Gas Chromatography

DETERMINATION OF VOLATILES IN WATER LILY FLOWERS USING GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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Volatile organic compounds in the stamens, petals, and pistils of 56 water lily cultivars were determined by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry. The stamens released a majority of the volatiles. A total of 117 volatile organic compounds were determined. Alkanes and alkenes were the most abundant, followed by aldehydes and ketones. Cluster analysis was used to divide the cultivars into three subsets characterized by specific aromatic compounds and associated aromas. Discriminant analysis confirmed the results of the cluster analysis. Three tropical water lily cultivars Colorata, 34, and Ai Ji Bai and one hardy water lily cultivar Somptuosa had particularly high aromatic compound concentrations and are recommended to produce fragrant, colorful, and hardy specimens.

Keywords: Gas chromatography–mass spectrometry; GC–MS; Solid-phase microextraction; SPME; Volatile compounds; Water lily

INTRODUCTION

The water lily (an aquatic herb of genus Nymphaea, family Nymphaeaceae) is a commercially valuable perennial aquatic flowering plant with global distribution. Based on geographic occurrence, the genus is divided into the two ecological groups of tropical and hardy water lilies (Huang et al. 2009; Knudsen and Tollsten, 1993; Knudsen, Tollsten, and Bergstrom 1993). Hardy water lilies are primarily found north of 25° N in subtropical, temperate, and boreal regions, while tropical water lilies...
lilies occur between $20^\circ$ S and $25^\circ$ N. Water lilies are widely used as ornamental plants in landscaping and hence are valuable economically and ecologically. An understanding of the composition and interaction of their metabolic products may allow the development of superior cultivars.

Plants may release volatile compounds from their flowers, leaves, fruits, and roots (Bertoli et al. 2003; Field et al. 1996). The most common volatile compounds produced by plants are terpenoids, phenylpropanoids, benzenoids, fatty acid derivatives, and amino acid derivatives (Dudareva, Pichersky, and Gershenzon 2004). These compounds have a low molecular weights (usually 100 to 250 Da), low boiling points, and low polarity values (Pichersky, Noel, and Dudareva 2006). Their primary functions are to defend the plant against herbivores and pathogens, attract pollinators, disperse seeds, and transmit signals in plant-plant interactions (Gang 2005). The composition and concentrations of these compounds provide information about the plant’s physiological condition (Millar and Sims 1998; Servili et al. 2000; Sides et al. 2000).

Gas chromatography–mass spectrometry (GC–MS) may be performed in the absence of standard samples (Sun and Wang 2005; Tao, Li, and Wang 2008). The technique has been further improved by combination with solid phase microextraction (SPME), which provides a representative method of collecting volatile compounds from living plants. To date, SPME has been used with food, air, soil, and water, and to determine volatiles from whole plants, flowers, and bark (Flamini, Cioni, and Morelli 2002; Rohloff and Bones 2005; Sha et al. 2004). It provides a fast and simple method for collecting volatiles based on adsorption and desorption from an inert fiber coated an absorbent. The concentration of analyte in the sample is proportional to the concentration of analyte adsorbed by the fiber (Eisert and Levensen 1996; Gyorgy and Karoly 2004; Lu and Liu 1998). Fernando and Grün (2001) analyzed volatile compounds in *Luffa acutangula* and *Momordica charantia* using SPME combined with GC–MS (SPME–GC–MS). They identified 16 volatile compounds in *L. acutangula* and 13 in *M. charantia*, and concluded SPME–GC–MS is useful at the ppt level (Fernando and Grün 2001). The SPME technique has several advantages, including high sensitivity, simplicity, and the ability to perform extraction, concentration, desorption, and injection in a single operation (Millar and Sims 1998; Servili et al. 2000; Sides et al. 2000).

Although volatile substances in plants have been thoroughly studied, little research has been conducted on water lilies. The results reported here on this species show that the stamen plays the central role in releasing volatiles. These findings provide a foundation for the future study of dynamic changes in flower development, as well as a theoretical basis for understanding the composition of volatiles in water lilies.

**MATERIALS AND METHODS**

**Plants**

Flowers were sampled from 34 hardy and 22 tropical water lily cultivars (Table 1). Plants were grown at the Beijing Botanical Garden in containers with a diameter of 40 cm and a height of 30 cm. Containers were placed in small square ponds 140 cm wide and 70 cm deep, with two cultivars in each pond. Plants were grown until flower collection (for at least three years) under constant conditions of fertilization, irrigation, disease prevention, and pesticide application. All flowers were collected on the first day of full flower opening.
Three flowers were collected from each cultivar. Flowers were collected between 5:00 and 7:00 a.m. and immediately processed. From each flower, 2.5 g of petals and 0.5 g each of stamens and pistils were sampled at random. Samples were placed individually in 40 mL brown glass vials sealed with a plastic cap with a polytetrafluoroethylene septum. To maximize the release of volatile compounds, vials were placed in a 45°C water bath for 1 h before analysis. Headspace SPME (HS–SPME) and 100 mm polydimethyl-siloxane-coated fibers were attached to a manual splitless injector (Supelco, USA) to adsorb the volatiles. Each sample was exposed to the fiber in the vial by inserting the injector into the sample headspace through the septum for 30 min at room temperature.

The fiber was then introduced into the injector of a GC–MS (GC6890N = MS5973; Agilent, USA). An HP-5MS capillary column (5% phenyl-methyl siloxane, 30 m × 0.25 mm, Agilent) was employed to conduct the analysis. Volatile compounds on the fiber were desorbed for 3 min in splitless mode. Helium was used as a carrier gas at a flow rate of 1.0 mL min⁻¹. The oven temperature was maintained at 40°C for 2 min and raised to 110°C at a rate of 4°C min⁻¹. This temperature was maintained for 2 min and increased to 150°C at a rate of 3°C min⁻¹, and held constant for 2 min. The temperature was then raised at 5°C min⁻¹ up to 200°C, which was maintained for 4 min. The temperatures of the injector, transfer line, ion source, and quadrupole were 250°C, 260°C, 230°C, and 150°C, respectively. The ionization potential of mass selective detector was 70 eV and the scanning range was 30–540 amu. The volatiles were identified based on comparison of their mass spectra with the NIST05 database using G1701DA ChemStation (Agilent).

The MSD Productivity ChemStation software (Agilent) was used to determine and identify the volatiles. Each component was identified using the NIST Mass Spectral Database 2008 spectral library. The 10 μL of 3-octanol at a concentration of 0.33 mg mL⁻¹ were used as the internal standard. Quantitative analysis was performed using the following formula: volatile component content (ng g⁻¹) = area

| Ecological group | Cultivar |
|------------------|----------|
| Hardy water lily  | Steven Strawn (1), Ray Davies (2), Lausanne (3), Arethusa (4), Perry’s Baby Red (5), Aflame (6), Alba (7), Princosi (8), Somptuosa (9), Venusta (10), Conqueror (11), Hollandia (12), Madame Wilton Gonnere (13), Sultan (14), Rembrandt (15), Virginia (16), Newton (17), Celebration (18), Rene Gerard (19), Ellisina (20), Marliacea Carnea (21), Aurora (22), Colorado (23), Mrs. Richmond (24), Mary Patricia (25), Sioux (26), Laydekeri Purpurata (27), Princess (28), Magnificent (29), Rosennymph (30), Laydekeri Fulgens (31), Indiana (32), Texas Dawn (33), Almost Black (34). |
| Tropical water lily | Capensis Thumb (R1), 42 (R2), 13 (R3), Ai Ji Bai (R4), 29 (R5), Colorata (R6), He Hua (R7), Albert Greenberg (R8), 49 (R9), General Perishng (R10), 22 (R11), Eldorado (R12), Lan He (R13), 9 (R14), 18 (R15), America (R16), Royal Purple (R17), Hu Die Lan (R18), Roxburgh (R19), Zanzibar (R20), Fo Shou Lian (R21), Mexicana Zuccarni (R22). |

**Notes:** Numbers in parentheses are the cultivar codes used in Figs. 1 and 3 and Supplementary Tables 1 and 2.
of each component × concentration of internal standard (mg mL\(^{-1}\)) × internal standard volume (μL) × 1000/(sample volume × internal standard peak area). Three measurements for each sample type were averaged.

Statistical analysis was performed on nine cultivars of water lily to test the hypothesis that the petals released the highest concentrations of volatile compounds. SPSS 11.5 (IBM, USA) was used to perform cluster analysis to compare cultivars based on the released volatiles. Discriminant analysis was employed to assess the classification.

RESULTS AND DISCUSSION

The water lily flowers contained up to 16,000 ng g\(^{-1}\) of volatiles. In most cultivars, the stamens contained the highest concentrations (p < 0.005; Fig. 1), accounting for up to 70% in tropical water lilies and for up to 90% in hardy water lilies. The volatile concentrations of other flower tissues had a maximum value of 1677 ng g\(^{-1}\). The volatile concentrations from pistils and petals were not significantly different in many cases, although they were generally smaller in pistils. In general, tropical water lilies contained greater concentrations of volatiles than the hardy cultivars.

Volatile Compounds

Listed in the order of elution, the principal compounds identified were benzene, 1,4-dimethoxy-2-methyl (324.52 ng g\(^{-1}\)), tridecane (20.47 ng g\(^{-1}\)), tetradecane (102.58 ng g\(^{-1}\)), pentadecane (683.37 ng g\(^{-1}\)), 7-hexadecene (30.91 ng g\(^{-1}\)), hexadecane (36.00 ng g\(^{-1}\)), 6,9-heptadecadiene (331.80 ng g\(^{-1}\)), heptadecane (170.70 ng g\(^{-1}\)), 9,17-octadecadienal (59.81 ng g\(^{-1}\)), 3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene(E,E,E) (62.64 ng g\(^{-1}\)), and alpha-farnesene (432.38 ng g\(^{-1}\)). Figure 2 presents an

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**Figure 1.** Volatiles in floral tissues of water lily flowers: nine hardy cultivars (coded 1–9) and nine tropical cultivars (R1–R9) as in Table 1 (n = 3); a, b, and c represent significant differences.
example chromatogram for the Fo Shou Lian cultivar. The first peak (labeled S) is the internal standard, 3-octanol.

A total of 117 volatile compounds were identified, composed of esters, alcohols, aldehydes, ketones, alkanes, and alkenes. Alkanes and alkenes were the most abundant, followed by aldehydes and ketones. Tropical water lilies released 79 volatile compounds while hardy water lilies released 71. The 39 alkenes, 15 alkanes, 2 esters, 10 alcohols, 5 aldehydes, and 1 ketone in hardy water lilies, and 43 alkenes, 22 alkanes, 7 esters, 2 alcohols, 5 aldehydes, and 3 ketones were identified in tropical water lilies. The concentrations and variety of volatiles were used to determine the compounds that contribute to the fragrance. In hardy water lilies, alcohols, aldehydes, and ketones (in decreasing order of importance) contributed most to the fragrance, but in tropical water lilies, esters, alcohols, terpenoids, aldehydes and ketones were more important. The cultivars therefore had different fragrances (Supplemental Tables S1 and S2).

Compared with the flowers of ornamental plants, water lilies released more volatile compounds but fewer aroma components. The variety of aroma components was also more limited. The primary aroma components were aldehydes and ketones in water lily with fewer terpenoids. Feng et al. (2008) detected fifty-three aroma components in *Rosa rugosa* ‘Tang Purple,’ and the main fragrant compounds were terpenoids. In the forty-nine aroma components of *Hedychium coronarium*, monoterpenes, sesquiterpenes, esters, ketones, and phenols contributed to the fragrance (Li et al. 2007).

Of the 117 volatile compounds found, 37 were common to tropical and hardy water lilies. Forty-two were unique to tropical water lily flowers, including 14 aromatic compounds. Hardy water lily flowers had 38 unique volatile compounds,
and 11 were aromatic. Of the compounds common to both cultivars, 19 were alkenes, 12 were alkanes, and the remaining 6 were aromatic. In hardy water lily flowers, most of the fragrant compounds were alcohols. Lilac alcohol, nerolidol, and 1,6,10,14-hedecatetraen-3-ol,3,7,11,15-tetramethyl were present in the highest concentrations. In tropical water lily flowers, esters, and ketones contributed the most to the fragrance, and of these ethyl benzoate, 2-heptadecanone, cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, acetic acid, and phenyl-methyl ester were the most common.

There were therefore important differences between the volatile compounds in the flowers of the two groups, with some difference in fragrances. The scent of hardy water lily flowers was characterized by nerolidol and lilac alcohol. In contrast, tropical water lily flowers derived their fragrance chiefly from ethyl benzoate, cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, acetic acid phenylmethyl ester, and 2-heptadecanone (F. Z. Zhang et al. 1999).

**Cluster Analysis**

For cluster analysis, within-group linkage and cosine clustering were used to ensure consistent results. The results were plotted as a dendrogram (Fig. 3). Based on the origin of the cultivars and the 117 volatile compounds produced, the 34 hardy water lily cultivars and 21 tropical water lily cultivars were divided into three clusters (W. T. Zhang and Yan 2004).

Cluster A was composed of 21 water lily cultivars characterized by the scents of rose and lilac. The volatile compounds contributing most to the formation of this cluster were heneicosane, undecane, tridecane, and octadecane. Of these, the most important were lilac alcohol B, 1,6,10,14-hedecatetraen-3-ol, 3,7,11,15-tetramethyl, and tetradecanal (Z. G. Li, Lee, and Shen 2006; Wang et al. 2012).

Cluster B consisted of 14 cultivars whose flowers smelled of lilac and oily. The volatile compounds contributing most to this cluster were 9-nonadecene, Z-5-nonadecene, 3-heptadecene, 6-tridecene, and 7-hexadecene. The two main aromatic compounds were 1,6,10-dodecatriene, 7,11-dimethyl-3-methylene, and lilac alcohol B.

Cluster C was composed of 21 tropical water lily cultivars with a sweet floral scent. The volatile compounds contributing most to this cluster were 3-(1,5-dimethyl-4-hexenyl)-6-methylene-cyclohexene, 1,6,10-dodecatriene, 7,11-dimethyl-3-methylene, kaurene, and bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-entenyl)-. The aromatic compounds 1,6,10-dodecatriene, 7,11-dimethyl-3-methylene, and 3-buten-2-one,4-(2,6,6-trimethyl-2-cyclohexen-1-yl) gave these cultivars their characteristic aroma (Mai 2000; Shu 2005). This cluster included 17 cultivars that released kaurene, a precursor of gibberellins.

The hierarchical cluster analysis separated the cultivars into those containing mainly alkanes (cluster A), mainly olefins (cluster C), and approximately equal quantities of alkanes and olefins (cluster B). This fits well with the differences between hardy and tropical water lilies discussed in the previous section, as clusters A and B comprised almost all hardy water lilies, while cluster C was composed of only tropical water lilies. The reason for the differences between clusters A and B requires further investigation.
Figure 3. Hierarchical cluster dendritic diagram of 56 water lily cultivars using within-group linkage and cosine clustering. Cultivars were categorized into clusters A, B, and C, and coded as in Table 1.
Discriminant Analysis

Discriminant analysis was used to divide the 56 water lily cultivars into three groups based on the 117 volatile compounds found in the flowers (Fig. 4). The results show that Sultan, Newton, Madame Wilfron Gonnere, Marliacea Carnea, Mrs. Richmond, Laydekeri Purpurata, Almost Black, Somptuosa, Rosennymph, Steven Strawn, Aflame, Perry’s Baby Red, 34, Arethusa, Sioux, Magnificent, Rembrandt, Texas Dawn, Mary Patricia, Princess, and Alba formed the first category. Princosi, Lausanne, Aurora, Venusta, Hollandia, Rene Gerard, Celebration, Ellisiona, Virginia, Laydekeri Fulgens, and Conqueror composed the second category. Ray Davies, Eldorado, Mexicana Zuccarni, Capensis Thumb, Colorata, Ai Ji Bai, 29, 13, 49, Albert Greenberg, 42, 22, General Pershing, Fo Shou Lian, 9, Lan He, 18, Hu Die Lan, Roxburgh, Royal Purple, and America were the third category. These results were similar to those of the cluster analysis, with groups 1 and 2 corresponding to clusters A and B and comprising mainly hardy water lilies, and group 3 corresponding to cluster C and consisting mainly of tropical water lilies.

Significance for Future Cultivars

Of studied cultivars, three had a larger variety of aromatic compounds in their stamens than the others. These were the tropical water lilies, Colorata, 34, and Ai Ji Bai. The principal fragrance compounds were esters such as acetic acid, phenylmethyl...
ester; cyclobutanecarboxylic acid, octyl ester; and benzoic acid, ethyl ester. Colorata and 34 flowers are pale blue, with a strong scent, while flowers of Ai Ji Bai are white. Colorata had a total anthocyanin concentration of 0.20 mg g\(^{-1}\). 34 had no anthocyanins, but flavonol and chalcone concentrations of 6.40 mg g\(^{-1}\), twice the value of Ai Ji Bai. This cultivar also contains the same types of pigments and glycosides as other water lilies with blue flowers, such as Fo Shou Lian and Lan He (Zhu et al. 2012). Anthocyanins contribute to the colors of flowers and provide anti-oxidant, anti-cancer, anti-allergic, and anti-ulcer functions, which has led to their introduction in the human diet. Anthocyanins produce red and purple petal colors. Flavonols belong to the anthoxanthins, which may cause the formation of yellow petals (S. W. Li 2001; Su 1996). Chalcone is a flavonoid which may be converted to a colorless flavanone under acidic conditions. The three cultivars mentioned above are therefore all potential candidates for producing blue fragrant water lilies. The hardy water lily Somptuosa may also be a good parent because it contains many fragrant compounds, such as 1,6,10-dodecatriene, 7,11-dimethyl-3-methylene, 9,12,15-octadecatrienial, \(cis,cis,cis\), -7,10,13-hexadecatrienial, lilac alcohol B, nerolidol, and tetradecane. This cultivar is white, but the tropical water lily parent may be selected to provide color and the fragrance, while Somptuosa may provide resistance to cold conditions. The selection of appropriate cultivars may allow the production of water lily varieties with desired characteristics of hardiness, an attractive color, and a desirable fragrance.

CONCLUSIONS

The volatiles in the flowers of 34 hardy and 22 tropical water lily cultivars were investigated. The stamen serves as the primary source for the release of volatile compounds. 117 volatile organic compounds were determined: alkanes were present in the highest concentrations, followed by alkenes, aldehydes, and ketones. Tropical water lily cultivars released greater concentrations of volatiles than hardy water lilies. Using within-group linkage in a cluster analysis, the 56 cultivars were divided into 3 clusters, each with its own fragrance compounds and its own characteristic aroma. The results were verified by discriminant analysis. The tropical water lily cultivars Colorata and 34, which have pale blue flowers and pleasant aromas, and Ai Ji Bai, which is white, may be used to produce blue fragrant water lilies. The hardy water lily cultivar Somptuosa may be employed to provide resistance to cold.

This study determined volatiles in water lily flowers by HS–SPME coupled with GC–MS. However, the mechanisms of formation and release of volatile compounds in plants, and of the activities of related enzymes, are complex and require further investigation. The use of plants for the commercial production of volatile compounds has the potential to be highly efficient and profitable, but many problems remain. As volatile compounds may affect fertilization, disease resistance, and productivity, the use of genetic engineering to alter flower traits to maximize production of these substances is potentially problematic. In-depth knowledge of associated processes and products is a prerequisite for further development.

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SUPPLEMENTAL MATERIAL

Supplemental data for this article can be accessed on the publisher’s website.

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