



Floral scent evaluation of segregating lines of *Alstroemeria caryophyllaea*



Danilo Aros^a, Natasha Spadafora^b, Michela Venturi^b, Gerardo Núñez-Lillo^c, Claudio Meneses^c, Lisa Methven^d, Carsten T. Müller^{b,*}, Hilary Rogers^{b,*}

^a Facultad de Ciencias Agronómicas, Universidad de Chile, Santa Rosa 11.315, La Pintana, Santiago, Chile

^b School of Biosciences, Cardiff University, Main Building, Park Place, Cardiff CF10 3AT, UK

^c Centro de Biotecnología Vegetal, Facultad de Ciencias Biológicas, Universidad Andrés Bello, República 217, Santiago, Chile

^d Department of Food and Nutritional Sciences, University of Reading, Whiteknights, PO Box 226, Reading, Berkshire RG6 6AP, UK

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ABSTRACT

Floral scent plays an important role in attracting and guiding pollinators and is composed of a bouquet of volatile organic compounds (VOCs). *Alstroemeria* is a commercially important cut flower, however breeding efforts have focussed on flower colour and size rather than scent. Recently analysis of two scented cultivars derived from the scented *Alstroemeria caryophyllaea* revealed a surprising divergence in VOC profiles. Here 13 scented lines of *A. caryophyllaea* derived from selfing were characterized including morphology, evaluation of the floral scent through GC–MS and sensorial analysis. Leaf shape, stem length, flower size, shape, colouration and productivity all varied between lines. Sensorial analyses indicated that two lines (C013 and C017) were most highly rated for their appearance and C017 was also scored highest for its scent contrasting with C004 which scored lowest. Analyses of scent bouquets from six of the lines revealed 23 terpenoid compounds. All lines showed the same most abundant compound putatively identified as β -trans-ocimene, and three further compounds were discriminatory amongst the lines following PCA. Genomic organization of *AlstroTPS*, a previously identified myrcene synthase, showed substantial polymorphism between lines. The multifactorial characterization performed in this study showed differences among the lines confirming parental heterozygosity.

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1. Introduction

The main function of floral scent is to attract and guide pollinators (Ando et al., 2001; Reinhard et al., 2004; Dudareva et al., 2004; Jürgens et al., 2003) although Dudareva and Pichersky (2000) suggest that flowers can also emit specific volatile organic compounds (VOCs) to repel non-beneficial insects, for example pollen or nectar ‘thieves’ or destructive insects. Furthermore some flowers are able to emit VOCs with anti-microbial or anti-herbivore activity to protect their reproductive organs (Friedman et al., 2002; Hammer et al., 2003; Farré-Armengola et al., 2013). Scent thus forms a key component of the ‘pollination syndrome’. This includes other pollination rewards and attractants such as petal colour and form, and nectar production, and has been an important driver in floral evolution (Fenster et al., 2004; Galliot et al., 2006).

Floral scent is composed of a bouquet of VOCs, including aromatic compounds, terpenoids and fatty acid derivatives (Knudsen et al., 2006). The composition and relative amounts of the VOCs in the final bouquet determines the overall scent perceived by the human olfactory system. Considering both the important role of floral scent in plant reproduction and its aesthetic value, composition of floral scent has been characterized in several ornamental species such as *Dianthus inoxianus* (Balaoa et al., 2011), snapdragon (*Antirrhinum majus*; Dudareva et al., 2005), carnation (*Dianthus* spp.; Jürgens et al., 2003) and petunia (*Petunia axillaris*; Kondo et al., 2006).

Sensorial analyses (Morinaka et al., 2001) provide information on the aesthetic qualities of the bouquet, based on the specific recognition and discrimination between a vast variety and combinations of odour molecules, by a large gene family of odorant receptors connected to the human olfactory system (Zhao and Firestein, 1999). Humans have very varied thresholds for different VOCs, so concentration in the mixture does not directly relate to what is perceived. For some compounds our olfactory system can be more sensitive than analytical tools, such as GC–MS, for

* Corresponding author. Tel.: +44 02920876352; fax: +44 02920874305.
E-mail address: rogershj@cf.ac.uk (H. Rogers).

evaluating floral scent (Hinterholzer and Schieberle, 1998). In addition, GC–MS alone cannot predict what the perceived odour type will be as combinations of compounds can have unique and specific aromas that are not the same as the individual compounds. On the other hand, GC–MS provides a very sensitive measure of bouquet composition including chemical identification of specific VOCs (Yeon Oh et al., 2008; Kondo et al., 2006; Klahre et al., 2011). Furthermore it is highly reproducible and a total of 1719 chemical compounds have been identified from samples of floral scent in 991 species of flowering plants to date (Knudsen et al., 2006). In addition to identification and quantification by GC–MS and sensory evaluation of what is perceived by the human, it is also important to determine whether the aroma is well liked. Therefore, selection of new ornamental lines benefits from a combination of sensory and analytical approaches.

Alstroemeria is a commercially important cut flower originating in South America with main centres of diversity in Brazil and Chile (Bayer, 1987; Muñoz and Moreira, 2003). Breeding has mainly targeted flower size and colour through interspecific hybridization (Buitendijk et al., 1995; Burchi et al., 1997), mutant selection (Aros et al., 2012a), development of polyploids (Lu and Bridgen, 1997; Takayuki, 1999) and more recently transformation (Akutsu et al., 2004). However little attention has been played to scent, as few native species are scented and breeding programmes have used a narrow genetic background (Aros et al., 2006). Recently there has been increased interest in scented flowers (Pichersky and Dudareva, 2007) and two scented *Alstroemeria* cultivars: ‘Sweet Laura’ and ‘Ajax’ have been developed by crosses between the Brazilian scented *Alstroemeria caryophyllaea* and non-scented lines (Pounders et al., 2003; R. Meijles personal communication). VOC analyses of these genetically related cultivars (Aros et al., 2012b) revealed a surprising divergence in VOC profiles. Both cv. ‘Ajax’ and *A. caryophyllaea* produce a single major terpenoid compound, though different to each other, while cv. ‘Sweet Laura’ produces at least three major and many more minor terpenoid compounds including (*E*)-ocimene, (*E*)-caryophyllene, humulene and myrcene. Bouquet composition has been previously reported to vary even between cultivars of the same species (e.g. in *Petunia*, Klahre et al., 2011), although the precise composition of VOC bouquets as a result of segregation has not been extensively studied (Andargie et al., 2014).

Terpenes are one of the most common VOCs in floral scent, particularly mono- and sesquiterpenes (Knudsen et al., 2006). Terpenoids comprise a large number of primary and mostly secondary metabolites with a wide variety of structural types and their biosynthesis is controlled by the action of terpene synthases (TPS). TPS genes have been identified from a wide range of species (Bohlmann et al., 2000; Dudareva et al., 2003; Yang et al., 2013) to examine evolutionary relationships within the gene family (Trapp and Croteau, 2001; Lee and Chappell, 2008). In *Alstroemeria* Aros et al. (2012b) identified a terpene synthase (*AlstroTPS*) with myrcene synthase activity, which is highly expressed in cv. ‘Sweet Laura’ tepals during peak VOC production. Gene structure of *AlstroTPS* placed it amongst class III terpene synthases according to the classification of Trapp and Croteau (2001), but with 5 introns instead of the 6 introns common for this class. Expression of *AlstroTPS* has also been confirmed in *A. caryophyllaea* tepals (Aros et al., 2012b), but its genomic structure has not been characterized in this species.

An analysis is presented here of a population of F1 lines produced through self pollination of *A. caryophyllaea* revealing morphological polymorphism followed by a multifactorial characterization of their floral scent, using both GC–MS and sensorial analysis to identify levels of variation in bouquet composition as well as aesthetic qualities. An analysis of genomic organization of *AlstroTPS* in these lines is also presented showing substantial polymorphism at the level of scent-related genes.

2. Materials and methods

2.1. Plant material

Self-pollination was performed on an *A. caryophyllaea* individual inflorescence. Seeds were collected from dried fruits 60–75 DAP and were soaked in warm water (30–40 °C initial temperature) for 48 h and then stratified at 4 °C for 2 weeks. Plants were grown at the University Botanical and Research Garden, Cardiff University (Cardiff, UK) in a greenhouse at a minimum of 14 °C. Humidity and light were not controlled. After the stratification, seeds were sown in pots containing a mixture of sand (25%), coarse grit (25%) and compost (50%). A total of 17 new lines were obtained of which 13 were morphologically characterized and the 5 most promising, from an ornamental point of view, were selected for further evaluation. Each line was vegetatively propagated in pots containing the same soil mixture as above.

2.2. Morphological characterization

Morphological characterizations were performed on 13 lines (C001, C002, C003, C004, C005, C006, C008, C009, C010, C013, C014, C016, C017) when plants were in full bloom during two flowering seasons (2008/2009 and 2009/2010). Leaf shape was assessed according to Hickey and King (1997). Stem length was considered as the distance between the soil level and the highest flower. Flower size was measured as height × width (cm). Width was considered as the maximum distance between the external margins of the two highest inner tepals or between the external margins of the two basal outer tepals. Height was considered as the distance between the margins of the highest outer tepal and the basal inner tepal. Flower shape was assessed as the ratio of height: width. Flower colour was assessed as the main two or three colours covering the six tepals. Flower markings were assessed by the identification of dots/stripes covering the tepals in terms of location (inner or outer tepals), abundance (rare, medium or abundant) and shape (dots or stripes). Pollen abundance was assessed as abundant, medium or poor.

2.3. Sensorial evaluation of scent

Seventy untrained participants were asked to evaluate five different scented lines of selfed *A. caryophyllaea* (C001, C004, C010, C013 and C017). The demographic composition represented both genders and five age ranges (Table 1). Participants were asked to score their flower purchasing frequency (Table 1) and their purchasing priorities in terms of the relative importance given to stem length, flower size, flower colour, floral scent and vase life by answering the question “when you buy/look at flowers the character you appreciate most is”. A 5-point category scale was used of “strongly disagree”; “disagree”; “neutral”; “agree” and “strongly agree”. Samples consisted of three floral stems cut in proportion to their natural length and placed in a 500 ml measuring cylinder in water. Lines were evaluated individually by each participant in terms of liking of appearance and scent. Both were rated using a standard 9-point hedonic category scale: “Dislike extremely”; “dislike very much”; “dislike moderately”; “dislike slightly”; “neither like nor dislike”; “like slightly”; “like moderately”; “like very much” and “like extremely”. Intensity of the scent was rated using a 9-point intensity category scale: “Extremely low”; “very low”; “moderately low”; “slightly low”; “neither high nor low”; “slightly high”; “moderately high”; “very high” and “extremely high”. Appearance was rated after scent characters and participants were asked to wait for about 1 min between evaluating each sample.

Table 1
Demographic distribution and consuming habits of volunteers in sensorial analyses.

| | % |
|------------------------------------|-----------------------|
| Age (years) | |
| 18–22 | 52 |
| 23–28 | 17 |
| 29–35 | 20 |
| 36–42 | 1 |
| 43+ | 10 |
| Gender | |
| Male | 34 |
| Female | 66 |
| Flower purchasing frequency | |
| Weekly | 1 |
| Monthly | 11 |
| Special occasions | 74 |
| Never | 14 |
| | Sensorial scale (1–5) |
| Character most appreciated | |
| Stem length | 2.75 a |
| Flower size | 3.28 ab |
| Vase life | 3.73 b |
| Floral scent | 4.35 c |
| Flower colour | 4.73 c |

Different letters indicate statistically significant differences $P < 0.05$.

2.4. GC–MS evaluation

VOC sampling was performed on six selfed *A. caryophyllaea* scented lines (C001, C003, C004, C008, C013 and C017) as described by Aros et al. (2012b). Briefly, three open flowers stage 4–5 (anthesis) were enclosed in 250 ml polystyrene bottle with 30 ml of distilled water and VOCs collected by solid phase microextraction (SPME). A 50/30 μm divinylbenzene/carboxene/PDMS composite SPME silica fibre on 2 cm fused silica (grey fibre, Sigma–Aldrich, Gillingham, UK) was exposed to the head space for 4 h. VOCs were desorbed from fibres for 2 min at 240 °C in the injection port of the gas chromatograph (GC 6890, Agilent, Wokingham, UK) and separated on a 30 m, 0.25 mm ID capillary column over 0.25 μm HP-5MS (Agilent) using the following temperature programme: initial temperature 35 °C for 2.5 min, first step increase 3 °C/min to 140 °C, second step increase 16 °C/min to 300, followed by 1.5 min at 300 °C constant. Electron Impact mass spectra were recorded in full scan mode from 35 to 500 m/z (70 eV, MSD 5873, Agilent) coupled to a GC (GC 6890, Agilent). To monitor system performance and provide retention time references for calculation of retention (Kovats) indices (RIs). A C_8 – C_{20} alkane standard was analyzed regularly (0.1 μL direct injection, Supelco, Gillingham, UK). Data were analyzed using AMDIS 2.71. Signals were integrated using total ion count and areas normalized. Putative identification was achieved by comparison of mass spectra with the NIST mass spectra library (v. 2.0, 2011) taking into account available information on Kovats indices.

2.5. Genomic organization

A DNeasy Plant mini kit (QIAGEN, Venlo, The Netherlands) was used to extract genomic DNA from young leaves of selfed *A. caryophyllaea*, scented lines C001, C003, C004, C006, C010, C013, C017 and the non-scented *Alstroemeria psittacina* which was included as a comparison. Three primer sets were used to amplify the full length sequence of the *AlstroTPS* gene: TPS-3' (Fw 5'-ATGGCTTCCCATCTTCTCTTC-3' and Rev 5'-CCCGTTGTTTCGT CATAGAACC-3'), TPS-I (Fw 5'-CGCCGAATGTTATGTTACGCATCA-3' and Rev 5'-CCCCTATCGAAATCCCTGCATTCT-3') and TPS-5' (Fw 5'-GCTTTGCACGATTACAGTGGT-3 and Rev 5'-AGGTTCCACCA-CATTGCCA-3) Amplification was carried out in a PTC-100 machine

Table 2
Leaf shape observed in 13 selfed lines of *A. caryophyllaea*.

| Line | Leaf shape |
|--------------|----------------------|
| DANCAR 001 | Lanceolate |
| DANCAR 002 | Lanceolate |
| DANCAR 003 | Linear |
| DANCAR 004 | Elliptic |
| DANCAR 005 | Elliptic |
| DANCAR 006 | Lanceolate |
| DANCAR 008 | Elliptic |
| DANCAR 009 | Lanceolate |
| DANCAR 010 | Lanceolate |
| DANCAR 013 | Lanceolate |
| DANCAR 014 | Lanceolate |
| DANCAR 016 | Lanceolate |
| DANCAR 017 | Linear |
| Parent plant | Linear to lanceolate |

(MJ Research, St Bruno, Canada) using GoTaq Master Mix (Promega, Madison, USA) following the thermal profile: 4 min at 95 °C; 35 cycles of 95 °C for 30 s, 57 °C for 20 s, and 72 °C for 40 s; and a final extension at 72 °C for 5 min. PCR products were purified from agarose gels using a GeneJET Gel Extraction Kit (Thermo Scientific, Loughborough, UK), sequenced and sequences aligned using Geneious 3.6.2. Variations on the conserved domains of the gene were detected using InterProScan software (The European Bioinformatics Institute, Cambridge, UK) available on line (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>).

2.6. Statistical analysis

The scales described for hedonic and intensity evaluation were translated into scores. For the hedonic scale the scores were: dislike extremely=1 and like extremely=9; for intensity scale the scores were: extremely low=1 and extremely high=9. Standard deviation (STEDV) and standard error (SE) were calculated. Analysis of variance (ANOVA) was performed through SPSS 17.0 for Windows, using Tukey's HSD (Honestly Significant Difference) test for multiple pairwise comparisons with a significance level of 0.05. Principal Component Analysis (PCA) (Jolliffe, 1986) was applied to the VOC dataset to summarize graphically differences in VOC abundance between the *A. caryophyllaea* lines and also to assess biological reproducibility. To prevent the VOC peaks of greatest intensity from dominating the PCA model, data were first standardized (value-average/STDEV) thus giving all VOCs a normal distribution. Univariate analysis (Kruskal–Wallis non-parametric N -way ANOVA) was also performed to test for significant VOC differences between the lines.

3. Results

3.1. Selfed progeny of *A. caryophyllaea* show morphological differences in both leaves and flowers

The most common leaf shape was lanceolate, observed in eight out of the 13 lines evaluated (C001, C002, C006, C009, C010, C013, C014 and C016). Elliptic leaves were observed in three of the lines (C004, C005 and C008), while linear leaves were observed only in C003 and C017 (Table 2). C004 showed the longest stem with an average of 70.6 cm ($n=49$), that was statistically different ($P < 0.05$) to six of the other lines, among them C001 and C003, which showed the shortest stems with averages of 35.5 ($n=34$) and 39.1 cm ($n=37$) respectively (Fig. 1). C004 had flowers of greatest height (7.48 cm, $n=49$) and shared greatest width (5.45 cm, $n=49$), with C001 (5.51 cm, $n=38$) (Fig. 2). The smallest flowers were observed in C005, C006 and C014. Both mean height and width were lowest in C005 with averages of 5.0 and 4.1 cm respectively

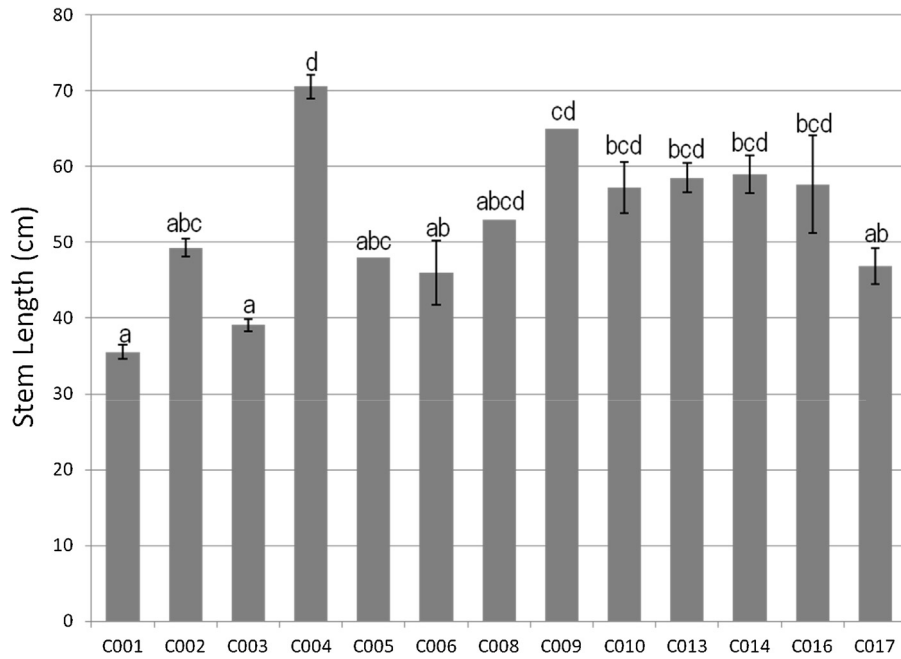


Fig. 1. Average stem length (\pm SE, $n=3-49$; the number of stems evaluated (n) depends on the productivity of each line. Different letters indicate statistically significant differences $P<0.05$) observed in selfed *A. caryophyllaea* lines, evaluated during two periods of flowering (2008/2009 and 2009/2010).

although there was a lot of variability especially in height and thus the flower size of C005 was not statistically distinguishable from the other two lines. The ratio between height and width showed average values of >1 in all cases indicating a vertical elliptical shape. C001 presented the most rounded flowers (ratio 1.13), although

not statistically different from six other lines, while C016 the most elliptical flowers (ratio 1.44) with no statistical differences with five other lines (Table 3). C006 produced the highest number of single flowers per floral stem (average value=6.5), significantly higher than all the other lines, excepting C009 and C014, both with an

Flower Size

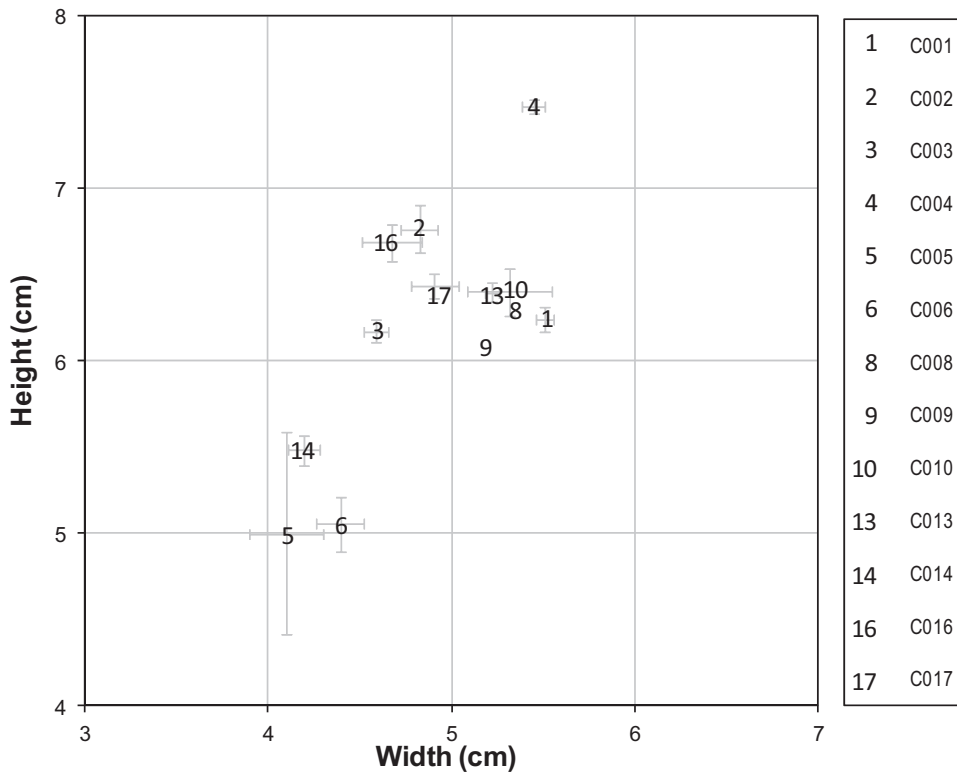


Fig. 2. Average flower heights and widths (\pm SE, $n=4-54$; the number of flowers evaluated (n) depends on the productivity of each line) evaluated on 13 selfed lines of *A. caryophyllaea*, during two periods of flowering (2008/2009 and 2009/2010).



Fig. 3. Flowers observed in 13 selfed lines of *A. caryophyllaea* and the parental line. Below each picture the average number of flowers per floral stems produced (\pm SE, $n=4-54$; the number of floral stem evaluated (n) depends on the productivity of each line.) is shown. Evaluations were performed during two periods of flowering (2008/2009 and 2009/2010). (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

Table 3

Average ratios calculated from the relationship between flower height and width (\pm SE, $n=4-54$)^a evaluated on 13 selfed lines of *A. caryophyllaea*, during two periods of flowering (2008/2009 and 2009/2010).

| Line | Ratio (height:width) | SE |
|------|----------------------|------|
| C001 | 1.13 a | 0.01 |
| C002 | 1.41 d | 0.01 |
| C003 | 1.35 cd | 0.01 |
| C004 | 1.38 cd | 0.02 |
| C005 | 1.22 abc | 0.14 |
| C006 | 1.15 ab | 0.03 |
| C008 | 1.21 abc | 0.00 |
| C009 | 1.17 ab | 0.00 |
| C010 | 1.22 abc | 0.04 |
| C013 | 1.23 abc | 0.01 |
| C014 | 1.31 bcd | 0.02 |
| C016 | 1.44 d | 0.05 |
| C017 | 1.32 bcd | 0.03 |

^a The number of flowers evaluated (n) depends on the productivity of each line. Different letters indicate statistically significant differences $P < 0.05$.

average of five flowers per stem. The poorest line was C017 producing only 3.6 flowers per stem, although only statistically different to the highest producer, C006 (Fig. 3).

All the lines were white in background colour, while different intensities of pink, red and green were seen as cover colour on the margins of the tepals (Fig. 3). Markings over the tepals were abundant in the majority of the lines, excepting on lines C001, C006 and C009 where they were scored as 'rare' (Fig. 3). Characterization of the anthers showed one sterile line with no pollen (C006), while anthers of the rest of the lines produce morphologically normal pollen (data not shown).

3.2. The selfed *A. caryophyllaea* lines differ in their sensorial attributes

Sensorial analysis was performed to determine whether there were any perceived significant differences in appearance or scent bouquet between five of the selfed *A. caryophyllaea* lines. The 70 participants in the analysis were first asked to rate their purchasing/rating priorities revealing that both flower colour and scent were highly rated and significantly different from stem length, flower size and vase life (Table 1).

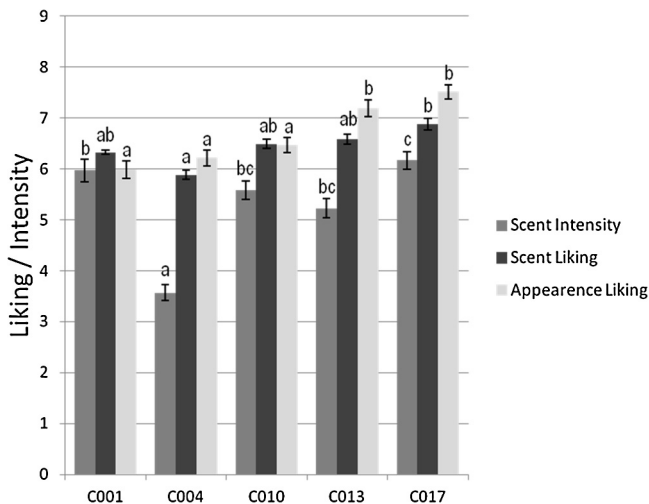


Fig. 4. Means of floral scent liking and intensity and appearance liking (\pm SE, $n=70$) evaluated on 5 selfed lines of *A. caryophyllaea*. The scale ranges from 'dislike extremely' (=1) to 'like extremely' (=9) for liking; and from 'extremely low' (=1) to 'extremely high' (=9) for intensity. Letters above same-shaded bars indicate statistically significant differences ($n=70$; $P < 0.05$).

C013 and C017 were most highly rated in liking for their appearance (significantly compared to the other lines), and C017 also scored most highly for liking and intensity of its floral scent bouquet although it was only significantly different from the lowest rated line, C004 (Fig. 4). C004 was rated lowest for both scent intensity and liking of scent as well as liking of appearance, although liking of scent was only significantly different to C017 and appearance was only significantly lower than C017 and C013.

3.3. Scent profiles differ amongst the selfed *A. caryophyllaea* lines

VOCs from four of the lines that had been evaluated sensorially (C001, C004, C013 and C017) were analyzed by GC–MS as well as two further lines (C003 and C008). In total 23 compounds, all terpenoids, were detected and putatively identified with high confidence (Table 4). All lines showed the same major peak as well as several minor peaks. The most abundant peak was identified as the

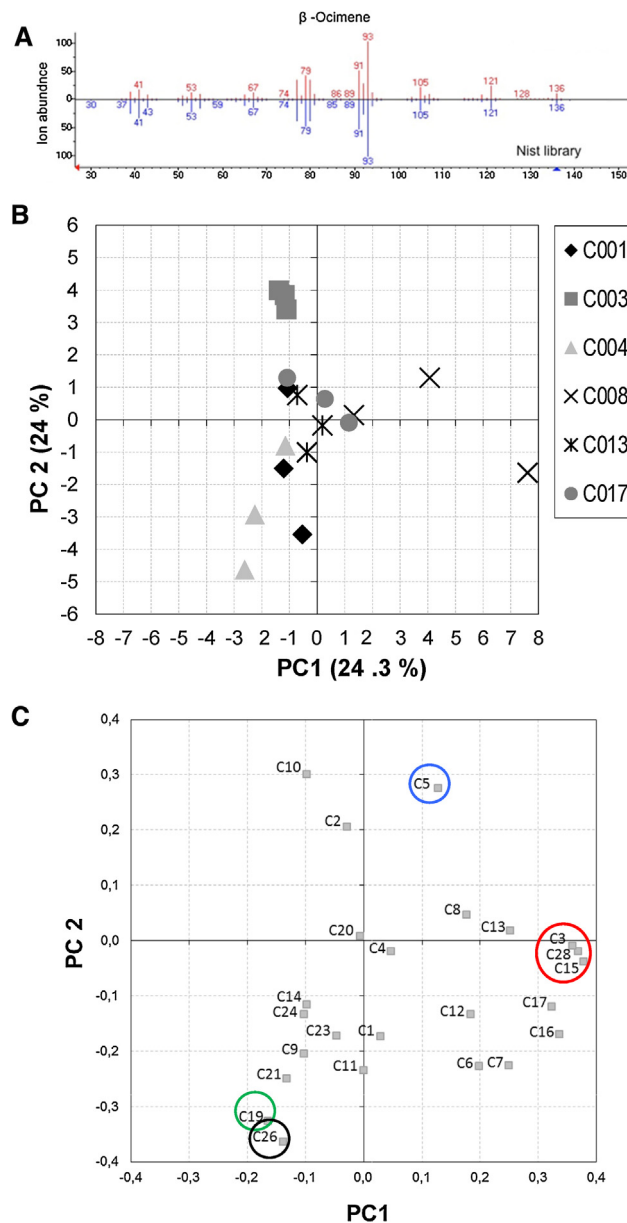


Fig. 5. VOC data from six selfed *A. caryophyllaea* lines analyzed by GC–MS. (A) Mass spectrum of the peak for β -ocimene from line C001 and the match from NIST library. (B) PC biplot based on three biological replicates. Identification names of the PC applied (C1–C28) are listed in Table 4.

monoterpene β -trans-ocimene (C10) (RI: 1038), based on a NIST library search and on the Kovats index (1040) reported by Porta et al. (1999) (Fig. 5A). Three compounds: 3-Thujene (C3), o/m Ethylanisol (C15) and an unidentified sesquiterpene (sesquiterpene 7, (C28) were only detected in line C008 ($P < 0.05$).

Sabinene (C5) was detected differentially in three lines, being most abundant in C003 followed by C008 and C017, but was below detection in lines C001, C004, and C013 ($P < 0.0005$). Another unidentified sesquiterpene, (sesquiterpene 2, C19), was detected in lines C001 and C004 but was below detection in the other lines. Eudesma-4(14),11-diene (C25, circled black in Figs. 5C and 6C) and sesquiterpene 6 (C26) were undetectable in C003 and higher in lines C001 and C004, though both were only significantly higher in C004 compared to C008, C013 and C017 ($P < 0.05$). No significant differences in levels of Myrcene were found between lines (Fig. 6E).

PCA analysis of VOC levels showed that 48% of variance of lines were explained by PC1 and PC2. The biplot of the components showed clear separation of lines C003, C004 and C017 along PC2, strong clustering of line C003 and separation of line C008 on PC1 (Fig. 5B). The same biplot showing the chemical components (Fig. 5C) reflected the role of compounds mentioned above; position of C3, C28 and C15 clustered strongly at the right end of PC1 (circled red in Fig. 5C), while sabinene (circled blue in Fig. 5C) was discriminatory for lines C003, C008 and C017. C19 (circled green) was strongly discriminatory for lines C001 and C004 while C26 (circled black) was a strong indicator for line C004.

3.4. Genomic organization

Although myrcene levels did not appear to differ between the lines, as this is the only terpene synthase gene available in *Alstroemeria*, it was used to investigate whether the differences in VOC profiles were reflected in polymorphism amongst the lines in the structure of scent-related genes. The genomic organization of the myrcene synthase is constituted by two domains, near the N terminus is the terpene synthase domain, and at the C terminal end of the protein is the terpene synthase metal binding domain (Fig. 7). This gene has six exons and five introns in *Alstroemeria* and for all the *A. caryophyllaea* lines and *A. psittacina* evaluated the presence of an insertion of 105 bp was observed in the fourth exon of the myrcene synthase genetic sequence (Fig. 7). The insertion was located at the same position in all seven of the selfed *A. caryophyllaea* evaluated

showing the same sequence. In the fifth exon a deletion of 61 bp was identified only in lines C001, C004, C006, C010 and C013 (Fig. 7), generating a stop codon in the middle of the terpene synthase metal binding domain of the gene and producing a shorter protein. Finally an insertion of 4 bp in *A. psittacina* was observed, changing the reading frame of the gene and generating a stop codon in the first part of the fourth exon of the gene. This insertion would produce a truncated protein lacking most of the terpene synthase metal binding domain of the myrcene synthase, and the insertion was not present in any of the *A. caryophyllaea* selfed lines.

4. Discussion

Although most of the lines evaluated were similar to the parental plant and to the botanical descriptions available for *A. caryophyllaea* (Assis, 2004; Foster, 1945), some characters showed segregation suggesting a possible underlying heterozygosity within the parent plant. This suggestion is supported by the natural outcrossing habit described for species of this genus (Aizen and Basilio, 1998; Cavieres et al., 1998; Valdivia and Niemeyer, 2005). Some of this segregation was observed in the differences in leaf shape, stem length, flower size, shape and colour. Line C004 appeared distinctive in several morphological characters including leaf size, stem length and flower size, however lines C006, C009 and C014 were more productive in terms of flower number compared to the other lines, and C017 flowers were of the most intense colour (Fig. 3). QTLs have been identified in *Alstroemeria aurea* for leaf length and width, flower colour and size (Han et al., 2002), and may be relevant to the characters seen in this study. The main pigments found in *Alstroemeria* flowers are anthocyanins, flavonoids and carotenoids, with the orange/red colours as seen in *A. caryophyllaea* due mainly to anthocyanins, specifically 6-hydroxypelargonidin glycosides (Tatsuzawa et al., 2003). An analysis of the inheritance of flower colour in *Alstroemeria* species and cultivars (Nørbæk et al., 1998) showed a complex composition and inheritance of various anthocyanin pigments. However Han et al. (2002) inferred from a QTL analysis of *A. aurea* that flower colour inheritance was relatively simple. The differences in the colour intensity and patterning seen here would support a more complex control given the variability revealed from selfing.

Although flower colour was the most important character influencing purchasing of flowers by the participants of the sensorial

Table 4

Volatile compounds detected in *Alstroemeria* danca flowers via solid phase microextraction, gas chromatography–mass spectrometry (SPME GC–MS).

| Compound | VOC# | Common name | RI (DB-5) | RT | RI present in terpenedata |
|--|------|-------------------------|-----------|---------|---------------------------|
| Hexanal | C2 | Hexanal | 799.9 | 4.2327 | 771 |
| 2-Methyl-5-(1-methylethyl)-bicyclo[3.1.0]hex-2-ene | C3 | 3-Thujene | 922.3 | 8.6598 | 932 |
| α -Pinene | C4 | α -Pinene | 926.8 | 8.8608 | 936 |
| α -Phellandrene | C5 | Sabinene | 967.5 | 10.705 | 973 |
| α -Pinene | C6 | Myrcene | 990.2 | 11.7346 | 987 |
| 3-Isopropenyl-5,5-dimethyl-cyclopentene | C7 | 3-Carene | 1006.1 | 12.4776 | 1000 |
| Unidentified Monoterpene | C8 | Monoterpene | 1028.2 | 13.4796 | |
| D-Limonene | C9 | D-Limonene | 1024.2 | 13.2675 | 1025 |
| 3,7-Dimethyl-, (E)-1,3,6-octatriene | C10 | β -Trans-ocimene | 1038.8 | 14.0722 | 1029 |
| 11-Methyl-4-(1-methylethyl)-4-cyclohexadiene | C12 | Moslene | 1057.4 | 14.957 | |
| (+)-4-Carene | C13 | Terpinolene | 1081.1 | 16.3223 | 1082 |
| 1-Ethyl-6-ethylidene-cyclohexene, | C1 | p-Ethylanisol | 1091.5 | 16.7012 | |
| 1-Ethyl-6-ethylidene-cyclohexene (20) | C15 | o/m-Ethylanisol | 1070 | 15.5046 | |
| 1,3,5,5-Tetramethyl-1,3-cyclohexadiene | C16 | Allo-ocimene | 1128.5 | 18.5267 | |
| 2,6-Dimethyl-, (E,Z)-2,4,6-octatriene (terpene) | C17 | Allocymene | 1140 | 19.0927 | |
| Unidentified sesquiterpene | C19 | Sesquiterpene 2 | 1387.6 | 30.6225 | |
| Unidentified sesquiterpene | C20 | Sesquiterpene 3 | 1410.9 | 31.1126 | |
| Caryophyllene | C21 | Caryophyllene | 1411.5 | 31.9224 | |
| Unidentified sesquiterpene | C23 | Sesquiterpene 5 | 1387.6 | | |
| α -Caryophyllene | C24 | α -Caryophyllene | 1446.3 | 33.2645 | |
| Eudesma-4(14),11-diene | C25 | Eudesma-4(14),11-diene | 1478.8 | 34.6524 | |
| Unidentified sesquiterpene | C26 | Sesquiterpene 6 | 1488.4 | 35.0607 | |
| Unidentified sesquiterpene | C28 | Sesquiterpene 7 | 1445.7 | 33.1326 | |

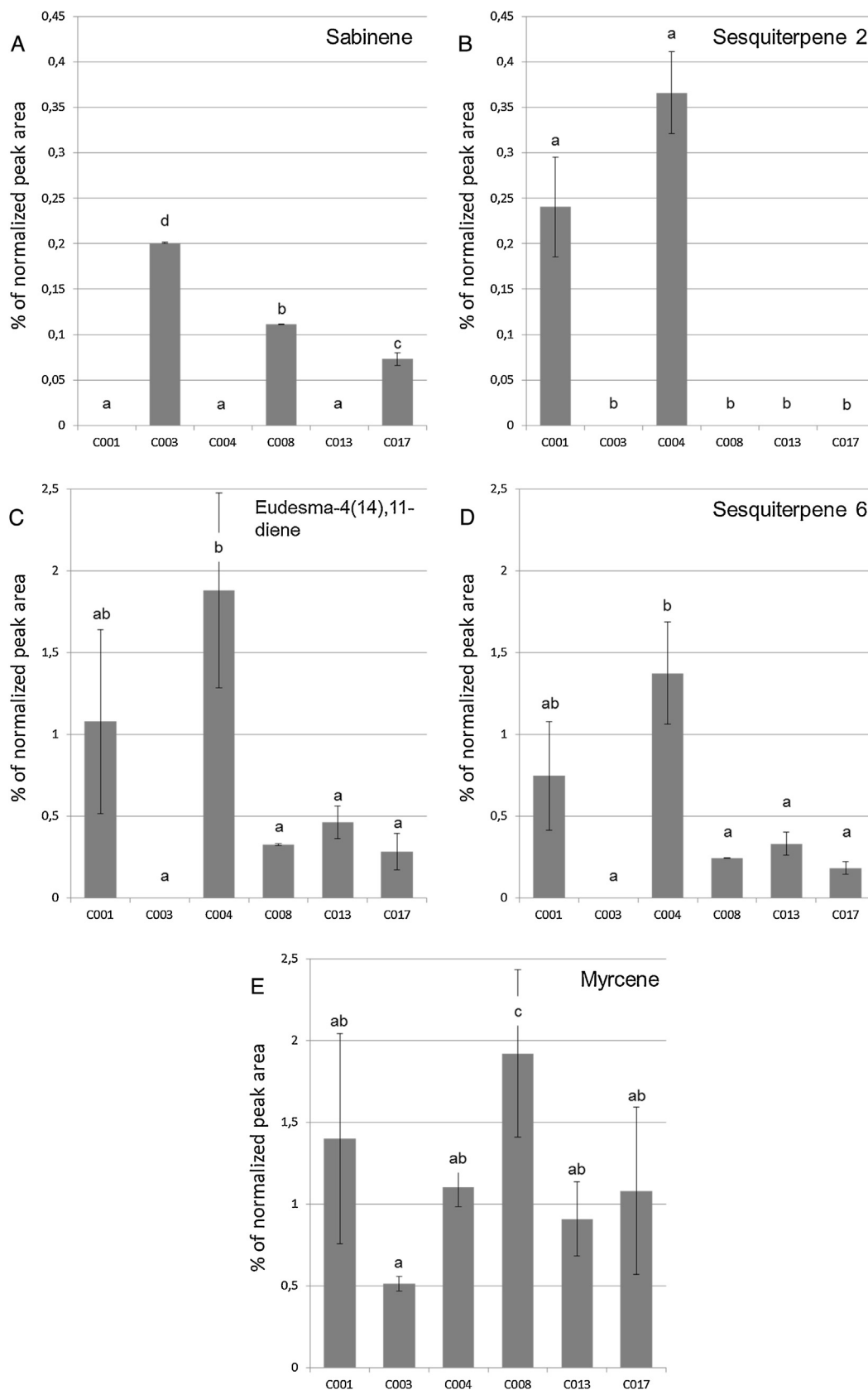


Fig. 6. Average relative abundance of selected compounds in selfed *A. caryophyllaea* lines (A–D) as identified by PCA and Myrcene (E). Letters above bars indicate statistically significant differences ($n=3$; $P<0.05$).

study here, floral scent (in terms of liking and intensity) also scored highly. Floral appearance, floral scent liking and scent intensity were therefore scored. Both genders were well-represented in the sample, which is important as olfaction is generally superior in females (Brand and Millot, 2001). The design of the sensorial tests asked participants to score the three criteria separately, however an interaction between floral scent and appearance cannot be excluded as the scent evaluation was performed in the presence of the visual stimulus and vice versa and the two stimuli are known to interact (Gottfried and Dolan, 2003). Line C017 was more valued in the sensorial analysis both for appearance and floral scent bouquet, and was one of the three lines rated highest for floral intensity suggesting a link between these three criteria. Furthermore, line C004 was distinct from C017 in all three criteria and was least valued in floral scent. VOC analyses were performed to explore further the differences in sensorial value. All five lines analyzed showed the same major peak that was identified as the monoterpene β -ocimene. This was distinct from the single peak previously identified from the parental *A. caryophyllaea* (Aros et al., 2012b) which could not be unequivocally identified, but which had a different RI value to β -ocimene. Based on PCA and ANOVA analysis distinctness of the VOC profiles of some of the lines tested could be ascribed to a small number of compounds. Of particular interest was the higher level of sabinine in line C0017 compared to line C004 and the higher levels of two sesquiterpenes putatively identified as eudesma-4(14),11-diene (also known as β -selinene) and selina-3,5-diene in line C004 compared to line C017. These three compounds are thus good candidates for a differential sensorial evaluation of these two lines. Sabinene is emitted by a number of scented species contributing to the spicy fragrance of nutmeg and some varieties of black pepper (Shulgin et al., 1967; Schenk and Lamparsky, 1981; Richard et al., 1971). β -Selinene is a minor component of celery seed oil VOCs (Macleod et al., 1988; Macleod and Ames, 1989) and is described as having a “herbal” or “green, fragrant” odour. Selina-3,5-diene is a minor component of cashew nut (*Anacardium occidentale*) leaf essential oils (Dzamic et al., 2009). Further sensorial analyses linked to GC separation would be needed to determine whether these compounds do in fact make an important contribution to the sensorial value of the lines. Myrcene was detected as a minor component of the VOC profile in all the lines analyzed, and although there were apparent differences between lines, these were not statistically significant and are thus unlikely to contribute to the sensorial value differences.

All the lines evaluated, including *A. psittacina*, showed the same genomic organization composed by 6 exons and 5 introns for the *Alstroemeria* myrcene synthase gene (*AlstroTPS*). This organization is the same shown by Aros et al. (2012b) for *A. cv.* ‘Sweet Laura’ which clustered as an anomalous member of class III according to the classification of Trapp and Croteau (2001). However differences in the size of exons and introns were observed among the lines: an insertion of 105 bp in the fourth exon was observed in both the scented *A. caryophyllaea* lines and the non-scented *A. psittacina*, but not in the scented *A. cv.* ‘Sweet Laura’. Therefore this insertion seems to be a common pattern for the Brazilian native species and not related to the scent character. Furthermore a deletion of 61 bp was identified in the fifth exon of the myrcene synthase only in five of the seven lines analyzed (Fig. 7). This indicates polymorphism at this gene locus, suggesting that this gene is heterozygous in the parent plant and the two alleles are segregating amongst the selfed F1 lines. The fact that the insertion noted in all the F1 lines introduces a stop codon, thus truncating the open reading frame suggests that this gene may not be functional. This is in contrast to *Alstroemeria cv.* Sweet Laura which was derived from a cross between Chilean non-scented *A. aurea* and scented *A. caryophyllaea* (Pounders et al., 2003; M. Bridgen, personal communication). This indicates that the functional *AlstroTPS* in *cv.* Sweet Laura may in fact derive from the

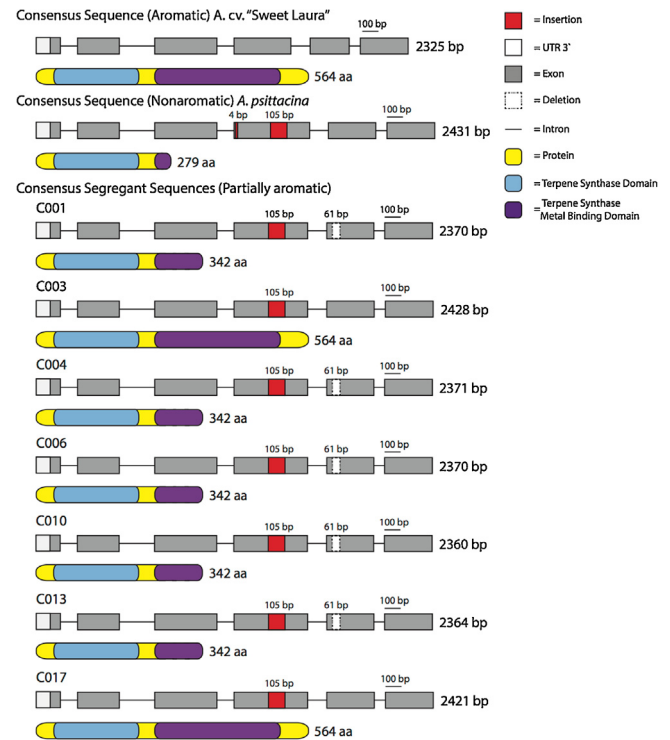


Fig. 7. Genomic organization of *AlstroTPS* observed in 7 selfed lines of *A. caryophyllaea*, compared to the scented *A. cv.* Sweet Laura and the non-scented *A. psittacina*.

unscented *A. aurea* parent, and perhaps have been activated in the new genetic background. It also indicates that other myrcene synthases are active in *A. caryophyllaea* since myrcene was detected in the scent bouquet by GC–MS.

5. Conclusions

In conclusion this work has shown that selfed progeny of the scented Brazilian *A. caryophyllaea* all resemble the parent plant but differ in a number of important morphological features including flower colour, size, productivity and markings, reflecting parental heterozygosity. All the F1 selfed lines are scented, but differ in their VOC profiles from each other and from their scented parent. Furthermore, the differences in flower morphology and scent result in differing sensorial value. Thus, even without performing crosses, some of the underlying diversity in *Alstroemeria* can be released for the development of new scented lines.

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