Fragrance Analysis of Two Scented *Hippeastrum* Species

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Abstract. We report the analysis of floral fragrance volatiles from two scented amaryllis species, *Hippeastrum brasilianum* and *Hippeastrum parodii*. Whereas the headspace of *H. brasilianum* is dominated by a large peak of (Z)- β -ocimene (88% of total peak area), *H. parodii* has two large peaks comprising 78%, identified as eucalyptol (1,8-cineole, 30%) and (Z)- β -ocimene (48%). The two species also differ in other constituent compounds. Overall, *H. parodii*, although producing less total volatile organic compounds (VOCs), has a more diverse bouquet. This species also exhibits an inverse pattern of emission between eucalyptol and β -ocimene, both daily and across the 4-day period from anthesis to senescence of the flower. We compare our results with reports for a complex amaryllis hybrid and a bat-pollinated species, *Hippeastrum calyptratum*. The hybrid had a very different complement of emissions. The bat-pollinated species shared some of the same constituent volatiles as *H. brasilianum* and *H. parodii*, but at lower percentages, and emitted a more diverse assortment of compounds. We conclude that both *H. brasilianum* and *H. parodii* attract lepidopteran pollinators but suggest that *H. parodii* may also attract other insects. We briefly discuss floral fragrance from the perspective of breeding amaryllis.

After color, fragrance is the second most important phenotypic character in consumer choice of floral products (Chandler and Brugliera, 2011). Flower fragrance is a complex character involving a combination of molecules synthesized endogenously by plants. Floral odors generally are a blend of several chemical substances that fall into three categories: terpenoids, phenylpropanoids/benzenoids, and fatty acid derivatives (Dudareva and Negre, 2005; Knudsen et al., 1993). Although the pathways leading to the final products have not been completely characterized, common modifications such as hydroxylation, acetylation, and methylation have been described (Amrad et al., 2016; Dudareva and Pichersky, 2000, 2006; Ramya et al., 2017).

As yet, there is no single model system for the elucidation of the genetics of floral fragrance synthesis or expression in plants (Guterman et al., 2002), although much progress has been made (Dudareva et al., 2013). Floral fragrance is not a character that can be observed visually and is rarely consistent biochemically (and thus, by inference, genetically as well). The flowers of *Arabidopsis*, a model for so many plant genetic pathways, do not produce many VOCs (Vainstein et al., 2001). Consequently, genetic characterization of flower scent is still incomplete (Dudareva and Negre, 2005; Dudareva and Pichersky, 2000, 2006). A few floral fragrance genes have

been identified from plants with very fragrant flowers (e.g., *Clarkia breweri* and *Antirrhinum majus*; Dudareva et al., 1996; Vainstein et al., 2001). Verdonk et al. (2005) isolated a transcription factor that regulates floral scent biosynthesis in *Petunia*, and the large MYB transcription factor family has been implicated in fragrance emission regulation in other plants as well (Ramya et al., 2017).

Flowers often contain a mixture of VOCs that combine to make a characteristic scent. For example, the four o'clock flower (*Mirabilis jalapa* L.) scent contained as its major component the terpenes (E)- β -ocimene with detectable amounts of β -myrcene, (Z)- β -ocimene, (E)-epoxy-ocimene, an aliphatic (Z)-3-hexenyl acetate, and a benzenoid benzyl benzoate (Effmert et al., 2005). Minor components included alloocimene, (Z)-3-hexenol, and methyl salicylate (Effmert et al., 2005). Pink snapdragon (*A. majus* L.; Dudareva et al., 2000), an orchid (*Calanthe sylvatica* Lindl.; Delle-Vedove et al., 2011), *Silene* L. spp. (Jurgens et al., 2002), tree peony (*Paeonia suffruticosa* Andrews; Li et al., 2012), *Hieracium* L. subgen. *Piloselloidea* (W.D.J. Koch) Peterm. (Feulner et al., 2009), the Peruvian lily (*Alstroemeria* L. hybrids; Aros et al., 2012), and *Dianthus inoxianus* Gallego (Balao et al., 2011) are examples of plants that emit some mixture of terpenoids, phenylpropanoids, benzenoids, or fatty acid derivatives as major components of floral scent.

Plants emit floral scents not only to attract pollinators but also to attract predators of insect herbivores or as repellents against herbivores (Dudareva and Negre, 2005; Gershenzon et al., 2000; Pare and Tumlinson, 1997). Different

insects exhibit a wide range of daily activity patterns (Herrera, 1990). In turn, many plants emit floral volatile compound in a rhythmic pattern (Fenske and Imaizumi, 2016). The bouquet of fragrant volatile compounds emitted may change over time and at different locations within an inflorescence. For example, *Hosta plantaginea* Asch. and *Protea* L. spp. emit a different combination of volatile chemicals as the flower ages (Liu et al., 2014; Steenhuisen et al., 2010). Day or night variation in scent components has been documented for several species. *Dianthus inoxianus*, snapdragon, *Odontoglossum constrictum* Lindl., *Citrus medica* L., *Hoya carnosae* R. Br., *Stephanotis floribunda* Brongn., and *Nicotiana* L. spp. all exhibit nocturnal or diurnal volatile emission pattern variations (Altenburger and Matile, 1988; Balao et al., 2011; Kolosova et al., 2001; Loughrin et al., 1990; Matile and Altenburger, 1988; Raguso et al., 2003).

Hippeastrum Herb. consists of 60–70 species distributed exclusively in tropical and subtropical America (Meerow, 2009). Most of the species do not produce noticeable floral fragrance, which is hypothesized to be a recessive trait in the genus (Meerow, 2009). Hummingbirds have been the most frequently observed flower visitors in *Hippeastrum* (Araujo and Sazima, 2003; Freitas and Sazima, 2006; Sazima et al., 1996). Fragrance has been observed notably in white (rarely yellow), long-tubed species such as *Hippeastrum brasilianum* (Traub and J.L. Doran) Dutilh, *Hippeastrum elegans* (Spreng.) H.E. Moore, *Hippeastrum fragrantissimum*, and *Hippeastrum parodii* Hunz. and Cocucci. Several pink-flowered, long-tubed species also produce fragrance (e.g., *Hippeastrum dorniae* (Traub) Meerow). The bat-pollinated (Vogel, 1969) epiphytic species *Hippeastrum calyptratum* (Ker Gawl.) Herb., has a very different floral odor, described variously as stale, sour, or fermented (Bestmann et al., 1997) and to date is the only species of the genus to have floral fragrance analyzed (Bestmann et al., 1997). Liu and Yeh (2015) reported fragrance volatiles for *Hippeastrum* ‘Blossom Peacock’ and its progeny ‘T.S.S. No.1-Pink Pearl’, a double-flowered complex hybrid (*H.* ‘San Remo’ $\times$ ‘Blossom Peacock’).

Fragrance has not been a major target of amaryllis breeders who traditionally select for color characteristics (Meerow, 2009). Introducing fragrance into nonscented varieties has the potential to increase demand for amaryllis plants and add to their value as cut flowers as well. The goals of this study were to determine the range of volatile compounds in two *Hippeastrum* species that produce fragrance, to study temporal patterns in scent emission, attempt to infer likely pollinators, and potentially help identify candidate genes for fragrance expression in the future from *Hippeastrum* transcriptome data.

Materials and Methods

Floral volatile compounds were analyzed from two fragrant species, *H. brasilianum*,

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a Brazilian *planalto* species from Minas Gerais and Espírito Santo states and *H. parodii*, a tropical desert species native to southwestern Bolivia and northwestern Argentina.

Volatile collection and analysis. Plants were grown in the United States Department of Agriculture/Agricultural Research Service greenhouse, Miami, FL (lat. 25°38.577815' N, long. 80°17.545062' W) and were transferred into the laboratory for volatile collection. Flowers, fully open for 24–48 h were enclosed in commercially available oven bags, loosely sealed with a twist tie, and kept at ambient temperature for 1 min before sampling. Volatile chemicals were sampled by solid-phase microextraction (SPME) with a 100- μ m polydimethyl siloxane coating (nonbonded) fiber (Supelco, Bellefonte, PA). Fibers were inserted through a small hole in the oven bag and exposed to headspace volatiles for 1 min. *Hippeastrum brasilianum* and *Hippeastrum parodii* flower's volatile samples were taken once every hour for 24 straight hours and repeated twice with different flowers from the same inflorescence. In addition, *H. parodii* flower volatiles were sampled daily at 10:00 AM from anthesis until senescence.

All chemical samples were analyzed using an Agilent 5975B gas chromatography (GC)-mass spectrometer (Agilent Technologies, Santa Clara, CA) in the electron impact mode. The column used was a DB5-MS 25 m $\times$ 0.25 mm $\times$ 0.25 μ m column (J&W Scientific/Agilent Technologies, Santa Ana, CA) and helium was used as a carrier gas at 1.2 mL·min⁻¹. The oven temperatures were programmed to increase from 45 °C to

130 °C at 10.0 °C min⁻¹, then from 130 to 220 °C at 15.0 °C·min⁻¹, and then kept at 220 °C for 2 min. Before each set of samples was collected, the SPME fiber was conditioned for 2 min at 220 °C in the GC-mass spectrometer injection port, and a fiber blank was recorded before initial sampling. A C₆ to C₁₆ alkane standard was analyzed for retention time reference data for Kovats indices calculations. Volatile chemicals were identified based on the comparison of mass spectra with the National Institute of Standards and Technology (NIST) Mass Spectral Program version 2.0d and NIST/EPA/NIH Mass Spectral Library (NIST11). Further confirmation was achieved by comparing the putative chemicals' mass spectra and Kovats indices with commercial standards (Sigma Aldrich, Milwaukee, WI) tested using the same GC-mass spectrometer and method used for flower volatile analysis. Where standards were not available, unknowns were tentatively identified by the best match from the NIST libraries. Minor peaks appearing in only one sample of the same species were ignored. A putative match for most major volatile compounds was found with a reverse match of 852 or higher.

Results

Peak identification. Five major volatile peaks were detected from *H. brasilianum* (Table 1; Fig. 1A). Among these, β -ocimene (including both *cis* and the much lower percentage *trans* stereoisomers) was the dominant constituent, occurring at all 24 sampling times in the highest amounts (Fig. 2A). Linalool, methyl salicylate, and

methyl benzoate occurred in most of the samples and each made up at least 1% of the total volatile content (Fig. 2B).

Seven volatile peaks were identified in *H. parodii* emissions over a 24-h period (Table 1; Figs. 1B and 3A). (Z)- β -Ocimene (49%), eucalyptol (30%), and myroxide (10%) were found in largest amounts. In addition to compounds named previously, nonanal, decanal, and a compound provisionally identified as 2,6-bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol appeared in the bouquet of volatile compounds consistently throughout the 24-h sampling period (Fig. 3B). Caryophyllene, comprising just more than 1% of total emissions, occurred in only 12 counts.

Temporal variation. The sum of all *H. brasilianum* volatile emissions were greater during daylight hours (2.9×10^6 counts) than after sunset (1.4×10^6 counts) (Fig. 4A). Linalool appeared throughout the 24-h sampling time span but was the only volatile that had its highest peaks during daylight hours (Fig. 2B). For *H. parodii*, a summation of all peak areas for each hour (Fig. 4B) revealed that there were greater total volatile emissions after sunset (2.1×10^6 counts) than during daylight (1.5×10^6 counts) hours (4 June 2015 sunrise 6:29 AM; sunset, 8:09 PM).

Total scent emissions in *H. brasilianum* came in pulses. During daylight hours, there was roughly 2 h between peak outputs (Fig. 4A). After sundown, frequency of the higher pulses' recurrence slowed. Evening pulses had higher peaks and lower troughs than the relatively steadier output during the day. Volatile emissions were slightly higher between 11:00 AM and 4:00 PM than they were

Table 1. Major volatile compounds, as a percent of total emissions over a 24-h period, from *Hippeastrum brasilianum*, and *Hippeastrum parodii*, mean of two analyses ($\pm$ SD).

Compound	Class	Daily emission		Sample	Standard	Count	NIST
		Percent	RT	RI	RI		RM
<i>Hippeastrum brasilianum</i>							
(E)-β-ocimene	Monoterpenoid	2.22 (±0.17)	5.59 (±0.23)	965.57 (±1.27)	965.96	19 (±2)	929
(Z)-β-ocimene	Monoterpenoid	88.32 (±1.57)	5.75 (±0.46)	971.13 (±2.13)	971.99	24 (±3)	969
Methyl benzoate	Aromatic ester	1.15 (±0.08)	6.49 (±0.38)	952.29 (±4.02)	953.21	18 (±2)	930
Linalool	Monoterpenoid	4.23 (±0.12)	6.55 (±0.26)	999.47 (±3.14)	999.29	21 (±3)	893
Methyl salicylate	Aromatic ester	2.51 (±0.21)	7.94 (±0.66)	1,084.86 (±5.27)	1,085.32	20 (±4)	936
<i>Hippeastrum parodii</i>							
Eucalyptol (1,8-cineole)	Monoterpenoid	30.35 (±1.03)	5.59 (±0.38)	965.60 (±4.26)	965.82	24 (±3)	903
(Z)-β-ocimene	Monoterpenoid	48.61 (±2.46)	5.75 (±0.51)	971.13 (±3.72)	971.99	24 (±2)	890
Nonanal	Aliphatic aldehyde	2.06 (±0.11)	6.61 (±0.42)	761.76 (±2.16)	767.76	24 (±3)	874
Myroxide	Monoterpenoid	10.15 (±0.31)	7.12 (±1.06)	n/a n/a	n/a	24 (±2)	870
Decanal	Aliphatic aldehyde	2.69 (±0.41)	8.09 (±0.16)	1,054.43 (±4.29)	1,054.43	23 (±3)	945
Caryophyllene	Sesquiterpenoid	1.10 (±0.08)	10.79 (±0.36)	1,415.43 (±5.05)	1,415.32	12 (±2)	930
2,6-bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	Phenolic	2.98 (±0.19)	12.59 (±0.51)	n/a n/a	n/a	24 (±2)	919

NIST = National Institute of Standards and Technology; RT = retention time; RI = Kovats Retention Index; RM = reverse match; and count = the number of occurrence in hourly samples over a 24 h period. Identification by comparison (mass spectrum electron ionization) to a commercial standard and RI. n/a = no standard available.

Fig. 1. Representative gas chromatography-mass spectrometer chromatograms of percent counts vs. acquisition time containing the most frequently reoccurring peaks for volatile compounds found in two fragrant *Hippeastrum* spp. (A) *Hippeastrum brasilianum*, 1:00 PM, 8 Apr. 2015. (a) (*E*)- β -ocimene, (b) (*Z*)- β -ocimene, (c) methyl benzoate, (d) linalool, (e) methyl salicylate. (B) *Hippeastrum parodii*, 2:00 PM, 4 June 2015. (a) Eucalyptol, (b) (*Z*)- β -ocimene, (c) nonanal, (d) myroxide, (e) decanal, (f) caryophyllene, (g) 2,6-bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol.

for the rest of the afternoon and throughout the night. At dawn, peak emission began to increase but with continued large differences between high and low point in the pulse.

The major volatiles of *H. parodii*, eucalyptol and (*Z*)- β -ocimene, had opposite emission patterns throughout the day (Fig. 3A). As relative counts for eucalyptol increased from

8:00 AM until 11:00 AM, those for (*Z*)- β -ocimene decreased. Eucalyptol emissions were higher beginning a little after 9:00 AM until after 4:00 PM when (*Z*)- β -ocimene became the dominant

Fig. 2. Occurrence of volatile compounds emitted from *Hippeastrum brasilianum* flowers over a 24-h period. (A) Major volatile compounds. (B) Minor volatile compounds.

fragrant compound. Among the minor volatiles, caryophyllene emissions began in the early morning and ceased shortly after sunset (4 June 2015 sunrise 6:29 AM; sunset, 8:09 PM). Decanal emissions were continuous throughout the day; however, they were notably higher from 8:00 AM through 4:00 PM with peak emission at 2:00 PM. In *H. parodii*,

the only species for which volatiles were collected from anthesis until senescence of the flower, six volatile compounds [eucalyptol, (*Z*)-β-ocimene, nonanal, decanal, myroxide, and 2,6-bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol] were emitted on all 4 d before the flower wilted (not shown). These six compounds accounted for 51% to 85% of

total emissions each day. (*Z*)-β-ocimene and eucalyptol, the two dominant components of *H. parodii* fragrance, had opposite emission patterns over the time span between flower opening and wilting. As the percentage of ocimene emitted increased, that of eucalyptol decreased; after day 2, this pattern reversed and ocimene decreased in favor of eucalyptol.

Fig. 3. Occurrence of volatile compounds emitted from *Hippeastrum parodii* flowers over a 24-h period. (A) Major and minor volatile compounds. Two additional minor volatiles are shown in a separate graph (B) for clarity. (B) Additional minor volatile compounds.

Hippeastrum parodii produced comparatively less scent than *H. brasilianum*. The difference was easily discernable by the human nose (as tested with all four authors). From 9:00 AM until 5:00 PM, *H. parodii* emissions were comparatively low with few high peak emissions (Fig. 4B). Scent emissions increased in the early evening (after 6:00 PM) with higher peaks and mostly higher troughs. This pattern lasted until 1:00 AM

when emissions began to decline. Excluding a large spike in emissions at 8:00 AM, after 4:00 AM, scent compounds were emitted at levels similar to that found during daylight hours.

Discussion

The floral bouquets of *H. brasilianum* and *H. parodii* differ in the relative richness of

VOC composition (greater in *H. parodii*; Fig. 1; Table 1). Headspace of *H. brasilianum* is dominated by (*Z*)-β-ocimene (Fig. 1A; Table 1), whereas that of *H. parodii* is dominated by both (*Z*)-β-ocimene and eucalyptol (Fig. 1B; Table 1). A previous report (Miyake et al., 1998) for the sphingophilous *Crinum asiaticum* L. (Amaryllidaceae) also found β-ocimene to be the greatest VOC emitted by the flowers (44.6%), in addition

Fig. 4. Relative amount of floral scent emissions from two *Hippeastrum* spp. over a 24-h period. (A) *Hippeastrum brasilianum*. (B) *Hippeastrum parodii*.

to methyl benzoate (12.6%), linalool (8.4%), and nerolidol (8%).

Both eucalyptol and β -ocimene are monoterpenes synthesized from the precursor geranyl pyrophosphate (Croteau et al., 1994; Dudareva and Pichersky, 2000; Peters and Croteau, 2003). Synthesis of eucalyptol at the expense of ocimene and vice versa may reflect an enzymatic/biochemical shift in conversion of geranyl pyrophosphate from one competitive pathway to another. Ocimene and myrcene emissions from snapdragon flowers were found to follow diurnal rhythms controlled by a circadian clock (Dudareva et al., 2003; Kolosova et al., 2001). Where snapdragon peak ocimene emission was between 11:00 AM and 6:00 PM, *H. parodii*'s ocimene emission in our study peaked between 1:00 and 3:00 AM. Nevertheless, a circadian control mechanism in *H. parodii* is possible.

During the course of a day, floral scent can vary both in the intensity of emission and the makeup of compounds that result in the final scent (Balao et al., 2011; Dötterl et al., 2012; Effmert et al., 2005; Loughrin et al., 1990). Changes in temporal scent patterns can be a means to attract specific pollinators at different times of day or night to optimize pollination success (Dötterl et al., 2012; Effmert et al., 2005; Knudsen et al., 2006; Raguso et al., 2003). Both *H. parodii* and *H. brasilianum* had emission patterns that changed throughout a 24-h cycle (Fig. 4). *Hippeastrum brasilianum* is thought to be pollinated by moths because the flowers are white, long-tubed, and sweetly fragrant, fitting a sphingophilous syndrome (Dobson,

2006), though no documented visitation reports are available. The only anecdotal reports of insect visitation to *H. parodii* are two bumblebee and one butterfly species (M. Saviello, personal communication). High-volatile emissions during daylight hours may indicate an alternative pollinator active during the day as these observations suggest.

Insect-pollinated flowers have much higher emission of VOCs than wind-pollinated flowers and a greater diversity of compounds in the scents that they produce (Farré-Armengol et al., 2015). Knudsen and Tollsten (1993) purport that flower adapted to hawkmoth (Sphingidae) pollination can be distinguished from those pollinated by noctuids (Noctuidae) by the presence of oxygenated sesquiterpenes in the VOC complement of sphingophilous species, but this observation has been refuted by later authors (Dobson, 2006). No sesquiterpenes were detected in *H. brasilianum*, but caryophyllene is a minor constituent of the VOCs of *H. parodii* (Table 1). Knudsen and Tollsten (1993) found that sphingophilous species were characterized by a major presence of farnesene, a VOC not present in either *Hippeastrum* species. Sphingophilous flowers also emit 1,8-cineole (i.e., eucalyptol), linalool, and nitrogen-containing VOCs when compared with flowers pollinated by noctuids (Knudsen and Tollsten, 1993). We detected linalool in *H. brasilianum* emissions, and eucalyptol was one of the two major peaks in *H. parodii*. We did not detect nitrogenous compounds in either species. Fatty acid derivatives, present in *H. parodii*, are associated with nocturnal settling moths (noctuids), rather than sphingids (Dobson, 2006).

Sphingid attraction to *H. brasilianum* may be suggested by the presence of linalool and methyl benzoate in the VOC emissions (Dobson, 2006), even though the species is not exclusively nocturnal (personal observation). Ultimately, it becomes difficult to predict the pollinators of plants by floral characteristics alone, including VOC emissions, in the absence of documented visitation (Dobson, 2006; Ollerton and Watts, 2000; Ramírez, 2003). The possibility that these species attract both diurnal and nocturnal lepidopteran visitors cannot be discounted.

VOCs reported for a fragrant complex hybrid of *Hippeastrum* (Liu and Yeh, 2015) and one of its parents were quite different from those reported here. They identified the emission constituents as cineole, 1,3,6-octatriene, β -linalool, 3-cyclohexene-1-methanol, and α -farnesene. Only linalool was shared with one of the species we examined, *H. brasilianum*. This may suggest that introgression between species of the genus may result in novel VOC emissions.

The bat-pollinated *H. calyptratum* emits an even more complex bouquet (Bestmann et al., 1997): the oxygenated terpenes 1,8-cineole (18.3%), perillene (13.1%), camphor (2.9%), and linalool (2.4%); the monoterpenes limonene (27.5%), γ -terpinene (10.2%), β -myrcene (8.7%), sabinene (3.7%), α -pinene (2.8%), δ -3-carene (2.6%); the aliphatic 3-hexanone (7.9%); and a few traces of other compounds. No sulfur-containing volatiles, implicated in many, but not all, bat-pollinated flowers (Bestmann et al., 1997; Dobson, 2006), were reported.

Other than *Hippeastrum* and *Crinum*, floral emissions of Amaryllidaceae have only been studied in *Narcissus* (Dobson et al., 1997). *Narcissus* spp. pollinated by insects that include butterflies and moths were characterized by VOCs typical of moth-pollinated flowers, particularly indole combined with high amounts of esters. Species visited exclusively by non-lepidopterans, especially bees and flies, had fragrances lacking this combination of volatiles. *Narcissus assoanus* Dufour displayed both fragrance chemotypes, perhaps indicative to the documented patterns of interspecific hybridization in the genus (Marques et al., 2010, 2011).

Floral fragrance and breeding behavior in Hippeastrum. The hypothesis that floral fragrance is a recessive character in amaryllis (Meerow, 2000, 2009) is based on inheritance patterns we have observed over the course of several decades of breeding *Hippeastrum*. F₁ hybrids of *H. brasilianum* and *Hippeastrum papilio* (nonfragrant) were uniformly without scent. Back-cross progeny (F₂ progeny were unavailable because of both self- and sibling incompatibility of the F₁) assort for fragrance. Others primary crosses between a fragrant and nonfragrant species also yield uniformly unscented progeny. In our experience, the F₁ of two fragrant species are uniformly scented.

In conclusion, volatile emissions of *H. brasilianum* and *H. parodii* are strongly suggestive of lepidopteran pollination. *H. parodii* in particular, with its biphasic alternation between eucalyptol and (Z)- β -ocimene (Fig. 3A), may have more than one pollinator. We have recently obtained targeted (base of the perigone) floral transcriptomes of both *H. brasilianum* and *H. papilio* and hope to be able to use these genomic resources to understand the genetics of fragrance expression in amaryllis hybridization. Characterization of the fragrance volatiles of *H. brasilianum* in particular may assist in the identification of candidate genes involved in differential expression of this character. A wider distribution of floral fragrance in amaryllis hybrids is a desirable goal of breeding efforts in the genus.

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