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ORIGINAL PAPER

Corolla structure and fragrance components in *Styrax tonkinensis*

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Abstract

Key message **The structure of petals and volatile compounds from fresh *Styrax tonkinensis* cut flowers were investigated by using micro-techniques and a headspace solid-phase micro-extraction technique coupled with GC–MS.**

Abstract *Styrax tonkinensis* is a fast-growing woody plant that is used for timber and as a medicinal plant. In the present study, the structures of the flower petals of *S. tonkinensis* were investigated and volatile compounds emitted from the petals were identified. Light microscopy, scanning and transmission electron microscopy were used to describe petal structure. The volatile constituents were analyzed using a headspace GC–MS technique. Results indicated that glandular hairs and 8–9 layers of parenchyma cells in the cream-white petals play a key role in emitting the fragrance. An analysis of the volatile components emitted by the cut flowers of *S. tonkinensis* at two stages of flower development (prior to and at anthesis) indicated that monoterpenes, such as 1,3,6-octatriene, 3,7-dimethyl-(*Z*), and α-pinene, were the most abundant volatile components in all samples.

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Keywords *Styrax tonkinensis* · Floral fragrance · Petal structure · Terpenes

Introduction

Flower fragrance of higher plants has not only ecological value but also many other values (Guterman et al. 2002; Raguso 2008). Pleasant and relaxing plant-derived aromas have been used therapeutically in psychological and physiological disorders (Corley 2007). Flowers are also planted in gardens and parks for their artistic appeal, thus increasing the quality and ornamental effect of the landscape. Cultures throughout history have placed a high value on landscape design and aromatic plants have always been an essential component in Chinese gardens (Sun et al. 2007). Although petals are the main source of flower fragrance (Dobson et al. 1990; Goodwin et al. 2003; Pichersky et al. 1994), the contribution of other floral organs, such as the calyx, stamens (pollen), and nectaries, cannot be ignored (Dobson et al. 1990, 1999; Bergström et al. 1995; Dudareva et al. 2004). While terpenoids are the main component of floral scent (Schilling et al. 2010), a variety of other compounds also contribute to fragrance as well. For example, 1,4 dimethoxybenzene is the main component of *Salix caprea* flowers (Dötterl et al. 2005). In actuality, floral aromas can be composed of several to hundreds of components (Knudsen et al. 1993; Dobson 1994; Dudareva and Pichersky 2000). Different chemical compounds are perceived as different fragrances and the chemical structures of the compounds play a critical role in the smell. The composition of compounds determined to make up floral scent can also be influenced by the stage of flower development (Goodwin et al. 2003; Ayasse et al. 2000), the time of sampling within a diurnal cycle (Pott

et al. 2002; Nakamura et al. 2006), environmental factors such as light intensity and temperature (Jakobsen and Olsen 1994), storage condition (Choi and Sawamura 2002), and the method of analysis (Schultz et al. 1997). Glover and Martin (2002) have also suggested that the shape and structure of petals can affect the diffusion of scent.

Since the components comprising floral scents are complex, techniques for the extraction and analysis of scent components are often complicated, including supercritical CO_2 extraction (Reverchon and Marco 2006) and headspace analysis (Knudsen et al. 1993). In some cases, these methods are coupled with gas chromatography/mass spectrometry (GC/MS) (Custódio et al. 2006), and gas chromatography–electro antennographic detection (GC–EAD) (Dötterl et al. 2005), to analyze floral constituents. Knudsen et al. (1993) and Knudsen and Gershenzon (2006) have reported that the floral headspace method has been the most widely applied strategy for the analysis of floral scent components.

Styrax tonkinensis, a fast-growing, oil-producing woody plant is used for timber and as a medicinal plant. This organism belongs to the family Styracaceae and is mainly distributed in Tonkin Gulf in Vietnam, and commercially produced in Yunnan, Guizhou, Guangxi, Guangdong, Fujian, Hunan and Jiangxi, China. The flowers of *S. tonkinensis* have white blossoms that bloom in May and June in Jiangsu province, China and emit a pleasant aroma. Therefore, *S. tonkinensis* germplasms represent a rich resource of aromatic plants. Numerous studies have been published regarding the wood, taxonomy, ecology, silviculture, chemical structure of its resin and the various uses of *S. tonkinensis* (Phuong et al. 2007; Pinyopusarerk 1994; Booth et al. 1999; Peng et al. 2013; Wang et al. 2006a, b). However, no reports have been published, pertaining to the composition of the volatiles produced by *S. tonkinensis* flowers. The objective of the present study was to identify the volatile components of *S. tonkinensis* flowers during two developmental stages using headspace-GC–MS analysis and to examine the anatomy and cytology of petal structure in order to provide some basic information about the essential oil that would be exploited. The flowers were examined at two different developmental stages, prior to and at anthesis.

Materials and methods

Plant materials

Fresh flowers of *S. tonkinensis* at two stages of development (prior to and at anthesis) were obtained from 4-year-old plants growing near the He Wang Ba reservoir, Maji town, Liuhe district, Nanjing City, China, in May 2013. Twenty cut flowers were collected at each stage of development. Five flowers were used for microscopic observation, five for sensory evaluation, and ten for the identification of aroma volatiles.

Evaluation of flowers

Flowers collected at each of the two stages of development were first evaluated for floral aroma, color, and other floral characteristics. Flowers were visually observed and then photographed using a stereomicroscope (Fig. 1; Table 1).

Petal microstructure

Samples of fresh flowers were fixed in 4 % glutaraldehyde in 0.2 M phosphate buffer (pH 7.2), rinsed with 0.1 M phosphate buffer solution, and then fixed again in 1 % osmium tetroxide and subsequently rinsed in phosphate buffer. For observations of the surface structure of petals, using scanning electron microscopy (SEM), some of the fixed samples were dehydrated in a graded series of ethanol, which was then replaced with anhydrous alcohol and isoamyl ester (1:1, 1:2), and finally pure isopropyl acetate. The samples were then dried in a Hitachi HCP-2 critical point dryer (Hitachi, Tokyo, Japan), and then coated with a

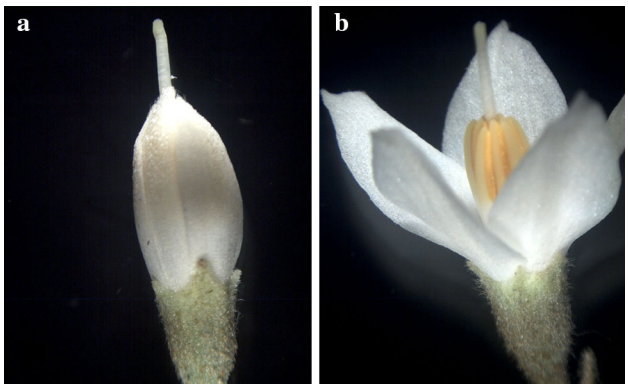

Fig. 1 Representative photographs of the stages of development of flowers of *S. tonkinensis* used in the present study to identify scent components. **a** Prior to anthesis. **b** At anthesis (0.7×)

Table 1 Sensory and morphological evaluation of flowers of *S. tonkinensis* at two stages of development, prior to anthesis and at anthesis

Flower state	Character and flavor
Prior to anthesis	Flowers just opening, corolla white, stigmas exerted from corolla, floral fragrance
At anthesis	Flowers in full bloom, corolla white, anthers not strongly separated, floral fragrance, no floral nectaries observed

mixture of gold/palladium in a Hitachi E-1010 sputter coater (Hitachi, Tokyo, Japan). Samples were subsequently observed and photographed in a FEI Quanta-200 SEM (FEI Company, USA).

In order to examine petal ultrastucture, some samples were dehydrated in a graded series of acetone, and then infiltrated, embedded, and polymerized in Epon812 epoxy resin. Thick (10 micron) and thin (50 nm) sections were obtained using an LKB-5 ultramicrotome (LKB Instruments Inc., Lucerne, Switzerland). Thick sections were observed in an Olympus CX 41 light microscope (Olympus Corporation, Tokyo, Japan). Thin sections were mounted on copper grids and stained with uranyl acetate and lead citrate, and observed and photographed in a JEM-1400 transmission electron microscope (TEM) (JEOL, Tokyo, Japan).

Detection of fragrance components

For fragrance analysis, ten whole, cut flowers at each stage were sealed in a 10-ml headspace vial, with flowers occupying approximately two-thirds of each vial, and then sealed using a sealing pad (temperature resistant to 180 °C) and an aluminum cap.

The conditions for headspace analysis were as follows: column box (85 °C); quantitative tube (100 °C); transmission line (115 °C); the time of sample vial balance was 30 min, the time of sample vial pressure (9 s), time of quantitative filling (0.15 min), time of quantitative ring balance (3 s), and time of injection (1 min).

A headspace auto sampler was used to capture the scent emitted by the flowers. The captured volatiles were analyzed using an Agilent 6890/5975B GC–MS gas chromatograph–mass spectrometer (J&W Scientific Inc., Folsom, CA, USA). The volatile compounds were separated using an Agilent HP-5MS (5 % Phenyl methyl siloxane, 30 m × 250 μm × 0.25 μm) capillary, and helium served as the carrier gas. The GC oven was programmed to run at 50 °C for 2 min followed by an increase of 4 °C per minute to 200 °C for 0.5 min, then 10 °C min^{-1} up to 280 °C, and then holding at 280 °C for 10 min. The inlet temperature was 230 °C. The amount of injected sample was 1 μl and the split ratio was 15:1. The ionization energy was 70 eV, and the ion source temperature was 230 °C. The mass spectra of eluted compounds were recorded for an *m*/*z* of 33–300. The acquisition mode was set to full scan.

The spectrum of each compound was compared to the Standards and Technology library of NIST05 (NIST Database, ChemSW Inc., Fairfield, CA, USA), and compounds were identified according to the retention time and NIST Database. The relative amount of each component was calculated using ion current peak area normalization method by workstation.

Results

General and ultrastructural observations of petals

Flowers collected at the two different stages (prior to and at anthesis) differed in several respects (Fig. 1; Table 1). In particular, the flowers smelled differently in the two developmental stages and, as would be expected, floral fragrance was much stronger when flowers were in full bloom. Results through anatomical observation indicated that: nectaries were not observed, the amount of pollen was very low, bell-shaped calyx was short, 16.876 ± 0.351 mm long, stellate hairs on the rough surface, and pistil was 1, style bar, 15.229 ± 1.060 mm long,0.636 ± 0.076 mm wide. Pollens, calyx and style had no fragrance. Therefore, the main source of the fragrance was the petals. Morphological and ultrastructural observations of *S. tonkinens*is petals are presented in Fig. 2. The petals consisted of an upper and lower epidermis, mesophyll, and vascular tissue. Many stellate hairs were present on the both epidermis of petals, and hairs were bigger and in greater number in the adaxial epidermis. In proximity to the root of stellate hair, there is a cystic structure which similar to a glandular hair (Fig. 2d). Petals comprised 8–9 layers of parenchyma cells. Ultrastructural observations of the parenchyma cells (Fig. 2c–f) indicated that they had large, central vacuoles, and peripheral cytoplasm containing organelles such as Golgi bodies and ribosomes. Numerous vesicles were also observed (Fig. 2e, f). Some of the vesicles were close to and fused to the plasmalemma, while others were observed external to the plasma membrane. Based on the common knowledge that Golgi are the terminus for packaging materials to be secreted, it appears that the parenchyma cells of the petals were involved in the production and secretion of the volatile, aromatic compounds emitted from the flowers of *S. tonkinensis*.

Floral scent constituents

A large number of different floral scent compounds, comprising different percentages of the total concentration, were produced in the flower petals at both stages of flower development sampled (Table 2). GC–MS analysis (Figs. 3, 4) detected 24 and 16 different compounds with a total number of 33 components emitted from flowers prior to and at anthesis, respectively. The composition of the components differed in the two stages of flower development.

A comparison of the total ion chromatograms from the two development stages (Figs. 3, 4) revealed that the peaks of stage 1 (prior to anthesis) were significantly higher than those observed in stage 2 (at anthesis). More specifically, four peak areas in stage 2, tr = 4.92, 8.72, 12.18, and

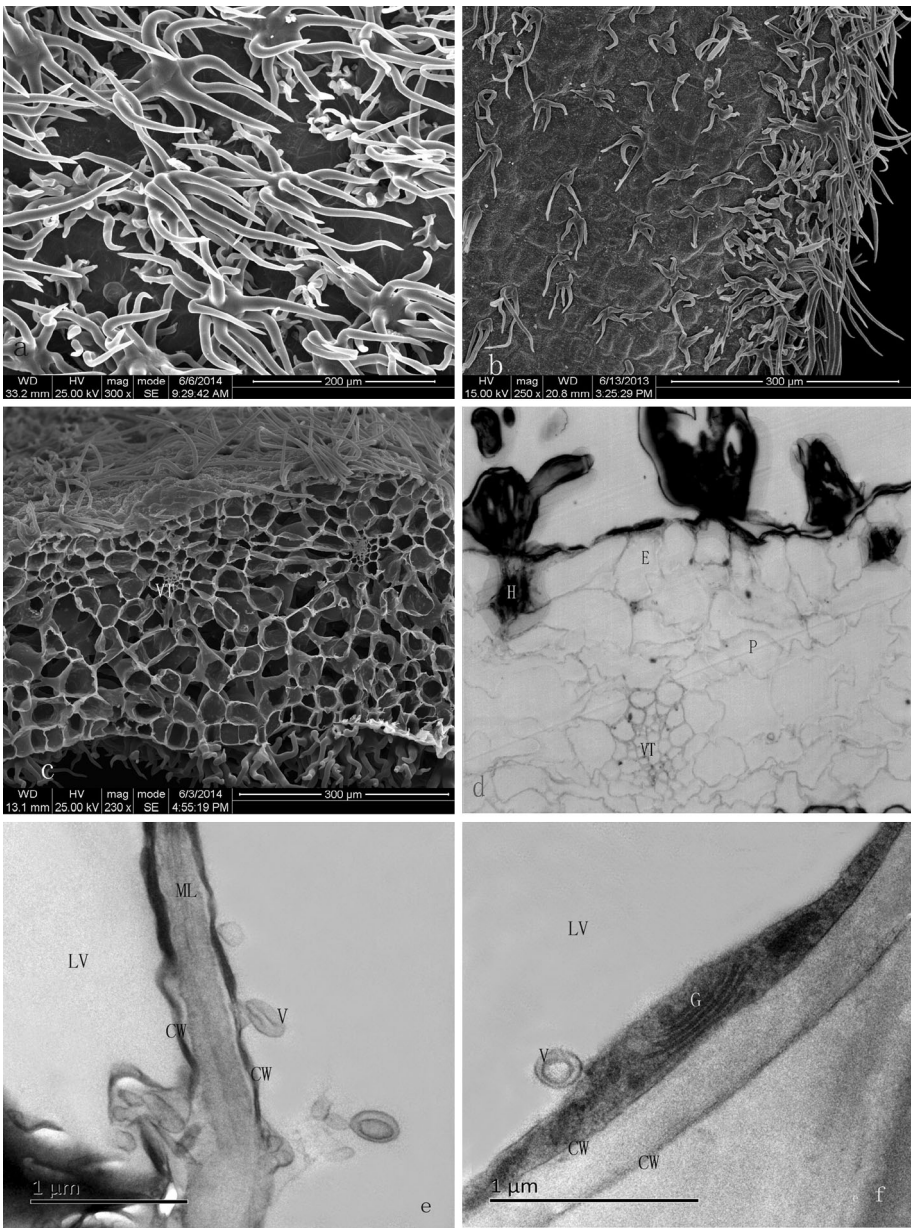

Fig. 2 Structure of *S. tonkinensis* petals. **a** SEM micrograph of the adaxial epidermis of flower petals, *bar* 200 µm. **b** SEM micrograph of the abaxial epidermis of flower petals, *bar* 300 µm. **c** SEM micrograph of a cross-section of a flower petal revealing that the mesophyll is composed of 8–9 layers of parenchyma cells, *bar* 300 µm. **d** Light micrograph of glandular-like cells at the base of stellate hairs, 10 × 40. **e**, **f** TEM of parenchyma cells in the mesophyll of flower petals. **e** Note vesicles external to the cytoplasm of the cell, *bar* 1 µm. Abbreviations used for figures. **f** Note the large, central vacuole, the peripheral cytoplasm, and presence of a Golgi body, *bar* 1 µm. *CW* cell wall, *H* hair, *E* epidermis, *LV* large vacuole, *ML* middle layer, *P* parenchyma, *V* vacuole, *VT* vascular tissue, *G* Golgi apparatus

12.52 min, were far less than the areas observed at stage 1. The two developmental stages had common peaks before 5 min but the peak areas were much smaller at stage 2. The detected compounds were mainly terpenes, esters, alcohols, alkanes, aldehydes, ketones and aromatic compounds. The most prominent and abundant group present in the volatiles emitted at both stage 1 and 2 was terpenes, such as 1,3,6-octatriene, 3,7-dimethyl-, (*Z*)- types of isomeric ocimene, that represented 38.7 and 72.4 % of the total amount of volatile compounds detected in stage 1 and 2 flowers, respectively. The second most abundant was 1S-α-pinene representing 17.85 and 9.10 % of the total constituents at stage 1 and 2, respectively. Estragole (15.69 %) was the third most abundant component prior to anthesis (stage 1), but hexanal (7.42 %) was the third most abundant at anthesis (stage 2). Along with these main compounds, there were also some minor components, such as limonene (7.69 %), β-phellandrene (6.78 %), benzene, 1-methoxy-4-(1-propenyl)- (3.30 %), hexanal (1.37 %), bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)- (1.21 %), 1,3,6-octatriene, 3,7-dimethyl-, (*E*)- (1.10 %), and bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-, (1*S*)- (1.05 %) detected from petals prior to anthesis. However, the number of minor components detected from petals at anthesis was fewer. For example, 1,3,6-octatriene, 3,7-dimethyl-, (*E*)- (1.97 %), ethyl acetate (1.92 %), dimethyl sulfide (1.54 %), bicyclo[3.1.0]hex-2-

Table 2 Fragrance components in *S. tonkinensis* in stage 1 (prior to anthesis) and 2 (at anthesis)

No.	Compound	RT (min)	Relative content (%)	
			Stage 1	Stage 2
1	Heptane, 1-(1-butenyloxy)-, (*Z*)-	1.805	0.23	–
2	Diaziridine,3,3-dimethyl-	1.805	–	1.22
3	Dimethyl sulfide	1.896	–	1.54
4	Furan, tetrahydro-3-methyl-	2.253	–	0.25
5	1-Butanol, 3-methyl-, carbonate (2:1)	2.344	0.20	–
6	Ethyl acetate	2.355	–	1.92
7	Hexanal	4.917	1.37	7.42
8	Styrene	7.417	–	0.33
9	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	8.477	1.21	–
10	Pyridine, 3-methyl-	8.494	–	0.27
11	1*S*-α-Pinene	8.726	17.85	9.10
12	Camphene	9.237	0.15	–
13	3-Thujen-2-ol, stereoisomer	9.407	0.23	–
14	β-Phellandrene	10.03	6.78	–
15	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	10.55	0.64	0.64
16	2-Norbornene	10.569	–	0.33
17	α-Phellandrene	11.09	0.70	–
18	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	11.52	0.38	–
19	Benzene, 1-methyl-3-(1-methylethyl)-	11.79	0.18	–
20	Limonene	11.95	7.69	0.60
21	Eucalyptol	12.066	–	0.54
22	1,3,6-Octatriene, 3,7-dimethyl-, (*E*)-	12.18	1.10	1.97
23	1,3,6-Octatriene, 3,7-dimethyl-, (*Z*)-	12.58	38.87	72.4
24	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	13.01	0.66	0.38
25	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	14.1	0.67	–
26	Nonanal	14.58	0.53	–
27	Benzene, 1-methyl-4-(1-methylethyl)-	14.764	–	0.30
28	Bicyclo[2.2.1]heptan-3-one, 6,6-dimethyl-2-methylene-	16.84	0.16	–
29	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	17.37	0.18	–
30	Estragole	18.06	15.69	–
31	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-, (1. α., 2. α, 5. α.)-	18.44	0.19	–
32	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-, (1*S*)-	18.56	1.05	–
33	Benzene, 1-methoxy-4-(1-propenyl)-	21.16	3.30	–

RT Retention time, – undetected

ene, 4-methyl-1-(1-methylethyl)- (1.42 %), and diaziridine,3,3-dimethyl- (1.22 %). In addition to the major and minor compounds, some other substances with a percentage of less than 1 % were detected.

Discussion

Since nectaries were not observed and calyx, style in the flowers of *S. tonkinensis* had no fragrance, petals appear to be the main source of the floral fragrance emitted from flowers. Our results on the structure of *S. tonkinensis* petals are similar to the results of the study conducted in *Clarkia* by Pichersky et al. (1994). The volatile compounds emitted from the petals prior to (stage 1) and at anthesis (stage 2) were analyzed by headspace GC–MS and the peaks obtained at the two stages were compared. Results indicated that terpenoids were the most abundant component at both stages of flower development. At anthesis 72.4 % of total fragrance consists of 1,3,6-octatriene, and 3,7-dimethyl-, (*Z*). Terpenoids have strong antioxidant activity. They protect plants against peroxidation and can inactivate reactive oxygen species (ROS) which are a main component of oxidative stress. In addition, they also play a key role in plant defense mechanisms (Loreto and Velikova 2001;

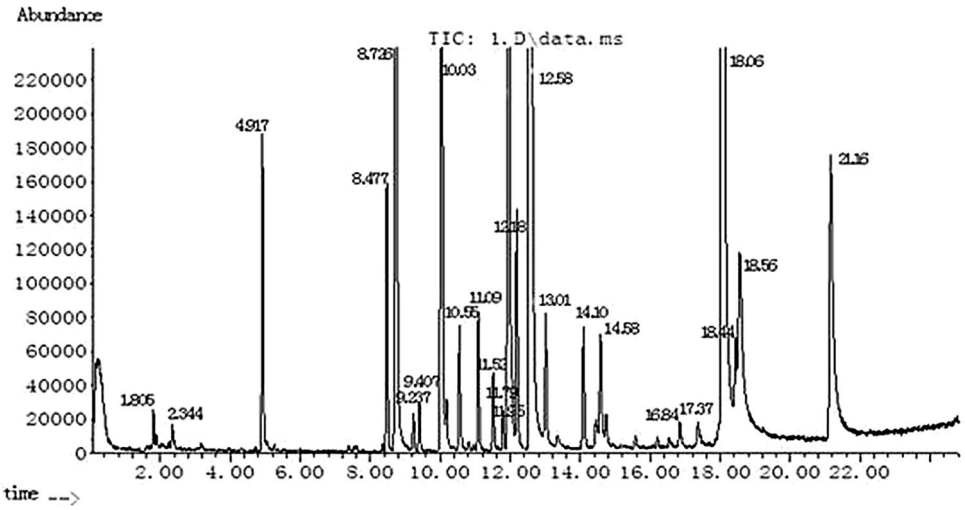

Fig. 3 Chromatogram of fragrance components identified in flowers of *S. tonkinensis* prior to anthesis (stage 1)

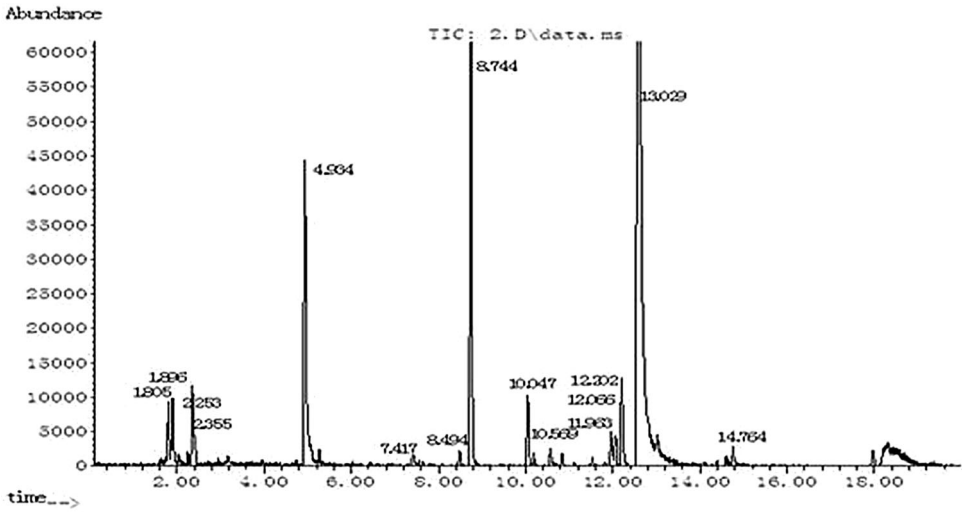

Fig. 4 Chromatogram of fragrance components identified in flowers of *S. tonkinensis* at anthesis (stage 2)

Loreto et al. 2000). Terpenoids in addition to their basic role in attracting pollinators have been used in commercial industries for a long time for many different purposes. Terpenoids have been attributed to numerous properties, such as cancer preventing, antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory, antiparasitic, and skin penetration enhancers. They have also been used as natural flavor additives in food and as fragrances in perfumers. This has made this class of compounds of great interest to the medical, food, and cosmetic industries (Takabayashi et al. 1994; Paduch et al. 2007; Brahmkshatriya and Brahmkshatriya 2013).

Our mass spectrometry analysis revealed many compounds with a molecular weight of 136.13. These substances are the isomers of monoterpenes (C10). The sedative effects, anti-cough, anti-asthma, and bacteriostatic properties of monoterpenes have been reported in many papers (e.g. Deng et al. 2004). The total content of monoterpenes in our study at the two stages of flower development was 76.67 and 85.87 %, respectively. In some plants, such as *Antirrhinum majus* (Nagegowda et al. 2008), and *Arabidopsis thaliana* (Chen et al. 2003), compounds such as linalool, ocimene, mycrene, nerol, and geraniol have been identified. In our study, 1,3,6-octatriene, and 3,7-dimethyl (*Z*), which are ocimene isomers, had the highest in quantity in both developmental stages, while α-pinene was second. Ocimene isomers, as aroma components, are present in a wide array of plant species, such as lilly (*Lillium* sp.), daffodil (*Narcissus* sp.) and citrus (Yoshiki and Nobuo 1991). (*E*)-β-ocimene, which can serve as an attractant to parasitoids, herbivores and pollinators, is a component of many floral scents (Fäldt et al. 2003). The characteristic smell of grass incense, many flower scents, orange flavor, and a wide array of spices are all due to ocimene. Ocimene is insoluble in water, but soluble in ethanol, ether, and chloroform. Some

essential oils, like lavender oil, and tarragon oil, contain it as a constituent. Ocimene and phellandrene are perceived to have a stimulating, refreshing effect (Wen and Yu 2005). In addition, terpene compounds, such as α-pinene and limonene, which have significant calming and other beneficial health effects on the human body, can be used as air fresheners. These compounds can also affect the human nervous system, producing a relaxing effect (Maria et al. 2009). Terpenes also exhibit a broad spectrum of antifungal, and anticonvulsant activity (Du et al. 2008).

Conclusion

In summary, scent emissions of *S. tonkinensis* vary depending on the stage of flower development. Thus, they exhibit a temporal and spatial mode of regulation (Schade et al. 2001; Dötterl and Jürgens 2005). The aroma components are dominated by high levels of monoterpenes. Therefore, *S. tonkinensis* has a strong potential use by the perfume, essential oil and food industries. It also can be used as an aromatic plant in landscape designs.

Author contribution statement Liping Xu is responsible for designing, finishing the experiment, data acquisition and analysis, manuscript preparation, and so on. Fangyuan Yu provided helpful suggestions in data analysis and final approval of the version to be published.

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Conflict of interest The authors declare that they have no conflict of interest.

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