Floral Scent Diversity is Differently Expressed in Emitted and Endogenous Components in Petunia axillaris Lines

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INTRODUCTION

Petunia axillaris is considered to be one of the parental species of garden petunia and is widely distributed in temperate South America. This species comprises three allopatric infraspecific taxa: subsp. axillaris, subsp. parodii and subsp. subandina. Petunia axillaris flowers emit a strong musty scent at night only. This property is thought to help attract nocturnally active hawk moths as specific pollinators, resulting in maintenance of reproductive isolation in the wild from syntopic scentless species such as P. integrifolia (Ando et al., 2001).

Among the subspecies of P. axillaris are various lines emitting sensorially different scents (Hoballah et al., 2005). The analysis of variations in floral scent among genetically close individuals is a powerful approach to understanding the mechanisms for generating scent diversity. Such studies have been carried out for Magnolia, Ficus and Silene, and the qualitative and quantitative variations among these plants’ scents have been discussed in relation to their pollinators (Azuma et al., 2001; Grison-Pigé et al., 2002; Dötterl et al., 2005). To discover the basis of scent diversity in the genus Clarkia, the gene for linalool synthase was cloned, and its expression was compared between the scented species C. breweri and the scentless C. connexa. The results suggested that this single enzyme could account for the difference in floral scent between these closely related species (Pichersky et al., 1994, 1995; Raguso and Pichersky, 1995). The success of these studies prompted the present investigation into the mechanisms leading to floral scent diversity in P. axillaris.

Floral scent is a mixture of volatile compounds, including aromatics, terpenoids and fatty acid derivatives, the composition of which determines the scent properties of a flower (Kudsen et al., 1993). In petunia, floral scents are composed of aromatic compounds biosynthesized from the common precursor phenylalanine (Batragna et al., 2004; Fig. 1). Recently, two enzymes involved in the metabolism of floral aromatic volatiles have been identified and characterized from Petunia hybrida ‘Mitchell’: S-adenosyl-Lmethionine:benzoic acid/salicylic acid carboxyl methyltransferase (PhBSMT1/2; Negre et al., 2003) and benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase (PhBPBT; Batragna et al., 2004). In addition, a novel R2R3-type MYB transcription factor, ODORANT1, has been identified that regulates the coordinate expression of genes encoding enzymes involved in diurnal fluctuation of
scent emission (PhODO1; Verdonk et al., 2005). Enzymes and regulatory factors such as these may be involved in generating the diversity of floral scents in Petunia axillaris.

The boiling points of most of the volatile compounds identified as floral scents range from 150 to 350 °C. These substances are present in floral tissues mostly as liquids or in solution, and only a portion is emitted as floral scents. Therefore, evaporation is regarded as an important process regulating floral scent emission. In a previous study, a comparison of the relative amounts of emitted and endogenous scent compounds of one line of Petunia axillaris subsp. axillaris indicated that emission was basically a physical phenomenon (Oyama-Okubo et al., 2005). Furthermore, the abundance of endogenous compounds in this line was determined by the rates of biosynthesis and degradation, and was reflected in the profile of emitted compounds according to the vapour pressure of each compound. These findings suggested that emitted and endogenous components must both be analysed independently to gain information about the relative contributions of evaporation and endogenous production to the final scent composition. Based on this concept, 13 specimens of Petunia axillaris of varying scents were chosen for analysis of the basis of floral scent diversity.

MATERIALS AND METHODS

Plant material

Plants of Petunia axillaris (Lam.) Britton, Stern & Poggenb. subsp. axillaris, subsp. parodii (Steere) Cabrera and subsp. subandina T. Ando (Ando, 1996) were raised from seeds collected from several natural populations in Uruguay, Brazil and Argentina. Thirteen individuals from among these plants were chosen at random and were found to vary in scent strength (Table 1 and Fig. 2).

In the specimen code, the initial letter and number designate the herbarium specimen code, with the letter indicating the country of the origin: A (Argentina), B (Brazil) or U (Uruguay). The subspecies abbreviation and the individual code are indicated by the following letters and numbers (AXI, subsp. axillaris; PAR, subsp. parodii; and SUB, subsp. subandina). These lines were vegetatively propagated in a greenhouse 2 months before the experiments. Just before the experiments, the plants were acclimated for at least a week in a growth chamber at a constant temperature of 25 °C under a photosynthetic photon flux density of about 250 μmol m⁻² s⁻¹ and a 12 h/12 h (0600 h–1800 h light/1800 h–0600 h dark) photoperiod.
Collection of emitted volatiles

Emitted volatile compounds were collected by the headspace method (Oka et al., 1999). Tedlar Bags (500 mL volume, GL science, Tokyo, Japan) were placed over the *P. axillaris* flowers to be sampled. A constant stream of air (approx. 500 mL min\(^{-1}\)) was filtered through activated charcoal (Oka et al., 1999) and then piped through the bag, and volatiles were collected with Tenax-TA (150 mg, GL science, Tokyo, Japan) traps. The relative humidity in the bags was approx. 50 %. Headspace collection was started at 2300 h and finished at 2400 h. Thereafter, the sampled flowers were harvested to determine the endogenous

### Table 1. Origins of the 13 specimens of *P. axillaris*

| Subsp. | Specimen code | Origin (Herbarium*) |
|--------|---------------|---------------------|
| 1      | *axillaris*   | B58AXI9 Brazil: Rio Grande do Sul, São Lourenço do Sul (MBM, GHSP, Ando) |
| 2      | *axillaris*   | B3120AXI10 Brazil: Rio Grande do Sul, Rio Pardo (GHSP, Ando) |
| 3      | *axillaris*   | B3120AXI13 Brazil: Rio Grande do Sul, Rio Pardo (GHSP, Ando) |
| 4      | *axillaris*   | U242AXI4 Uruguay: Canelones (MVFA, Ando) |
| 5      | *axillaris*   | U1AXI Uruguay: Montevideo (MVFA, Ando) |
| 6      | *parodii*     | B298PAR7 Brazil: Rio Grande do Sul, Santana do Livramento (GHSP, Ando) |
| 7      | *parodii*     | U220PAR9 Uruguay: Artigas (MVFA, Ando) |
| 8      | *parodii*     | U220PAR10 Uruguay: Artigas (MVFA, Ando) |
| 9      | *parodii*     | U238PAR11 Uruguay: Paysandú (MVFA, Ando) |
| 10     | *parodii*     | U238PAR15 Uruguay: Paysandú (MVFA, Ando) |
| 11     | *subandina*   | A130SUB9 Argentina: San Juan, Valle Férril (BAB, Ando) |
| 12     | *subandina*   | A139SUB2 Argentina: La Rioja, Famatina (BAB, Ando) |
| 13     | *subandina*   | A203SUB8 Argentina: Salta, Santa Victoria (BAB, Ando) |

*Abbreviations of herbaria are after Holmgren et al. (1990) except GHSP (Centro de Pesquisas História Natural, São Paulo, Brazil) and Ando (temporary collection of Toshio Ando).
concentrations of scent compounds, following the experimental design of the previous study (Oyama-Okubo et al., 2005).

Preparation of emitted volatiles

Scent compounds were extracted from the Tenax-TA four times, using pentane and diethyl ether (5 mL each) alternately (Oyama-Okubo et al., 2005). After addition of ethyl decanoate (20 mg) as an internal standard, the extract was dried over anhydrous sodium sulfate and concentrated at 40°C in a water bath.

Preparation of endogenous volatiles

Flowers were harvested at 2400 h after collecting the emitted volatile compounds, and the flower tissues (excluding the calyx) were frozen in liquid nitrogen and ground in a mortar. The resulting powder was extracted twice with pentane (5 mL each) in a microwave oven (700 W) for 20 s. Ethyl decanoate was added as an internal standard. The extract was then dehydrated with anhydrous sodium sulfate and concentrated at 40°C in a water bath.

GC–MS and GC analysis

Capillary gas chromatography–mass spectrometry (GC–MS) was performed using an Agilent 6890N gas chromatograph coupled to an Agilent 5973N Mass Selective Detector (Agilent Technologies, Wilmington, DE, USA) (Oyama-Okubo et al., 2005). The GC was equipped with a splitless injector and a DB-WAX capillary column (30 m in length, 0.25 mm i.d. and 0.25 μm film thickness). The column oven temperature was kept at 45°C for the first 2 min, then increased by 3°C min⁻¹ to 220°C and maintained at 220°C for 10 min. Injection, interface and ion source temperatures were 250, 280 and 250°C, respectively. Helium was used as the carrier gas. GC analysis was performed using an Agilent 6850 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) monitored by a flame ionization detector (FID). Analytical conditions were the same as for GC–MS described above. The amounts of each volatile were calculated by comparison with the peak area of the internal standard. The compound identifications were made by NIST02 library search provided with the GC–MS software. Identification was substantiated on the basis of mass spectra and retention time by subjecting authentic samples to GC–MS analysis under the same conditions.

RESULTS

Components of emitted volatiles

Benzaldehyde, methyl benzoate, phenyl acetaldehyde, benzyl acetate, benzyl alcohol, 2-phenylethanol, iso-eugenol and benzyl benzoate were detected as emitted volatiles in many of the Petunia axillaris lines (Fig. 3A; details are shown in Table 2). The total amounts ranged from 0.6 nmol per flower in A130SUB9 to 228.1 nmol per flower in U242AXI4. Methyl benzoate was the most predominant compound in all lines and accounted for 34–100% of the total amount of volatiles emitted. In other words, the observed variation in the total amount of emission was almost entirely accounted for by the variation in the amount of methyl benzoate emission. The second most

![Graph A](https://example.com/graphA.png)

![Graph B](https://example.com/graphB.png)

**Fig. 3.** Composition of volatile compounds in 13 specimens of *P. axillaris*. (A) Emitted volatiles. (B) Endogenous volatiles. Specimens are listed in order of the total amount of emission. Specimens are defined in Table 1.
# Table 2. Amounts of floral scent compounds emitted per flower from 13 specimens of P. axillaris

| Specimen   | Benzaldehyde (bp 179°C) | Phenyl acetaldehyde (bp 195°C) | Methyl benzoate (bp 200°C) | Benzyl alcohol (bp 205°C) | Benzyal acetate (bp 206°C) | 2-Phenyl ethanol (bp 221°C) | iso-Eugenol (bp 266°C) | Vanillin (bp 285°C) | Benzyal benzoate (bp 324°C) | Total |
|------------|--------------------------|---------------------------------|-----------------------------|---------------------------|-----------------------------|----------------------------|------------------------|-----------------|----------------------------|-------|
| B58AX19    | 41 ± 0.9                 | nd                              | 23.6 ± 3.0                  | 1.6 ± 0.4                 | nd                          | 1.3 ± 0.3                  | 10.2 ± 3.7             | nd              | 1.1 ± 0.2                 | 41.8 ± 7.0 |
| B1320AXI10 | 0.7 ± 0.4                | nd                              | 4.2 ± 2.1                   | 0.2 ± 0.2                 | nd                          | 0.9 ± 0.5                  | 0.2 ± 0.2              | nd              | nd                         | 6.3 ± 3.0 |
| B1320AXI13 | 1.2 ± 0.2                | nd                              | 7.6 ± 1.6                   | 1.4 ± 0.5                 | nd                          | 1.9 ± 0.7                  | 2.0 ± 0.8              | nd              | nd                         | 14.2 ± 1.5 |
| U242AXI4   | 19.2 ± 2.0               | 4.4 ± 0.9                        | 182.3 ± 13.9               | nd                        | nd                          | 9.2 ± 1.1                  | 3.8 ± 0.7              | nd              | 8.9 ± 1.2                 | 227.8 ± 16.5 |
| U1AXI      | 17.5 ± 5.3               | 7.1 ± 4.4                        | 104.3 ± 29.2               | 2.6 ± 0.3                 | nd                          | 2.0 ± 0.8                  | 12.8 ± 1.7             | nd              | 4.9 ± 1.3                 | 151.4 ± 38.5 |
| B298PAR7   | nd                       | 53.0 ± 9.1                       | 1.4 ± 1.4                  | 1.0 ± 1.0                 | nd                          | 1.8 ± 0.4                  | 0.5 ± 0.5              | 57.8 ± 12.3     |                            |       |
| U220PAR9   | 3.7 ± 2.1                | 0.3 ± 0.3                        | 21.4 ± 8.9                 | 7.6 ± 4.3                 | 9.7 ± 6.0                   | 0.6 ± 0.1                  | 0.1 ± 0.1              | nd              | 43.5 ± 21.2              |       |
| U220PAR10  | 4.2 ± 1.6                | nd                              | 19.8 ± 6.0                 | 1.8 ± 0.4                 | nd                          | 0.4 ± 0.1                  | 0.2 ± 0.1              | nd              | 26.4 ± 7.2               |       |
| U238PAR11  | 12.7 ± 4.3               | nd                              | 52.5 ± 18.2                | 20.7 ± 7.2                | 5.8 ± 2.1                   | nd                        | 3.9 ± 0.8              | nd              | 92.2 ± 31.7              |       |
| U238PAR15  | 0.8 ± 0.8                | nd                              | 50.7 ± 11.4                | 4.7 ± 2.5                 | nd                          | nd                        | 3.5 ± 0.2              | nd              | 63.6 ± 14.4              |       |
| A130SUB9   | nd                       | 0.6 ± 01                         | nd                          | nd                        | nd                          | nd                        | nd                    | nd              | 0.6 ± 0.1                |       |
| A139SUB2   | 2.1 ± 0.8                | nd                              | 4.5 ± 1.8                  | 5.8 ± 3.2                 | nd                          | 0.4 ± 0.2                  | nd                    | nd              | 1.2 ± 0.4                | 14.1 ± 5.2 |
| A203SUB8   | 10.0 ± 3.9               | nd                              | 46.1 ± 7.6                 | 15.4 ± 4.7                | 4.6 ± 0.7                   | nd                        | nd                    | nd              | 76.1 ± 16.6              |       |

Mean value ± s.e.m. (nmol per flower). bp, boiling point; nd, not detected.

# Table 3. Endogenous levels of floral scent compounds per flower from 13 specimens of P. axillaris

| Specimen   | Benzaldehyde (bp 179°C) | Phenyl acetaldehyde (bp 195°C) | Methyl benzoate (bp 200°C) | Benzyl alcohol (bp 205°C) | Benzyal acetate (bp 206°C) | 2-Phenyl ethanol (bp 221°C) | iso-Eugenol (bp 266°C) | Vanillin (bp 285°C) | Benzyal benzoate (bp 324°C) | Total |
|------------|--------------------------|---------------------------------|-----------------------------|---------------------------|-----------------------------|----------------------------|------------------------|-----------------|----------------------------|-------|
| B58AX19    | 0.2 ± 0.2                | nd                              | 12.3 ± 1.2                 | 7.9 ± 1.9                 | nd                          | 6.9 ± 0.5                  | 85.2 ± 17.4            | 0.2 ± 0.2       | 19.9 ± 29                  | 132.5 ± 22.9 |
| B1320AXI10 | 0.2 ± 0.8                | nd                              | 20.8 ± 0.8                 | 0.3 ± 0.2                 | nd                          | 5.5 ± 1.5                  | 13.1 ± 3.4             | nd              | nd                         | 53.7 ± 12.1 |
| B1320AXI13 | 0.5 ± 0.1                | nd                              | 26.0 ± 0.3                 | 1.6 ± 0.5                 | nd                          | 10.9 ± 1.0                 | 6.8 ± 1.4              | nd              | 2.2 ± 0.1                 | 24.5 ± 2.1 |
| U242AXI4   | 4.2 ± 1.1                | 2.8 ± 1.1                       | 105.0 ± 11.7               | 10.4 ± 1.7                | nd                          | 28.8 ± 3.7                 | 38.1 ± 6.8             | nd              | 176.6 ± 28.1              | 365.9 ± 46.2 |
| U1AXI      | 4.9 ± 1.0                | 5.3 ± 2.3                       | 120.7 ± 24.0               | 7.2 ± 0.6                 | nd                          | 22.1 ± 4.2                 | 173.6 ± 30.0           | 2.2 ± 2.2        | 183.8 ± 38.4              | 519.9 ± 82.2 |
| B298PAR7   | nd                       | nd                              | 55.1 ± 5.8                 | 5.9 ± 2.1                 | nd                          | 3.5 ± 1.8                  | nd                    | 24.7 ± 7.1       | 891 ± 15.5                |       |
| U220PAR9   | 0.6 ± 0.1                | nd                              | 146.1 ± 1.1                | 14.8 ± 3.0                | nd                          | 2.6 ± 0.8                  | 12.0 ± 0.2             | nd              | 0.2 ± 0.1                 | 33.9 ± 4.4 |
| U220PAR10  | 0.7 ± 0.1                | nd                              | 141.0 ± 1.5                | 4.1 ± 1.1                 | nd                          | 2.7 ± 1.1                  | 0.8 ± 0.2             | nd              | 23.2 ± 1.1                |       |
| U238PAR11  | 1.9 ± 0.6                | nd                              | 399.1 ± 10.5               | 80.6 ± 24.2               | 2.8 ± 0.9                   | 1.0 ± 0.5                  | 1.6 ± 0.7             | nd              | 127.8 ± 34.8              |       |
| U238PAR15  | nd                       | nd                              | 361.0 ± 3.7                | 20.8 ± 4.6                | nd                          | 1.4 ± 0.3                  | 33.9 ± 4.0             | nd              | 31.4 ± 5.0                | 1236 ± 16.7 |
| A130SUB9   | nd                       | nd                              | nd                          | nd                        | nd                          | nd                        | nd                    | nd              | nd                         |       |
| A139SUB2   | 0.9 ± 0.1                | nd                              | 12.0 ± 0.3                 | 14.3 ± 9.3                | nd                          | 4.1 ± 0.7                  | nd                    | nd              | 8.4 ± 3.3                 | 28.8 ± 16.3 |
| A203SUB8   | nd                       | nd                              | 184.2 ± 2.1                | 25.1 ± 3.2                | nd                          | nd                        | nd                    | nd              | 43.6 ± 5.3                |       |

Mean value ± s.e.m (nmol per flower). bp, boiling point; nd, not detected.
Fig. 4. Relationship of boiling points to the natural logarithms of the ratios of the emitted to endogenous concentrations of volatile compounds in *P. axillaris* subsp. *axillaris* U242AXI4. 1, Benzaldehyde (boiling point 179 °C); 2, phenyl acetaldehyde (boiling point 195 °C); 3, methyl benzoate (boiling point 200 °C); 4, 2-phenyl ethanol (boiling point 221 °C); 5, *iso*-eugenol (boiling point 266 °C); 6, benzyl benzoate (boiling point 324 °C). Bars indicate the s.e.m.

abundant compound was benzaldehyde in U220PAR10, benzyl alcohol in A203SUB8 and U238PAR15, and *iso*-eugenol in B58AXI9. 2-Phenylethanol and the other compounds were minor components in all lines.

**Components of endogenous volatiles**

Most compounds that were detected in the emitted samples were also detected as endogenous components in the flowers harvested at 2400 h (Fig. 3B; details in Table 3). Benzaldehyde was detected as an emitted component only in A203SUB8, B1320AXI10, B298PAR7 and U238PAR15, as was methyl benzoate in A130SUB9. Conversely, some compounds were detected only in the endogenous samples, such as benzyl benzoate in B1320AXI13 and vanillin in U220PAR10. For each line, the endogenous composition was markedly different from the emitted profile. The proportion of higher boiling point compounds, e.g. *iso*-eugenol or benzyl benzoate, was higher in the endogenous composition than in the emitted profile; the opposite was observed for the lower boiling point compounds, e.g. benzaldehyde and methyl benzoate. The total amounts of endogenous volatiles ranged from undetectable in A130SUB9 to 519.9 nmol per flower in U1AXI. No direct correlation was found between the total amounts of the emitted and endogenous components.

**Relationship between boiling points and emission ratios**

Plant lines that yielded relatively stable qualitative data for at least three compounds were chosen for further analysis. For each compound, the logarithm of the ratio between the amount emitted and the endogenous amount was plotted against the boiling point. The compounds included in the analysis were benzaldehyde, phenyl acetaldehyde, methyl benzoate, benzyl alcohol, 2-phenyl ethanol, *iso*-eugenol and benzyl benzoate. As predicted from physical theory, the analyses showed a nearly linear negative correlation between he emission ratio and boiling point for all the plant lines. The data for U242AXI4 are a typical example (Fig. 4). The regression equations for the various lines are as follows: y = −0.014x + 2.969 (A139SUB2), y = −0.014x + 1.954 (U238PAR15), y = −0.024x + 4.763 (U220PAR10), y = −0.028x + 5.539 (B58AXI9), y = −0.030x + 5.635 (U1AXI), y = −0.031 + 7.005 (U238PAR11), y = −0.032x + 6.713 (U242AXI4), y = −0.037x + 7.482 (B1320AXI13) and y = −0.037x + 7.429 (U220PAR9).

**DISCUSSION**

Similar to results previously obtained for the line U1AXI (Oyama-Okubo *et al*., 2005), all of the floral scent compounds detected in the headspace and flower tissue of the 12 additional lines of *P. axillaris* used in the present study were again aromatic compounds. The mixture of emitted volatiles contained predominantly methyl benzoate, resulting in qualitative homogeneity with quantitative variation (Fig. 3A). In other words, the total emission of each line was greatly affected by the amount of methyl benzoate emission. The correlation between sensory strength at 2400 h and the total amount of emitted volatiles seems to be accounted for by this property (data not shown).

No association was found between the morphological characteristics of the subspecies of *P. axillaris* and their floral scent properties (discussed below). Climatic conditions may not exert the same selection pressure on floral scent as they do on plant morphology (Fig. 2). Interestingly, U242AXI4 and U1AXI, which emitted relatively large amounts of scent compounds, are the only self-incompatible lines among the specimens studied here. This finding suggests an ecological role for floral scents in helping to attract pollinators to cross-pollinate self-incompatible lines (Ando *et al*., 1998; Tsukamoto *et al*., 1998; Kokubun *et al*., 2006).

For each line, the endogenous volatile composition was markedly different from the emitted composition (Fig. 3A, B). Both qualitative and quantitative variation was found among the endogenous profiles. Based on the observed endogenous profiles, the C6–C1 pathway leading to methyl benzoate and benzyl benzoate is active in all lines (Fig. 1). The qualitative variation probably arises from differences in the activity of the C6–C2 pathway, leading to 2-phenyl ethanol and phenyl acetaldehyde, and the C6–C3 pathway, leading to *iso*-eugenol. The quantitative variation seems to depend mainly on the activity of a common pathway upstream of these three branched pathways. This possibility is currently being investigated by analysing the concentrations of phenylalanine and other precursors.

The boiling points of the substances detected in this study range from 179 to 324 °C (Tables 2 and 3). The ratio of the emitted amount to the endogenous amount of each...
compound is correlated with its vapour pressure (Fig. 4). A negative correlation between the emitted/endogenous ratio and boiling point was obtained for all examined lines, indicating that emission of floral scents can be understood as primarily a physical phenomenon in *P. axillaris*, as the previous study concluded (Oyama-Okubo et al., 2005). Lower boiling point compounds were always over-represented in the emission profile relative to their endogenous concentrations, reducing the qualitative variation in the emitted component among the lines. Variation in the regression equations describing the relationship between emission ratios and vapour pressures was observed among the *P. axillaris* lines. These differences may suggest the operation of a physiological mechanism that regulates the evaporation process.

This study demonstrates that floral scent diversity is generated by variation in both endogenous production and evaporation of volatile compounds. So far, studies of floral scent have tended to concentrate on biosynthesis of the compounds, including the isolation of biosynthetic enzymes and the cloning of their genes. An understanding of the evaporation process in each plant is also necessary for a full comprehension of floral scent production.

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