monthly. Irrigation was done regularly using overhead sprinklers. Mature plants that had inflorescences with open flowers were sampled randomly at each site and used for volatile collection.

Table 1: Sampling location, time and weather conditions

Location	Altitude m asl	Sampling time (pm)	Ambient Temp. °C and Rainfall
Sagana	1214	5.45–6.45	17.5
		7.03 -8.03	17.5 -17.0
			Rain ceased ½ hr- wet soils
Juja	1350	5.30-6.30	21 -19.0
		6.46 -7.46	19.0 – 18.0
			Dry
Tigoni	1850	5.52–6.52	10.5
		7.14 -8.14	10.5 -10.3
			light drizzle throughout sampling
Meru	2068	6.00 -7.00	9.0 – 8.0
		7.20 -8.20	8.0 -7.0
			Dry

Volatile collection technique and pollinator monitoring

The floral volatiles of tuberose were collected using the push and pull technique. The portable pump for collecting volatile emissions in vivo is illustrated in Figure 1A. The pump was connected to flow meters controlling the air flow into and out of the chamber and Super Q volatile trap was used for collection of the volatile by vacuum suction. The volatile trap was made of a glass cartridge (7 mm internal diameter (id)) filled with 100 mg of Super Q (Alltech Associates, State College, Pennsylvania, USA) adsorbent. A double pump (Figure 1B) was used in this study and two samples were collected simultaneously ***(****a* and *b****)*****,** a makeshift tent for pump was used during rain and drizzle **(**Fig. 2A). The collection chamber was made of a polyvinyl acetate bag (406 x 444 mm) sealed with a plastic twist tie (Fig. 2B). The sampling was conducted from 1730 to 2100 hours, when a high volatile emission was expected. Tuberose flowers exhibit nocturnal maxima (Dudareva et al. 2006). At each site a control (blank no inflorescence enclosed to determine extraneous substances within the chamber) and three

samples were taken. Each sampling included three samples from individual inflorescence. Three open flowers on each inflorescence were sampled. The first hour of sampling was referred to as *Time1* and the 2nd hour as *Time2* at all sites. After collection, the filter was eluted with 3 mL of dichloromethane and the extracts were stored at –18°C. Sampling time was exactly 1h. Pollinators visiting the flowers were monitored through observation during the time of volatile collection.

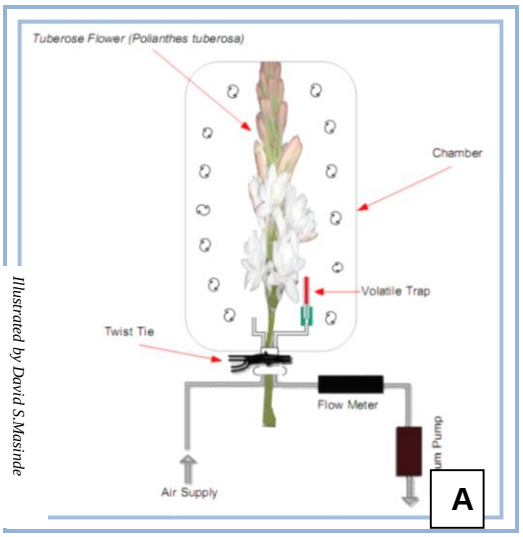

Figure 1A: Push and pull device showing the inflorescence in the chamber.

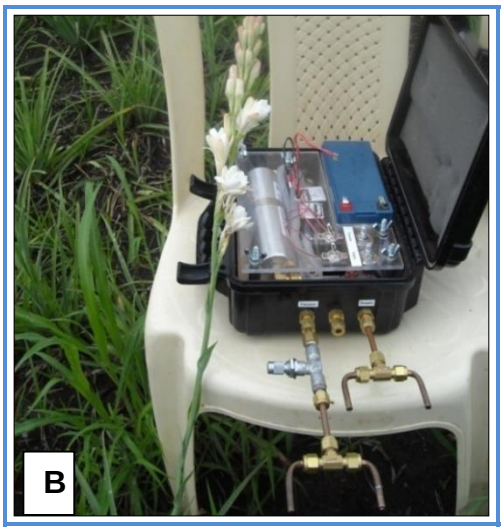

Figure 1B: VOC pump set up for *in vivo* volatile collection

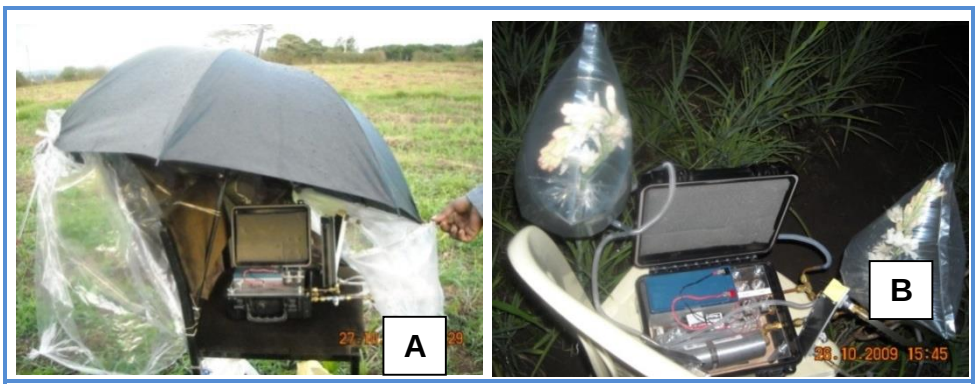

Figure 2A: Makeshift tent for pump when there was drizzle in the field.

Figure 2B: The polyvinylacetate bag inflates and forms the chamber when air is pumped in.

Gas chromatography mass spectrometry (GC–MS) analysis of floral volatiles

Volatiles were analyzed using GC HP 5890 series II GC equipped with a flame ionization detector (FID) and an HP-5 column (30× 0.25 mm internal diameter (ID)×0.25 µm film thickness). Nitrogen was used as carrier gas, with a column pressure of 46 psi and injection temperature of 250°C. One µL of sample was injected in the splitless mode, with the oven temperature programmed from 60°C for 5 min to 280°C at 10°C/min, and held at this temperature for 15 min. GC- MS analysis was carried on an Agilent Technologies 7890A GC equipped with an HP-5 MS capillary column (30×0.25 mm ID×0.25 µm film thickness) coupled to 5795C MS. One microliter of each sample was injected in the splitless mode, and helium was used as carrier gas at 1.0 ml min^{-1}. The oven temperature was from 35°C for 5 min, increased to 280°C at 10°C min^{-1} and then held at this temperature for 15 min. Spectra were recorded at 70 eV in the electron impact (EI) ionization mode. Compounds were identified by comparing mass spectra and retention times with those of reference compounds as well as with mass spectra in different computer libraries.

Statistical analyses
There were three replicates per site. The control values were subtracted from the sample values before data analysis. The means were presented with the standard deviation. Variation in data and percentages of the peak area were determined using Excel Analysis Toolpak, 2007 for Windows.

RESULTS
There were no beetles on the tuberose flowers at 17.14 hrs but many beetles were present on the inflorescence by 18.17 hrs (fig. 3).

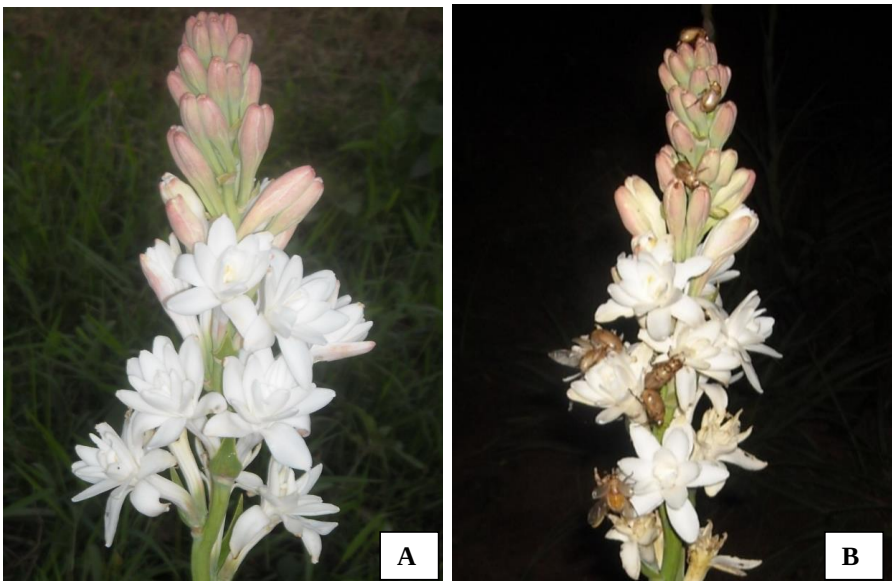

Figure 3: Visitation of beetles driven by scent emission; A) 17.14 hrs no beetles and B) 18.17 hrs with beetles

Fragrance compounds
GC-MS analysis revealed more than 28 compounds from the *in vivo* emission of tuberose flowers (table 2). Twenty one compounds were found in sufficient abundance to allow quantification in all flowers at all altitudes.

Table 2: Mean percentages of peak areas of 28 identified chemicals emitted in vivo from tuberose flowers in different tuberose growing locations.

Most compounds could be classified according to terpenoid biosynthetic pathways. The major compounds were methyl benzoate, 1, 8-cineole, alpha terpineol and methyl anthranilate with average abundances of 47%, 29%, 5%, and 3% respectively. Of the volatiles from tuberose, 14 were oxygenated compounds, 9 were monoterpenes, 3 were sesquiterpenes, and 1 was an aromatic compound (table 3).

	Chemical identified	Mean percentage area under peaks of chemicals				Classification
No	Compound	Sagana	Meru	Juja	Tigoni	Functional group
1	Thujene<alpha->	0.08±0.1	0.00±0.0	0.11±0.1	0.00±0.0	Monoterpene
2	Pinene<alpha->	1.19±0.0	0.82±0.3	1.68±0.5	0.88±0.0	Monoterpene
3	Benzaldehyde	1.51±1.3	1.97±2.2	1.22±1.5	1.69±1.7	Aromatic aldehyde
4	Sabinene	1.97±0.1	1.40±0.4	2.76±0.5	1.56±0.1	Monoterpene
5	Myrcene	0.34±0.0	0.28±0.1	0.51±0.1	0.33±0.0	Monoterpene
6	Cineole <1,8->	27.24±2.1	24.97±12.9	29.26±3.5	28.47±9.4	Alkene
7	Ocimene<(E)-beta->	0.08±0.0	0.10±0.0	0.11±0.0	0.13±0.0	Monoterpene
8	Sabinene hydrate<trans->	0.08±0.1	0.14±0.1	0.11±0.1	0.16±0.1	Monoterpene
9	Methyl benzoate	42.71±11.2	45.77±16.6	45.55±8.7	42.63±14.8	Aromatic ester
10	Terpineol<alpha->	3.35±2.9	1.93±3.3	6.45±2.8	7.80±0.5	Monoterpenes
11	Methyl salicylate	1.66±2.9	5.95±5.2	0.00±0.0	0.00±0.0	Aromatic ester
12	[1,1'-Bicyclopentyl]-2-one	1.43±0.7	0.00±0.0	0.00±0.0	1.96±0.1	Aromatic ketone
13	Indole	1.30±0.4	1.51±0.6	1.57±1.0	1.06±0.3	Aromatic amine
14	Anthranilate<methyl-	4.11±0.8	2.92±0.9	3.46±2.0	1.96±0.7	Aromatic ester
15	Eugenol	0.49±0.1	0.63±0.2	0.65±0.4	0.55±0.1	Aromatic alcohol
16	Methyl eugenol	0.00±0.0	0.00±0.0	0.67±0.5	0.00±0.0	Aromatic alcohol
17	Longicyclene	0.02±0.0	0.05±0.1	0.00±0.0	0.00±0.0	Monoterpene
18	Cyclopentanone, 2-cyclopentylidene	0.05±0.1	0.00±0.0	0.00±0.0	0.00±0.0	Aromatic ketone
19	Bourbonene<beta->	0.04±0.1	0.45±0.2	0.00±0.0	0.31±0.0	Alkene
20	Methyl eugenol	0.54±0.2	0.63±0.3	0.00±0.0	0.00±0.0	Aromatic alcohol
21	Longifolene	0.02±0.0	0.12±0.0	0.13±0.1	0.00±0.0	Sesquiterpene
22	Himachalene<gamma->	0.05±0.1	0.35±0.2	0.21±0.1	0.00±0.1	Sesquiterpene
23	Isoeugenol<Z->	0.93±0.1	0.85±0.2	0.62±0.2	0.00±0.1	Aromatic alcohol
24	Germacrene D	0.18±0.3	1.17±1.4	0.00±0.0	0.00±0.0	Sesquiterpene
25	Methyl isoeugenol<E->	1.16±2.0	0.54±0.9	1.77±2.1	0.22±0.0	Aromatic alcohol
26	Farnesol<2E,6E->	0.32±0.1	0.05±0.1	0.17±0.1	0.82±0.0	Aliphatic alcohol
27	Benzyl benzoate	2.21±0.4	2.76±1.2	1.86±1.2	0.00±0.6	Aromatic ester
28	Isopropyl tetradecanoate	0.13±0.0	0.00±0.0	0.11±0.2	0.47±0.0	Aliphatic ester

±SD refers to means of area under peak with the standard deviation.

Table 3: Emitted *in vivo* volatiles of tuberose flowers grouped according to functional groups.

No.	Monoterpenes C_{10}	Sesquiterpenes C_{15}	Oxygenated Compounds	**Aromatic Compounds**	Total
1	Alpha Thujene>	Longifolene	1,8-cineole	**Indole**	4
2	Alpha Pinene	Himachalene	Benzaldehyde		3
3	Sabinene	gamma	Methyl benzoate		3
4	Myrcene	Gemacrene D	Methyl salicylate		2
5	Ocimene		1,1-bicyclopentyl-2-one		2
6	Sabinene hydrate		Methyl anthranilate		2
7	Terpineol		Eugenol		2
8	Longicyclene		Methyl eugenol		2
9	**Bourbonene**		cyclopentanone		2
10			Isoeugenol		1
11			Methyl Isoeugenol		1
12			2E,6E Farnesol		1
13			Benzyl benzoate		1
14			Isopropyl tetradecanoate		1
Total	**9**	**3**	14	**1**	**27**

Table 4: Volatiles emitted at sampling locations

Location	Altitude masl	Emitted volatiles identified	Ambient Temp. °C
Sagana	1214	27	17.5 17.5 -17.0
Juja	1350	21	21.0 -19.0 19.0 – 18.0
Tigoni	1850	20	10.5 10.5 -10.3
Meru	2068	26	9.0 – 8.0 8.0 -7.0

Environmental variation in floral fragrance

The weather conditions under which the volatiles were collected were not similar in the different areas. Sagana and Tigoni were wet while Meru and Juja were dry. Temperature during sampling ranged 7-9°C in Meru, 10.3-10.5°C in Tigoni, and 17-21°C in Sagana and Juja. The tuberose field in Juja was adjacent to a bright security and beetles started foraging when the floral scent filled the air. The number of floral fragrance compounds at the four altitudes where tuberose volatiles were collected is shown in Table 4.

The two principal compounds 1, 8-cineole and methyl benzoate contributed to 69-84% of the total floral fragrance data, based on 27 compounds. The total number of volatiles compounds emitted was 28 for the three locations but individual locations had different numbers of volatile compounds identified. **Meru, Tigoni and Sagana regions had one or two volatiles that were only found in these regions** (table 5).

There was no clear relationship between altitude and the peak area of the key volatiles methyl benzoate and 1,8-Cineole (Fig. 4). The peak area ranged between 40 and 50% for Methyl benzoate and 20 and 30% for 1,8-cineole and the samples were not significantly different with very high standard errors. The methyl benzoate emission decreased during the second hour while 1,8-cineole increased (Fig. 5).

Table 5: Volatiles specific to each location

Location(s)	Chemicals	Ambient Temp. ^{0}C during collection
Meru	• Muurola-4(14),5-diene<trans • 2-Pyrrolidinone, 1-methyl-	• 9 -7
Tigoni	• Copaene<beta-> • Methyl geranate	• 10.5 -10.3
Meru & Tigoni	• Bourbonene<beta->	• 10.5- 7
Juja	• None	• 21 – 18
Sagana	• Cyclopentanone, 2-cyclopentylidene	• 17.5 -17

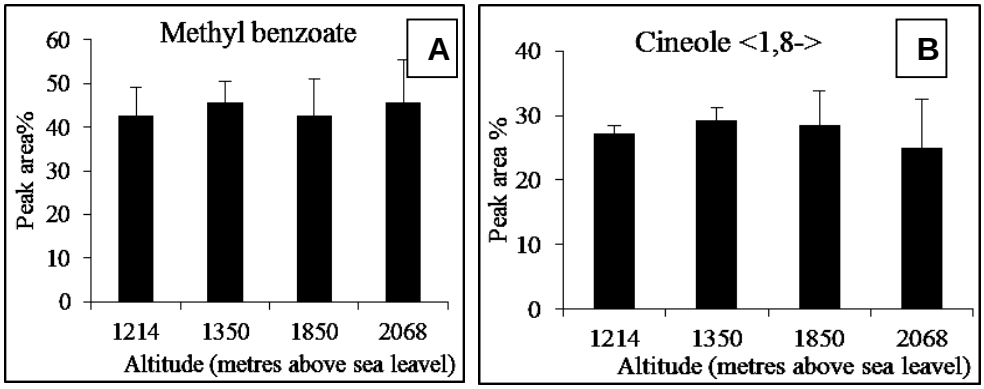

Figure 4: Emission at main volatile compounds at various altitudes A) Methyl benzoate and B) 1,8-cineole

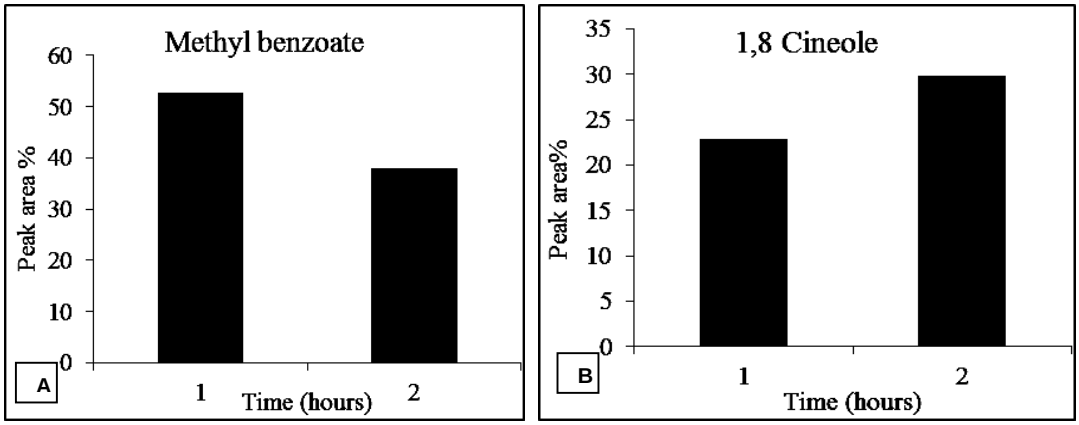

Figure 5: Total emissions of the two main compounds at all locations: A). Methyl benzoate; and B). 1, 8 - cineole emission over time.

DISCUSSION

Attractants and Volatile Composition

Beetles present on the flowers in Juja (fig. 3) were observed after fragrance emission was evident. The air was filled with the strong sweet fragrance of tuberose and beetles are known to be attracted these scent (Knudsen and Tollsten 1993). Though the initial attraction of the beetles may have been the bright security lamp but they ended up on the flowers due to the floral scent. Such strong scents belong to specific classes of volatile compounds commonly used in perfumery (Knudsen and Tollsten 1993). There is no documentation of tuberose pollinators.

The tuberose volatiles identified were 28 with varying amounts in abundance. The floral fragrance in tuberose was observed to vary over time within the collection period. Most of the compounds found in the tuberose volatiles (Table 2) are common floral volatile compounds that are present in flowers of many other plant species (Knudsen *et al.*, 1993; Knudsen and Gershenzon 2006). The oxygenated monoterpene 1,8-cineole was the dominant compound of the monoterpenes, comprising 29%. Methyl benzoate, an aromatic ester, comprised 47% of the total peak area of volatile compounds emitted in all the sites. There was more methyl benzoate emitted within the first hour than the second hour of trapping. The emission of 1,8-cineole increased with time (fig 4). Methyl benzoate has been associated with nocturnal maxima rhythm (Kolosova *et al*., 2001). In *Mahonia japonica* it was reported that emission of monoterpenes and aromatic compounds was controlled by diurnal changes in light levels (Picone et al. 2002).

Methyl benzoate and 1,8-cineole abundance and potential products

Though cultivation practices and inflorescence stage were similar, the temperatures at the various locations were different. Methyl benzoate and 1,8, cineole were the most abundant at all altitudes, other components were not all present at all locations. The abundance of the volatile ester methyl benzoate is similar to findings by Rakthaworn et al. (2009) who found methyl benzoate was the most abundant compound regardless of extraction method. Other authors relate the abundance to pollinator activity as shown in snapdragon, passion fruit, orchids and petunia (Dudareva et al. 2000; Kolosova et al. 2001; Varassin et al. 2001; Negre et al. 2003; Salzmann et al. 2006). Variation in floral fragrance composition has been indicated in flowers of a similar species that have different pollinators at different altitudes (Knudsen 2002). Commercially, methyl benzoate is known to possess flavor/aroma qualities giving a sweet floral scent with a fruity undertone, it is used to flavor berry and cherry condiments. 1,8-cineole, also known as eucalyptol, found naturally in eucalyptus genus and is used as a food additive (baked goods, confectionery, meat products and beverages), medicinal additive (mouthwash and cough suppressant), pesticide (Nidiry and Babu 2005; Sangita et al. 2006)), and a perfuming agent and tonic

as well. The compound 1,8-cineole is typical of many essential oils from bay leaves, tea tree, rosemary and sage. It is used to give a minty, spicy or peppery flavor to condiments.

Environmental influences on fragrance

The emission patterns were not similar and there were unique volatiles emitted exclusively at some locations. Juja was the warmest location (18- 21°C) and did not emit the following: methyl salicylate, [1,1'-bicyclopentyl]-2-one, longicyclene, cyclopentanone, 2-cyclopentylidene, bourbonene<beta-> and germacrene D (Table 7.4). Sagana second warmest location (17 -17.5°C) had the highest number of volatile compounds identified 27, Tigoni was cool at 10.3°C to 10.5°C Tigoni had 20; and Meru which had the lowest temperature (7-9°C) had 26 compounds. Tuberose volatiles are known to be unstable with variable volatility and concentration. They are easily oxidised by contact with air or degraded by heat (Guenther, 1948). The advantage of using the push and pull is that though there is a possibility for oxidation of the volatiles during trapping, there is no exposure to high temperature; therefore the fragrance may be a close representation of the natural fragrance of the tuberose flower.

According to the Food and Chemical Toxicology registry (Anon 2000), the tuberose concrete under this registration has the main compounds as farnesol, methyl anthranilate, eugenol, methyl benzoate, benzyl benzoate, methyl anthranilate, benzyl alcohol, geraniol, and nerol. Our study showed that the four altitudes were different in fragrance profile but there was no specific trend associated to the altitude/temperature or weather (wet or dry). The solvent extracted fragrance had more volatiles identified and some were in trace amounts. Methyl benzoate and anthocyanin biosynthesis originate from the same phenylpropanoid pathway and when methyl benzoate emission increased it is possible that the metabolic flow was diverted from anthocyanin biosynthesis to benzoic acid production (Dudareva and Pichersky 2008). The volatile profile of the solvent extracts was not similar to the *in vivo* emitted profile.

A total of 28 compounds from intact flowers of *P.tuberosa* were separated and identified. alpha -pinene, sabinene, myrcene, *cis*-ocimene, benzaldehyde, terpinolene, were the characteristic compounds of the fragrance tuberose flowers. These results demonstrated that push pull technique using the portable volatile collection pump SPME–GC–MS is a simple, method suitable for the analysis of volatile compounds emitted from intact flowers of *P. tuberosa* in different localities. It is a useful method to distinguish the difference of volatile compounds emitted from flowers at different locations. As demonstrated in this study, twenty of the twenty –seven, were consistently identified with a purity of ≥90%. However, in this case we observed an overwhelming dominance of hydrocarbon derivatives, probably because of the low volatility of oxygenated compounds (Bouvier-Brown et al. 2009).

Conclusions and recommendations

The influence of location on biochemical compound composition of tuberose floral volatiles shows that at least 28 compounds were detected at all altitudes though there were differences between samples and not all the compounds were detected in all locations. The presence of methyl benzoate and 1,8, cineole in large amounts indicates the potential for industrial exploitation. This is the first reported study of floral fragrances emitted *in vivo* for intact tuberose flowers. We have chemically characterized the floral fragrance in tuberose and analyzed the floral fragrance between altitudes. The study shows that the mobile volatile collection pump can be used to collect and identify volatiles. The studies on floral volatiles of tuberose show the potential for product diversification through value addition. Some products could be processed locally through the cottage industry. Interventions include building capacity of farmers in processing, packaging, and branding and risk mitigation. This information can be used by stakeholders in the floriculture industry, including policy makers to in place strategies that promote new products.

Manufacture of fragrance from tuberose flowers by extraction of volatiles would add value to the crop and make it more profitable as recommended for Rwanda in their agribusiness development. However, more studies are necessary to establish extraction protocols and economical volumes of tuberose flowers for commercial volatile extraction and the prerequisite climatic conditions associated with good quality fragrance compounds.

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