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The Receptacular Nectar Tubes of *Pelargonium* (Geraniaceae): A Study of Development, Length Variation, and Histology

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APPROVAL PAGE

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The Receptacular Nectar Tubes of *Pelargonium* (Geraniaceae): A Study of Development, Length Variation, and Histology

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ABSTRACT

The nectaries of *Pelargonium* are unusual in the Geraniaceae in that the flowers only have one deep dorsal tube as opposed to five shallow canals. The genus is also known for having great length variation in nectar tubes, variation that has been suggested to play an important role in the diversification of this genus. Interpretations of *Pelargonium* nectaries contradict each other as some describe the nectar tube as an adnate sepal spur while others describe it as an hypanthial tube as a result of an elongate receptacle. In addition, the developmental basis of differing nectar tube lengths is unknown.

SEM analysis of *P. ionidiflorum* and *P. odoratissimum* dissections revealed that displacement of the dorsal antesealous stamen by the flanking antepetalous stamens anticipates the space where the nectar tube will develop before the tube begins to form. Soon after all floral organ primordia have been initiated, the concavity of the nectar tube of *Pelargonium* initiates and then elongates through intercalary growth of the receptacle. Early stages of development up to a bud length of 4.0 mm are similar in both species. Functional Data Analysis of longitudinal growth of buds 0.25 mm to maturity showed that the tubes of *P. ionidiflorum* become longer through both higher rates of elongation and a longer duration of elongation than those of *P. odoratissimum*. Nectar tube elongation also continues for nearly a week after anthesis.

Evidence suggests that the nectar tube of *Pelargonium* is not evolutionarily derived from a sepal spur that becomes fused to the pedicel. No involvement of the spur is observed at any stage in the development of the nectar tube in *Pelargonium*. Furthermore, the development of the spur begins late in floral ontogeny in contrast with the initiation of the nectar tube, which begins early in development of *Pelargonium* flowers. This contrasts with the pattern in species with free nectary perianth spurs, such as *Aquilegia olympica* or *Impatiens columbaria*. Finally,

sections along the length of mature *P. ionidiflorum* tubes revealed a lack of vascular pattern that would support an interpretation of a spur fused to the pedicel. *Pelargonium* is more accurately classified as having a deep receptacular nectary.

INTRODUCTION

Many animal pollinated flowers produce nectar, a reward that is synthesized in and secreted from specialized glands called floral nectaries (Caspary, 1848; Fahn, 1952). As pollinators forage for nectar, they may also actively forage for pollen, in either case pollen is deposited on their bodies and transferred to another flower. Floral nectaries can occur on any whorl of the flower (Bernardello, 2007). Flower shape can greatly influence accessibility of nectar (Bernardello, 2007). Less-exposed or hidden nectar can be successfully consumed only if the shape and size of the animal's mouthparts are compatible with those of the flower. For example, the nectar of a dish or bowl-shaped flower is more exposed than that of a bell or funnel-shaped flower, making it easier for bees to access the reward. The mouthparts of birds and long-tongued insects, on the other hand, can access long narrow perianths (Bernardello, 2007). The diversity of flower shapes and sizes mediates reproductive isolation, limiting pollination to specific animals (Bernardello, 2007).

Some angiosperm families, e.g., the Ranunculaceae, Orchidaceae, and Tropaeolaceae, have species with nectar spurs, hollow tubular perianth outgrowths that secrete and contain nectar. These structures have independently evolved numerous times and are thought to be influential in shaping plant-pollinator interactions (Hodges, 1997; Bernardello, 2007). Charles Darwin initially proposed that the length of nectar spurs tends to match the length of the pollinator's tongue (Darwin, 1862; Darwin, 1877). This led to his famous prediction of a hawkmoth with a long tongue capable of foraging nectar from the Malagasay star orchid (*Angraecum sesquipedale*) with its nearly foot long nectar spur (Darwin, 1862). His idea was validated many years later by discovery that this orchid was indeed visited by a moth with a 22 cm tongue (Wasserthal, 1997). Long tube lengths limit the morphology of pollinators that can access the nectar, and may also increase the likelihood of physical contact with reproductive structures because the pollinator must probe deeply, or in particular ways, to reach the nectar.

The development of nectar spurs has been characterized anatomically and morphologically in many species, including, for example, *Aquilegia* (Tucker and Hodges, 2005; Puzey et al. 2012; Antoń and Kamińska, 2015; Yant et al., 2015) and *Impatiens* (Caris et al., 2006). In some species of *Aquilegia* and in *Impatiens columbaria*, the nectar spur forms as an outpouch of a perianth organ. In contrast, the genus *Pelargonium* possesses a unique nectar tube (Fig 1A, Vogel 1998c) that has been frequently described as “a spur adnate to the pedicel” (Almousslem and Tilney-Bassett, 1989; Bernardello, 2007; Ringelburg, 2012; R. schenbleck et al., 2014), although this classification is contradicted by descriptions of the tube as an hypanthial nectar tube (Japp, 1909; Webberling, 1989; Miller, 1996; Struck, 1997). Japp (1909) described the development of the nectar tube as a “stagnation of growth in an adaxial-extrastaminal area of the [receptacle]” (translated by Link, 1994). Among the 280 species of *Pelargonium*, the length of the nectar tube varies from less than 10 mm to greater than 20 mm (Struck, 1997) with variation in the length of the tube presumably correlating with pollinator proboscis length (Struck, 1997; Bakker, 2005). Based on that diversity, Bakker (2005) and others (Struck, 1997; Ringelburg, 2012) have suggested that nectar tube length and associated changes in floral morphology played a critical role in diversification within the genus. And the diversification in *Pelargonium* is extensive. For instance, this genus constitutes the seventh largest radiation in the hyperdiverse Greater Cape Floristic Region of Southern Africa (Linder, 2003).

Pelargonium is one of five (Price and Palmer, 1993), or six if we include *Hypseocharis* as suggested by Boesewinkel (1998), genera of the Geraniaceae, all of which have nectaries (Kubitzki, 2007). While all other genera are characterized by five nectaries (Link, 1994; Kubitzki, 2007), *Pelargonium* has only one. In *Geranium*, radial flower symmetry (polysymmetry) is associated with five uniformly sized shallow nectaries (Fig 1B; Vogel, 1998c). In *Erodium*, slight dorsiventrality (monosymmetry) of the flower is associated with the greater size of the three

dorsal nectaries relative to the remaining two (Fig 1C). The center dorsal nectary is the largest of the five (Vogel, 1998c). In *Erodium hymenodes*, however, there is one single dorsal nectary (Link, 1994). *Pelargonium* is distinguished from the other genera in the family by having both highly monosymmetric flowers and a single deep, dorsal nectary (Fig 1A, 1D). It is tempting to consider *Erodium* as an intermediate between *Geranium* and *Pelargonium*, but *Pelargonium* is sister to the other genera in the family (except *Hypseocharis*: Weng et al., 2014), rendering the monosymmetric flowers in *Pelargonium* and *Erodium* convergent rather than homologous (R schenbleck et al., 2014).

Despite the potentially important role of nectar tube length in species diversification (Struck, 1997; Bakker et al. 1999; Ringelburg, 2012), and the apparent confusion regarding the morphology of the nectar tube in *Pelargonium*, research that emphasizes the development or anatomy of the nectar tube in the genus is limited (Japp, 1909; Sauer, 1933; Labbe, 1964). Labbe (1964) examined the anatomy of *P. endlicherianum*, *P. peltatum*, and *P. zonale* and presented anatomical evidence in the form of sketches. Sauer (1933) interprets the nectar tube as a formation of the dorsal sepal, which conflicts with the receptacular tube interpretation Japp (1909) proposed.

Endress (2010) examined the flower development of *Geranium robertianum*. The flowers of *G. robertianum* have five evenly distributed nectar canals. Remarkably, each canal is formed by the coordination of six floral organs such that the edges overlap tightly leaving a tubular space that will serve to guide the pollinators' mouthparts to the nectar secreting structure situated below a stamen opposite the sepal (antesepalous) (Figure 2). The canals develop gradually as the floral organs form and finally become apparent near the full maturity of the flower (Figure 3). In contrast, *Pelargonium* nectaries are situated deep in the receptacle and do not involve synorganization of independent floral organs away from the floral organs.

In this study, I compared development of flowers of two species differing in nectar tube lengths. The specific questions posed were: (1) when during development and how do differences in nectar tube length arise, and (2) is there morphological or anatomical evidence to support the assertion that *Pelargonium* nectaries arose evolutionarily from sepal spurs adnate to the pedicel (Almousslem and Tilney-Bassett, 1989; Bernardello, 2007; Ringelburg, 2012; R. schenbleck et al., 2014), or as a hypanthial nectar tube (Japp, 1909; Miller, 1996; Struck, 1997). The former interpretation implies that the tube is part of the calyx, where the basal portion of the dorsal sepal outpouches to form a tube. The latter interpretation suggests that the tube is part of the receptacle. Evidence for either of these interpretations is lacking. In addition, Weberling (1989) describes the nectar tubes as “axial spurs,” and considers them “combined with the flower stalk,” but provides no developmental evidence for his interpretation. Thus, the goals of this study are to (1) understand the developmental basis of contrasting nectar tube lengths, (2) explain the development of the nectary in the context of floral development in *Pelargonium*, and (3) explain the anatomy of the nectary in *Pelargonium*. Based on the data, I propose models for nectar tube development and anatomy.

MATERIALS AND METHODS

The study system

The genus *Pelargonium* L'Hér consists of about 280 species, 90% of which are endemic to southern Africa. The highest species diversity in the genus is found in the south-western region of South Africa called the Greater Cape Floristic Region (GCFR). About 200 species occur in the GCFR, making it the third largest angiosperm genus of this region (citation). *Pelargonium* is also an economically important genus. Cultivated species are found in gardens and homes all over the world and essential oils are widely used in the perfume industry. *Pelargonium* is known for its large variety of vegetative and floral morphologies. Growth forms

range from herbaceous annuals, subshrubs, and shrubs to stem succulents and geophytes. Leaf shapes vary from entire to dissected to midrib, and the extent of leaf lobing is evolutionarily labile (Jones, 1999). Petal number, hypanthium length, petal color, nectar guide types, nectar guide distribution, and the number of fertile stamens also vary from species to species (R schenbleck *et al.*, 2014).

In this study, I will investigate development of the nectar tubes in the flowers of *P. ionidiflorum* and *P. odoratissimum*. Both species are perennials in the perennial clade (Clade B), and have similar native ranges (Jones et al., 2013). The former species have long nectar tubes with a median length of 30.2 mm whereas the median length of the tubes of *P. odoratissimum* is 8 mm (Ringelburg, 2012). Both species were chosen for this study because in spite of their similar native ranges, their nectar tube lengths are significantly different.

Flower Size Analysis

In order to characterize variation in mature flowers of each species, 19 mature *P. ionidiflorum* flowers and 21 mature *P. odoratissimum* flowers were collected from two plants per species at the UConn EEB Research Greenhouse. I measured the left dorsal petal and nectar tube lengths of each flower. A Welch's t-test was performed in R to compare differences in mean dorsal petal sizes and nectar tube lengths for both species.

Scanning Electron Microscopy Analysis

Developing flower buds between 0.5 to 4.0 mm were dissected such that for each bud, the calyx was removed and nectar tube was exposed on the dorsal side. Dissected buds were transferred to distilled water and rinsed by gentle swirling to rehydrate the tissue that potentially dehydrated during dissection. The dissections were gradually dehydrated to a 50% ethanol solution to prevent shrinking and to wash off any residue prior to fixation. Buds were then fixed under FAA solution (Berlyn and Mykschke, 1976) under refrigeration for one day.

The buds were dehydrated to 100% ethanol and critical point dried (Model 931, Tousimis, Rockville, Maryland, USA) for scanning electron microscopy (SEM). They were mounted onto SEM stubs with doubled sided tape, sputtered coated with gold-palladium for under 3 minutes (E5100, Polaron, acquired by Quorum Technologies, East Sussex, UK) and imaged with a scanning electron microscope (NOVA NanoSEM 450, FEI, Eindhoven, The Netherlands).

Nectar Tube Histology

Sections of a *P. ionidiflorum* nectar tube were cut at a thickness of 9 μm using the RMC ET-920 microtome and a glass knife. Cross sections were cut 2 mm above, 1 mm above, and through the base of the nectar tube as well as the pedicel below the nectar tube. The sections were stained with aqueous 0.05% Toluidine Blue O. Sections were examined using a light microscope at 50x, 100x, and 400x magnifications, using bright field.

Growth Analysis

To measure the rates of perianth and nectar tube elongation, twenty 0.25 – 0.75 mm buds per species (10 per plant) were tagged. Each bud was tagged on a different inflorescence. Perianth and nectar tube measurements were made daily between 12:00 and 1:00 pm, with more precise measurements of the smallest buds made with 0.25mm and 0.5mm thick insect pins. Measurements continued until the perianth and the nectar tube stopped growing.

In the following analysis, we introduce a novel method to the plant growth literature, Functional Data Analysis (FDA) (Ramsay & Silverman, 2005), in order to display informative aspects of growth data that are not visible through standard methods of fitting preconceived functions to growth (Hunt, 1982). FDA is a branch of statistics that estimates functions from the data itself with a number of smaller basis functions determined either by the researcher or derived quantitatively. This allows continuous curves to be treated as discrete units instead of a

collection of data points, allowing for more rigorous statistical analysis typical of discrete datasets.

In the context of this study, lengths of floral structure were measured over a series of days for *P. ionidiflorum* and *P. odoratissimum*. Growth curves for each individual flower were converted into a functional format using the R package “fda” (Ramsay et al., 2014). Because growth was non-cyclical and monotonic (i.e., no re-occurring patterns and always expanding), functions of growth curves were estimated with B-spline basis systems, positioning knots at each day. First and second derivatives were calculated from these growth curves in order to describe rates of change along growth times. Growth averages with 95% confidence intervals were estimated for tube and perianth by species for both growth and higher order functions.

RESULTS

Mature flowers of *P. ionidiflorum* and *P. odoratissimum* have 5 sepals, 5 petals, 7 stamens, and a gynoecium. The base of the nectary is visible as a small bump on the receptacle, some distance distal to the pedicel (see arrows on Fig 4A and 4C). Flowers of *P. ionidiflorum* have longer nectar tubes (mean = 29.5 ± 3.7 mm) compared to *P. odoratissimum* (mean = 9.7 ± 1.1 mm) ($p < 0.05$). Flowers of the two species also differ in flower and tube color: petals of *P. ionidiflorum* are pink and the tube is magenta while the petals of *P. odoratissimum* are white with green tubes. The dorsal sepal of both species is the largest of the five. Unlike the other four sepals that are reflexed towards the pedicel, the dorsal sepal extends forward. The corolla of both species is monosymmetrical with two petals dorsal and three ventral. The dorsal petals are shorter than the ventral petals and have distinct violet markings. Dorsal petals of *P. ionidiflorum* are significantly larger (14.1 ± 2.4 mm) than those of *P. odoratissimum* (10.5 ± 1.3 mm) ($p < 0.05$). Both species have seven stamens that occur in two whorls; five are opposite the sepals and the other two are opposite the dorsal petals (see labels in Fig 5).

Initiation and Early Development of the Nectar Tube

In 0.5 mm buds of both species, all floral organs have been initiated (Fig 6A, 6B). In all SEM images shown, all sepals have been removed. Petal primordia are delayed in development relative to sepals and stamens. Stamens have distinct filaments and anthers in both species. Dorsal views of buds reveal virtually no expansion of the internode between the petals and antepetalous stamens, or between the two stamen whorls. The dorsal antesealous stamen (SS in Fig 6A, 6B) is displaced acropetally toward the gynoecium and flanked by two antepetalous stamens (SP). The synorganization of these three stamens creates a relatively wide space between the two dorsal petals (P), that anticipates the location of the nectar tube. At this 0.5 mm stage, however, a concavity indicating the initiation of the nectar tube is not yet evident in this region in either species. Vertically aligned files of rectangular cells at the base of the dorsal antesealous stamen (Fig 6B) suggest the initiation of elongation growth below the point of petal insertion.

As development progresses, there is some variation among buds within each species in the degree of elaboration of the nectar tube. In general, however, slightly larger stages (0.9 mm) reveal little evidence of the nectar tube initiation in either species. The absence of vertically aligned files of rectangular cells is evidence for the lack of intercalary growth of the receptacle surrounding the cavity in *P. ionidiflorum* (Fig 6C) and while vertically aligned cells maybe slightly more obvious in *P. odoratissimum* (Fig 6D), this difference is not consistent across different samples at this stage. The petal primordia do not appear to have elongated relative to the 0.5 mm buds in either species.

Slightly more mature buds (1.1 mm) reveal that concavities have begun to form in both species as indicated by vertically aligned files of elongating cells beneath the position of petal insertions (Figs 7A). That this region is elongating is shown by the increasing distance between

the base of the nectar tube (Fig. 7A, 7B insert) and the region of petal insertion. In contrast to the elongate, rectangular epidermal cells lining the tube wall, cells on filaments are polyhedral, indicating that rapid elongation has not yet begun. For *P. ionidiflorum*, theca within anthers are becoming more clearly differentiated, and the antesealous stamens have elongated slightly more than the antepetalous stamens. Petal primordia have begun to elongate in both species, as well (compare Fig 6C, 6D and Fig 7A, 7B with reference to scale bars).

By the time floral buds reach 1.5 mm in length, the distance from the position of insertion of the petals to the base of the cavity has increased (Fig 7C, 7D); note that the dorsal side of the nectar tube has been dissected away to reveal the base of the tube. Vertically aligned files of cells that line the nectar tube are apparent. Compared to the 1.1 mm buds, there are more columns of vertically aligned cells (22 to 26 in *P. ionidiflorum*; 15 to 18 in *P. odoratissimum*) (compare Fig 7A, 7B to Fig 7C, 7D). Anther number can vary among flowers and in Fig 7D, the lack of anther-filament differentiation in the antepetalous anthers of *P. odoratissimum* suggests that they are in the process of aborting.

In dissections of 2.0 mm buds, there is a distinct difference in cell shape between the top of the tube and the bases of the stamens for both species (Fig 8A, 8B). The polyhedral cells at the base of the stamens have become more differentiated from the vertical files of the nectar tubes, indicating that elongation growth in this region is no longer occurring; all subsequent growth in the stamens is distal to their base (Fig 8A, 8B insert). Prior to this stage, the boundary was not as well-defined (compare Fig 7C and Fig 8A; Fig 7D and 8B). Externally, the swollen base of the nectar tube is easily distinguished from the more cylindrical pedicel (Fig. 8A).

Subsequent development of buds from 2.5 mm to 4 mm in length is similar in the two species. The nectar tubes continue to expand in length through intercalary growth of the receptacle, including the removed dorsal wall, above the base of the tube. For both species, the

three ventral antesealous stamens elongate faster than the dorsal antesealous stamen or the antepetalous stamens (Fig 8C, 8D).

In 4 mm buds of both species, the petals have grown large relative to the antepetalous stamens. The petals of *P. ionidiflorum* are shorter than the antepetalous stamens (SP) (Fig 9C) whereas those of *P. odoratissimum* are longer than the antepetalous stamens (Fig 9D). The nectar tubes have elongated significantly, reaching almost 1 mm in length on samples of both species. In summary, the mode and timing of early stages of nectar tube development are nearly identical on the two species.

Perianth and Nectar Tube Elongation Rates

Development of nectar tubes in buds >4 mm was examined quantitatively relative to the total length of the visible perianth (sepals initially, then petals after emergence and anthesis). All buds measured were 0.25 to 0.5 mm in length on day 1. Bud growth is similar in both species until around day 18 (Fig 10A). In each species early stages, the perianth (i.e., sepal) expansion rates peak around day 5, and then begin declining (Figure 11A) until dramatic increases in perianth expansion begin again with petal anthesis, which occurs earlier in *P. odoratissimum*. The rate of corolla expansion on *P. odoratissimum* peaks at about 1 mm/day on day 15, the day following petal emergence. Flowers reach anthesis approximately 15.5 days after the beginning of measurements. The nectar tubes continue to elongate post-anthesis until day 17 (Fig 11A), reaching at a mean final length of approximately 11.5 mm. In contrast, the timing of anthesis in *P. ionidiflorum* is highly variable with some buds opening as early as day 12 or as late as day 23. On average, the petals emerge from the calyx 17.2 days after the beginning of measurements and are fully reflexed at 20 days. Peak corolla expansion rate is similar to that of *P. odoratissimum*, but the relatively high rate of corolla expansion continues for up to seven days following petal emergence, resulting in a final length of approximately 18 mm.

By day 5 of bud growth, nectar tubes of buds of both species have begun elongating visibly. On average, tube lengths and growth rates are similar between species until a day after petal emergence of *P. odoratissimum* (day 15, Fig 10B), when its nectar tube declines in growth rate (day 15, Fig 11B). In contrast, the growth of the nectar tube in *P. ionidiflorum* increases dramatically just prior to petal emergence (day 17, Fig 11B), reaching a rate that is approximately 3X faster (Fig 11B) than the peak expansion rate of the corolla during anthesis that occurs a day or two later. Once anthesis occurs in *P. ionidiflorum*, growth of the nectar tube begins to decline (day 17, Fig 11B). Despite these average patterns, large 95% confidence intervals resulting from considerable variation in nectar tube expansion rates within species result in overlapping confidence intervals (Figure 11B). Nevertheless, the overall differences in growth patterns indicate that the greater nectar tube length of *P. ionidiflorum* results from both a greater rates of growth and longer duration of growth that occur late in flower development. Most nectar tubes in *P. ionidiflorum* do not stop elongating until day 27, 10 days after anthesis, reaching a mean of approximately 30 mm.

Nectar Tube Anatomy

Serial sections reveal that the region of the pedicel just proximal to the nectary contains six vascular bundles (see labels in Fig 12A). A subtle bump on the dorsal side anticipates the location of the nectar tube. At the base of the nectar tube, the number of vascular bundles has increased from six to nine (see labels in Fig 12B). Cross sections just below the nectar tube base (Fig 12B, 13B) cut the dorsal vascular bundle longitudinally, as indicated by the orientation of the tracheary elements (see labels in Fig 14A). This orientation suggests that the bundle is oriented perpendicular to the long axis of the nectar tube in this location. A section 1 mm above the base of the nectar tube base (Fig 12C) shows the cavity of the tube. The number of vascular bundles in this region has increased from eight to 11. Eight of them have formed a ring with

fibers surrounding the ring (see labels in Fig 12C). Another two bundles are located between the ring and ventral side of the cavity (Fig 12C, 13C). The last vascular bundle is adjacent to the dorsal side of the tube (Fig 12C, 13C). The epidermis of the nectar tube is lined with papillate epidermal cells and stained parenchyma cells (Fig 14B). Two mm above the base of the nectary, some of the vascular bundles in the ring have fused together (see labels in Fig 12D, 13D). Fibers can be found surrounding the vascular ring (Fig 12D, 13D). Two vascular bundles are located between the ring and ventral side of the cavity and another bundle is located adjacent to the dorsal side of the cavity (Fig 12D, 13D). The epidermis of the nectar tube is still lined with papillate cells, but in that location, the parenchyma cells are more lightly stained (Fig 14C).

DISCUSSION

Nectary development and differences in tube length

In *P. ionidiflorum* and *P. odoratissimum*, the nectar tube develops as an invagination in the receptacle formed in the region of the internode between sepals and petals followed by intercalary growth of the receptacle below this region. For both species, all floral whorls are initiated before the elongation of the nectar tube. However, even before the tube has begun to elongate, the acropetal displacement of the dorsal antesealous stamen creates a space that anticipates the location of the nectar tube. The inception of the nectar tube occurs in buds of similar sizes for the two species. It is recognized as a concavity in the area centripetal to the dorsal sepal (note that this sepal has been removed in all of the SEM figures) (Fig 15A). The presence of vertically arranged cell files below the insertion of the petals indicates that intercalary growth of the receptacle is responsible for subsequent elongation of the tube (see Region 2 in Fig 15B as an example). Nectar tube elongation is qualitatively similar in the two species up through 4 mm in length.

Variation in tube length both among and within *Pelargonium* species (Struck, 1997; Ringelburg, 2012) has been well documented. Variation in nectar tube lengths

could arise because the inception of the tube occurs at earlier stages of development in a long-tubed species, or via differences in growth rates or durations. SEM analysis revealed that the time of origin and early growth of the nectar tube lengths are similar between *P. ionidiflorum* and *P. odoratissimum*. Quantitative analysis of tube growth shows that the longer nectar tubes of *P. ionidiflorum* result from both a greater rate and duration of growth. Tube growth was similar for the two species until a day after *P. odoratissimum* reached petal emergence. At this time, elongation of *P. odoratissimum* nectar tubes decelerated while tubes of *P. ionidiflorum* not only continued to grow, but the velocity of growth increased. The biological outcome of nectar tube elongation that continues at a rate of 3 mm/day after flower opening is significantly longer nectar tubes.

Development and Characterization of the Nectar Tube

The nectar tubes of *Pelargonium* flowers are often described as sepal spurs adnate to the pedicel (Almouslem and Tilney-Bassett, 1989; Bernardello, 2007; Ringelburg, 2012; R schenbleck et al., 2014) or as receptacular in origin (Japp, 1909; Miller, 1996; Struck, 1997). My data show that that the *Pelargonium* nectar tubes are not spurs derived from sepals. In general, nectar spurs are described as hollow, slender, saclike outgrowths of the perianth (Bernardello, 2007; Koopman and Ayers, 2005). Nectar spurs extend freely from their organ of origin and are not adnate to other floral whorls. The development of nectar spurs is shown clearly in the work on *Aquilegia olympica* (Tucker and Hodges, 2005) and *Impatiens columbaria* (Caris et al, 2006). The spurs in *Aquilegia* are established via outpocketing at the base of each petal (Figure 16). A similar pattern in development can also be found in the sepal spurs of *Impatiens columbaria*. The distal end of the spur bulges out from the base of the dorsal sepal (Figure 17). In contrast, I find no evidence that the nectar tube of *Pelargonium* arises as an outgrowth of the dorsal sepal. Rather, it is integral to the formation of the receptacle. Moreover,

no other members of the Geraniaceae have nectar spurs. Although *Geranium robertianum* has nectar canals formed via synorganization due to position and shape of multiple floral organs, each of which arises independently (Endress, 2010), nectaries in the sister clade to *Pelargonium* are small, superficial glands opposite sepals. But, these are not spurs. *Hypseocharis*, the likely genus sister to *Pelargonium* and the rest of the Geraniaceae (Boesewinkel, 1988; Price and Palmer, 1993), also does not have a nectar spur or a tube.

Examination of the vascular tissue associated with the nectar tube matches the vasculature sketches from Labbe (1964) and also provides evidence that it is not a spur. There are three precepts of vascular conservatism, the third of which states that “orientations of bundles demonstrates homologies” (Schmid, 1972). Moseley (1967) remarked that vasculature “may reveal the former boundaries, relative positions, numbers, and categories of organs, or their parts, which may now be obscured by reduction, connation, and adnation”. Figures 18A and 18B shows a hypothetical derivation of a nectar tube by fusion of a nectar spur to a pedicel. According to this hypothesis the vascular bundle that supplies the dorsal sepal would take a convoluted path. From a longitudinal perspective, the vascular bundle that supplies the dorsal sepal will theoretically travel up the pedicel, loop down the ventral side of the spur, and loop back up the dorsal side of the spur (see Figure 18B). Consequently, there would be two vascular bundles (Fig 18D). The two bundles on the ventral side of the spur should have opposite orientations of xylem and phloem (Fig 18D). However, cross sections revealed that the vasculature does not coincide with the hypothetical orientation of a fused spur. Instead, it is likely that the vascular bundle that supplies the dorsal sepal travels up the pedicel and branches off near the nectary (Fig 18C, 18E), a concept that Japp (1909) also proposed in his study. In summary, the nectar tubes of *Pelargonium* are deep single dorsal receptacular nectaries, most accurately described earlier by Weberling (1989) and Japp (1909).

Conclusions

From developmental and anatomical standpoints, the nectar tube of *Pelargonium* is not an adnate sepal spur, but a deep receptacular nectary. Theoretically, the base of the tube is established during early ontogeny and the tube elongates via intercalary growth of the surrounding receptacle. The contrast in tube lengths is caused by the difference in elongation rates and duration; the divergence of tube lengths does not occur until post-anthesis. Ecological and evolutionary consequences of post-anthesis changes in nectar tube lengths are unexplored.

Past studies have shown that growth of different flower parts such as petal spurs or the pedicel is controlled by the KNOX gene, *HIRZ*, which promotes elongation (Golz et al., 2002; Box et al., 2011; Wang et al., 2015). These studies present the intriguing idea that differences in nectar tube elongation among *Pelargonium* species may be controlled by mutation of a few genes that act late in development after anthesis, if they exist.

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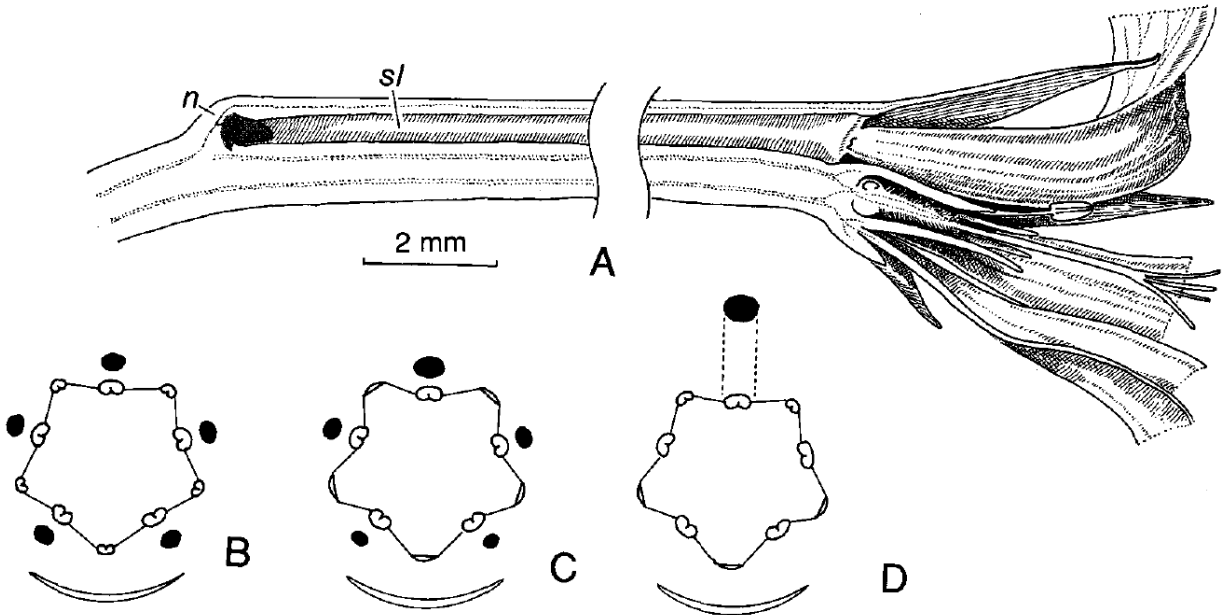


FIG. 1. Nectary of *Pelargonium* and its sister clade from Vogel (1998). (A) Longitudinal view of the nectary of *P. peltatum*. (B-D) Cross sections of androecia and nectar glands (black) in *Geranium* (B), *Erodium* (C), and *Pelargonium* (D).

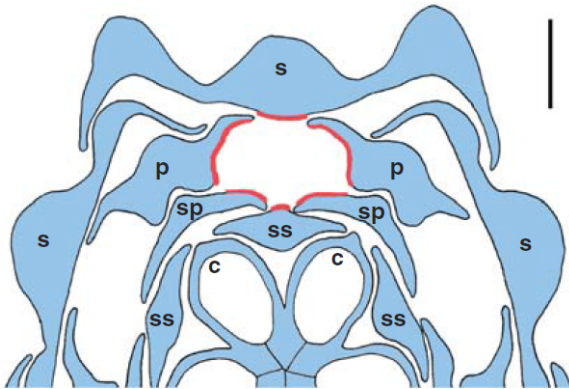


FIG. 2. A schematic drawing of a cross section of a *Geranium robertianum* flower from Endress (2010). The participation of six organs creates a nectar canal (red lines). C, carpel; P, petal; S, sepal; SP, antepetalous stamen, SS, antesepalous stamen. Scale bar = 200 μ m.

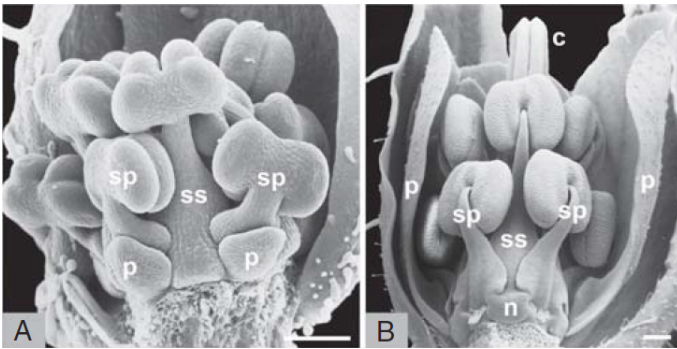


FIG. 3. SEM micrographs of *Geranium robertianum* flowers from Endress (2010). (A) An early stage where petal primordia are minute relative to the antepetalous stamens. (B) A flower at late ontogeny. The petals exceed the stamens in length. The nectary is finally apparent at the bases of the antepetalous stamens. C, carpel; P, petal; S, sepal; SP, antepetalous stamen, SS, antesepalous stamen; N, nectary. Scale bars: (A) = 100 μ m; (B) = 200 μ m.

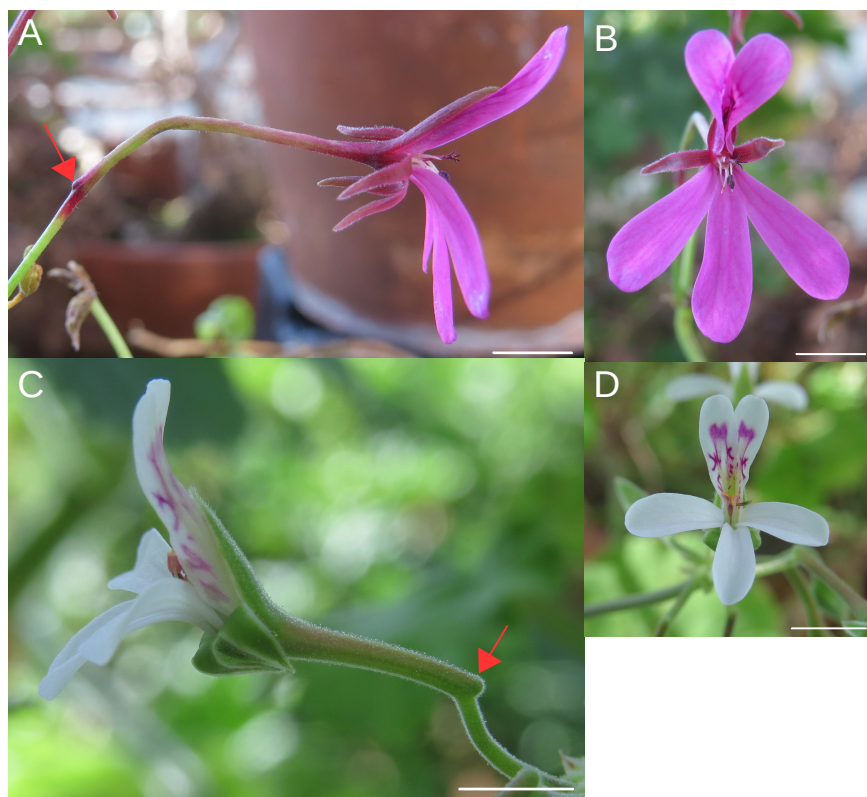


FIG. 4. *Pelargonium ionidiflorum* and *Pelargonium odoratissimum*. Mature flowers. (A,C) Longitudinal view of a flower of *P. ionidiflorum* and *P. odoratissimum*, respectively. The nectary is located closer to the base of the pedicel as a pronounced hump (red arrows). The tube is significantly longer on *P. ionidiflorum*. (B,D) Front view of the flowers of *P. ionidiflorum* and *P. odoratissimum*, respectively. The two dorsal petals are separated from the three ventral petals. Scale bar = 5 mm.

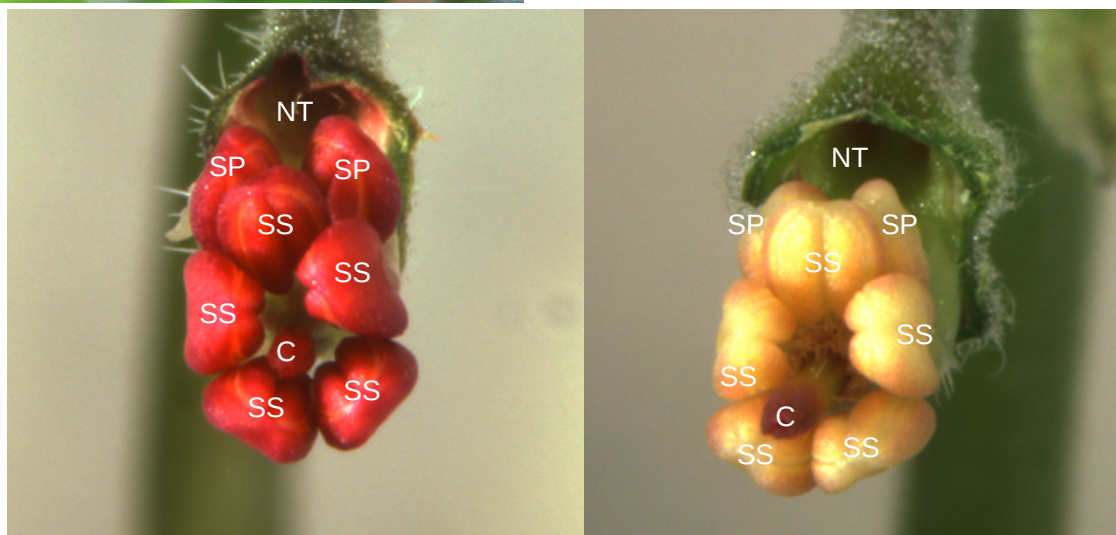


FIG. 5. *P. ionidiflorum* (left) and *P. odoratissimum* (right). Top views of dissected flower buds prior to anthesis. Both species have a total of seven stamens, five of which are antesepalous and two of which are antepetalous. The two antepetalous anthers flank the dorsal antesepalous anther on both species, anticipating the opening of the nectar tube. C, carpel; SP, antepetalous stamen, SS, antesepalous stamen; NT, nectar tube.

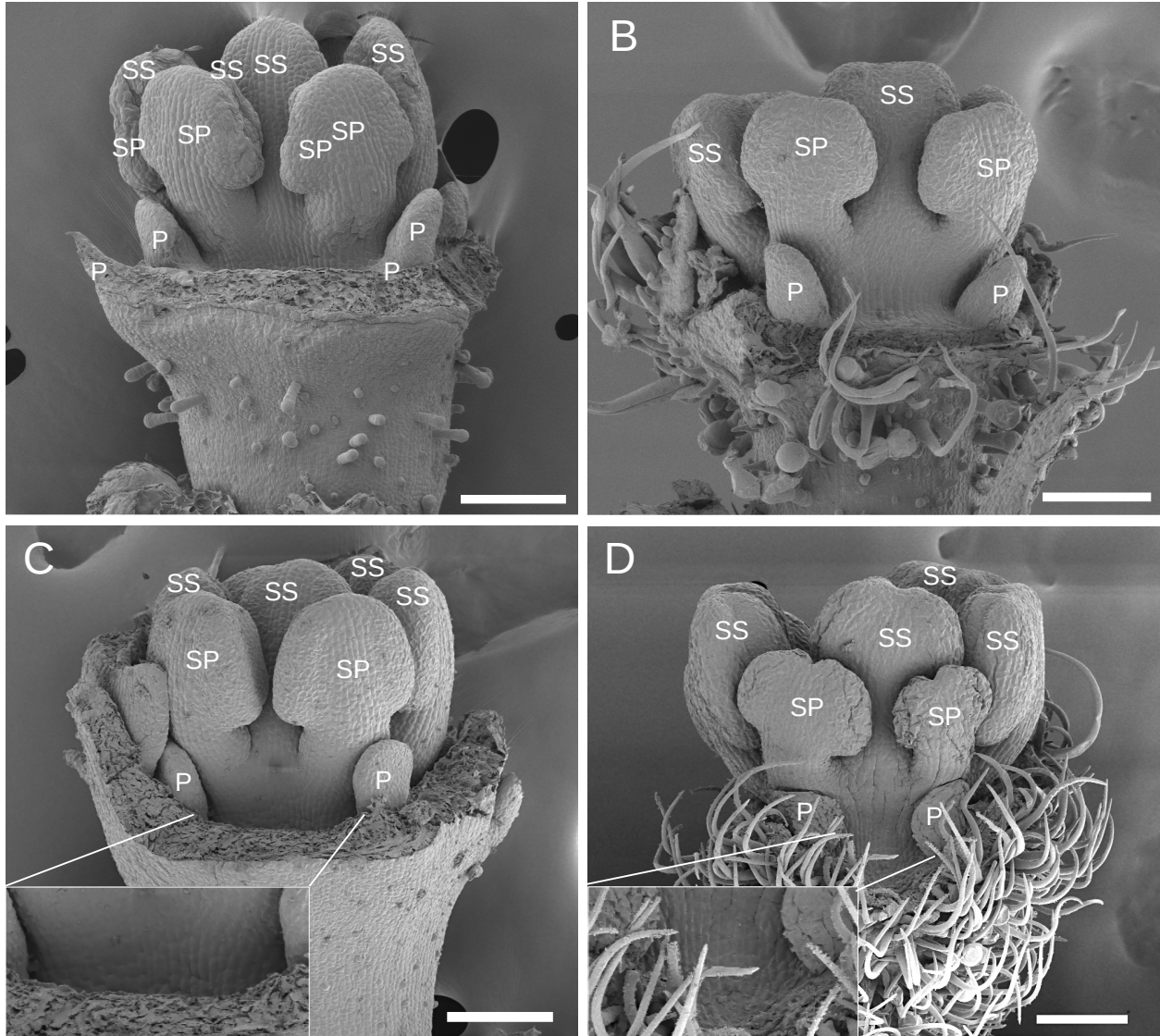


FIG. 6. *Pelargonium ionidiflorum* and *Pelargonium odoratissimum*. SEM micographs of flowers. (A, B) 0.5 mm long *P. ionidiflorum* and *P. odoratissimum* buds, respectively, from the dorsal side. All floral organs have been initiated with the dorsal antesealous stamen flanked by two antepetalous stamens, anticipating the location of the nectar tube. The nectar tube is absent on both species. (C, D) 0.9 mm long *P. ionidiflorum* and *P. odoratissimum* buds, respectively. The nectar tube is still absent on the former species (see left box). In contrast, concavity is being established at the base of the receptacle on *P. odoratissimum* (see right box). Radial files of vertically rectangular cells are present below the region of stamen and petal insertion on both species, suggesting for cell division. *P. odoratissimum* also has more trichomes on the pedicel than *P. ionidiflorum*. Box, base of receptacle; P, petal; SP, antepetalous stamen; SS, antesealous stamen. Scale bars (A-D) = 100 μ m.

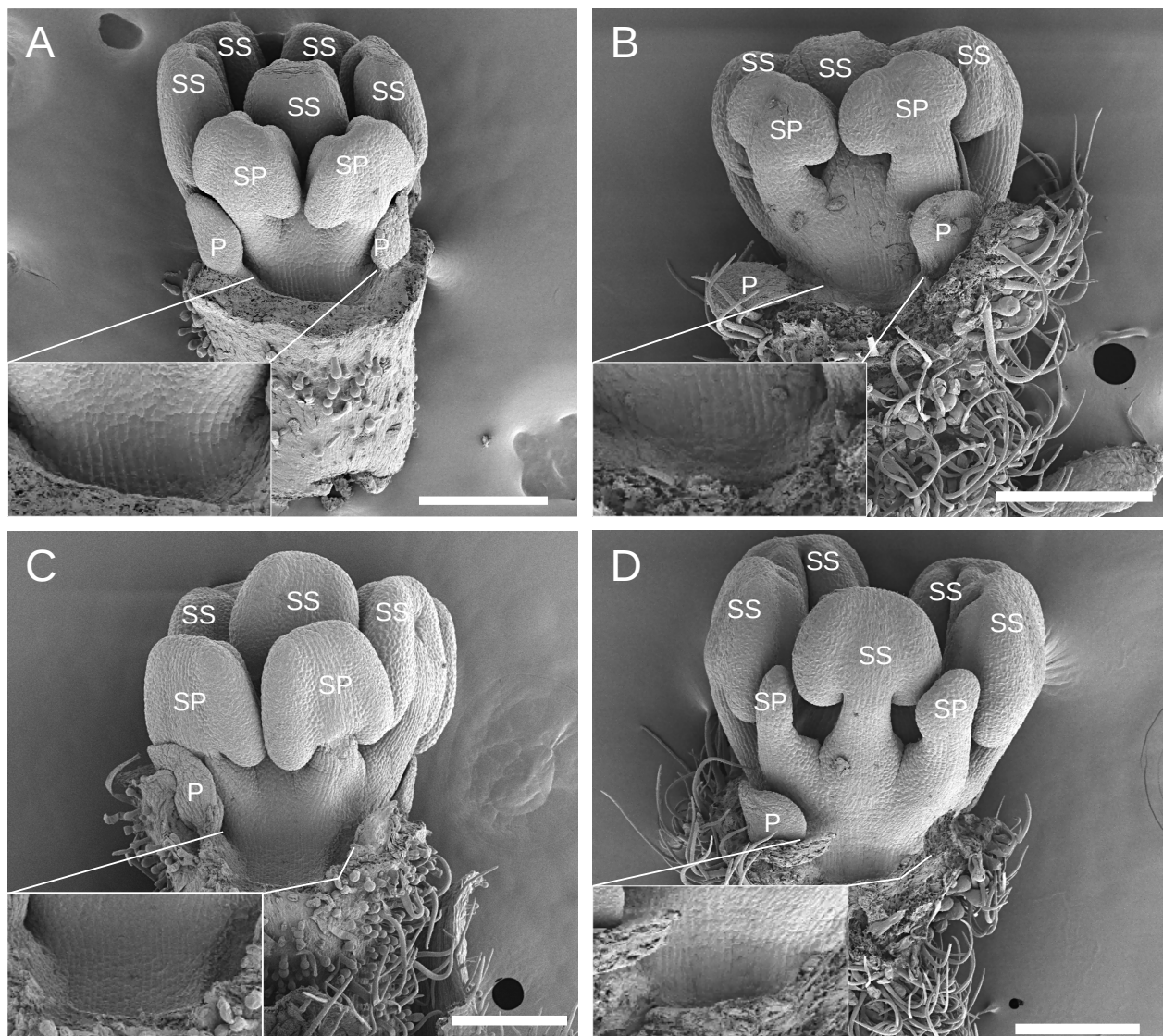


FIG. 7. *Pelargonium ionidiflorum* and *Pelargonium odoratissimum*. SEM micographs of flowers. (E,F) 1.1 mm long *P. ionidiflorum* and *P. odoratissimum* buds, respectively, from the dorsal side. Concavity has begun to form on *P. ionidiflorum* as indicated by the radially filed vertical cells beneath the petal insertions (see box). On both species, the antesepalous stamens are elongating slightly more than the antepetalous stamens. Petal primordia have also begun to elongate. (G,H) 1.5 mm long *P. ionidiflorum* and *P. odoratissimum* buds, respectively, showing the nectar tubes becoming more pronounced than the buds in the previous stage. The number of columns of radial vertical cells have become more numerous on both species (see boxes). The antepetalous anthers in *P. odoratissimum* have aborted, demonstrating that anther number can vary among flowers. Box, nectary; P, petal; SP, antepetalous stamen; SS, antesepalous stamen. Scale bars (A-D) = 200 μ m

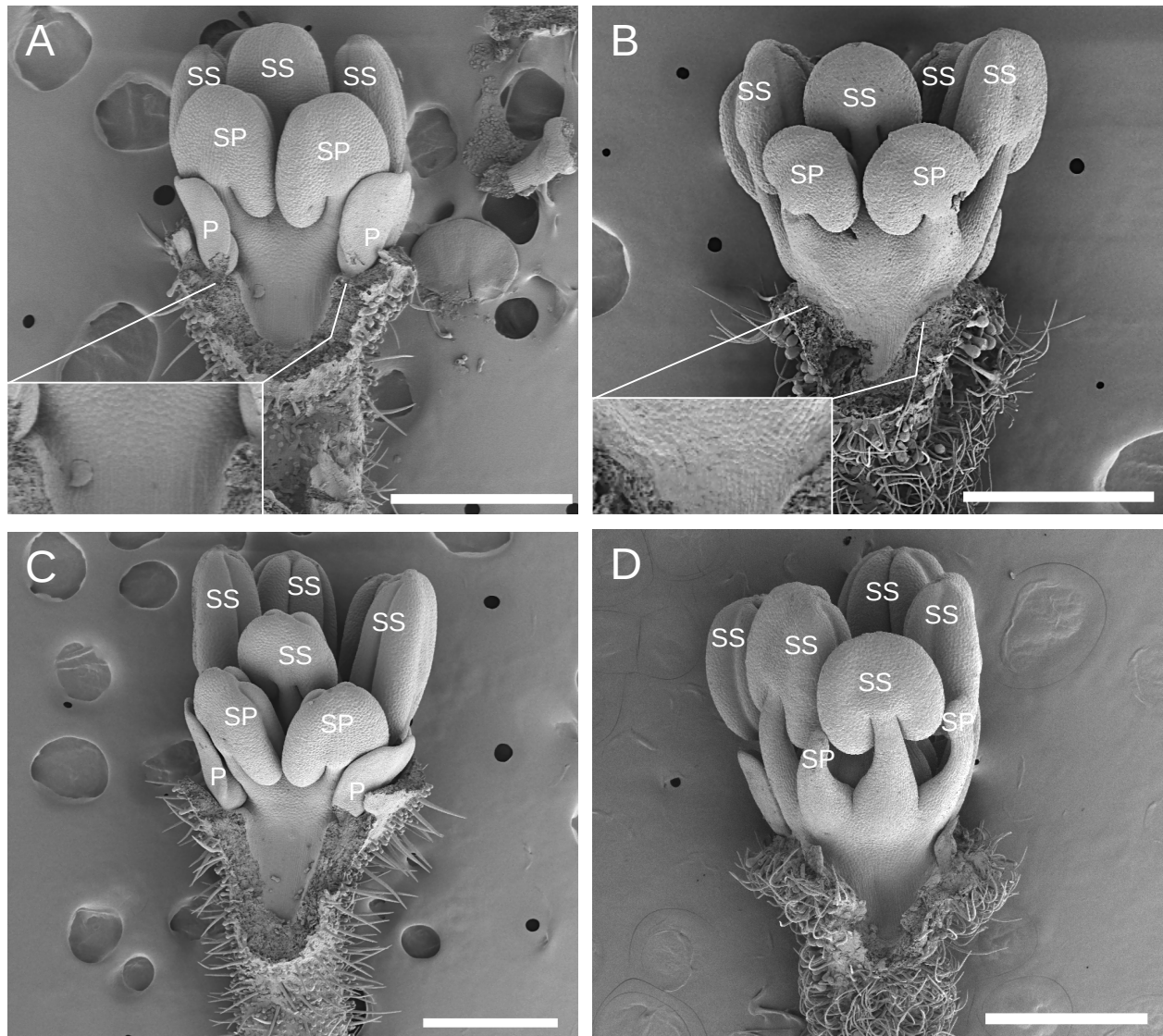


FIG. 8. *Pelargonium ionidiflorum* and *Pelargonium odoratissimum*. SEM micographs of flowers. (I,J) 2.0 mm long *P. ionidiflorum* and *P. odoratissimum* buds, respectively, showing the nectar tubes continuing to elongate. The polyhedral cells at the base of the stamens have become more differentiated from the vertical files of the nectar tubes of both species (see boxes). The petals of *P. odoratissimum* were removed to better visualize the tube. The base of the nectary is easily distinguished from the more cylindrical petiole. (K,L) 2.5 mm long *P. ionidiflorum* and *P. odoratissimum* buds, respectively, showing the nectar tubes continuing to elongate through acropetal growth of the receptacle above the tube. The petals in *P. ionidiflorum* are elongating relative to the antepetalous stamens. The petals in *P. odoratissimum* were removed and the antepetalous anthers have aborted. P, petal; SP, antepetalous stamen; SS, antesepalous stamen. Scale bars = 500 μ m.

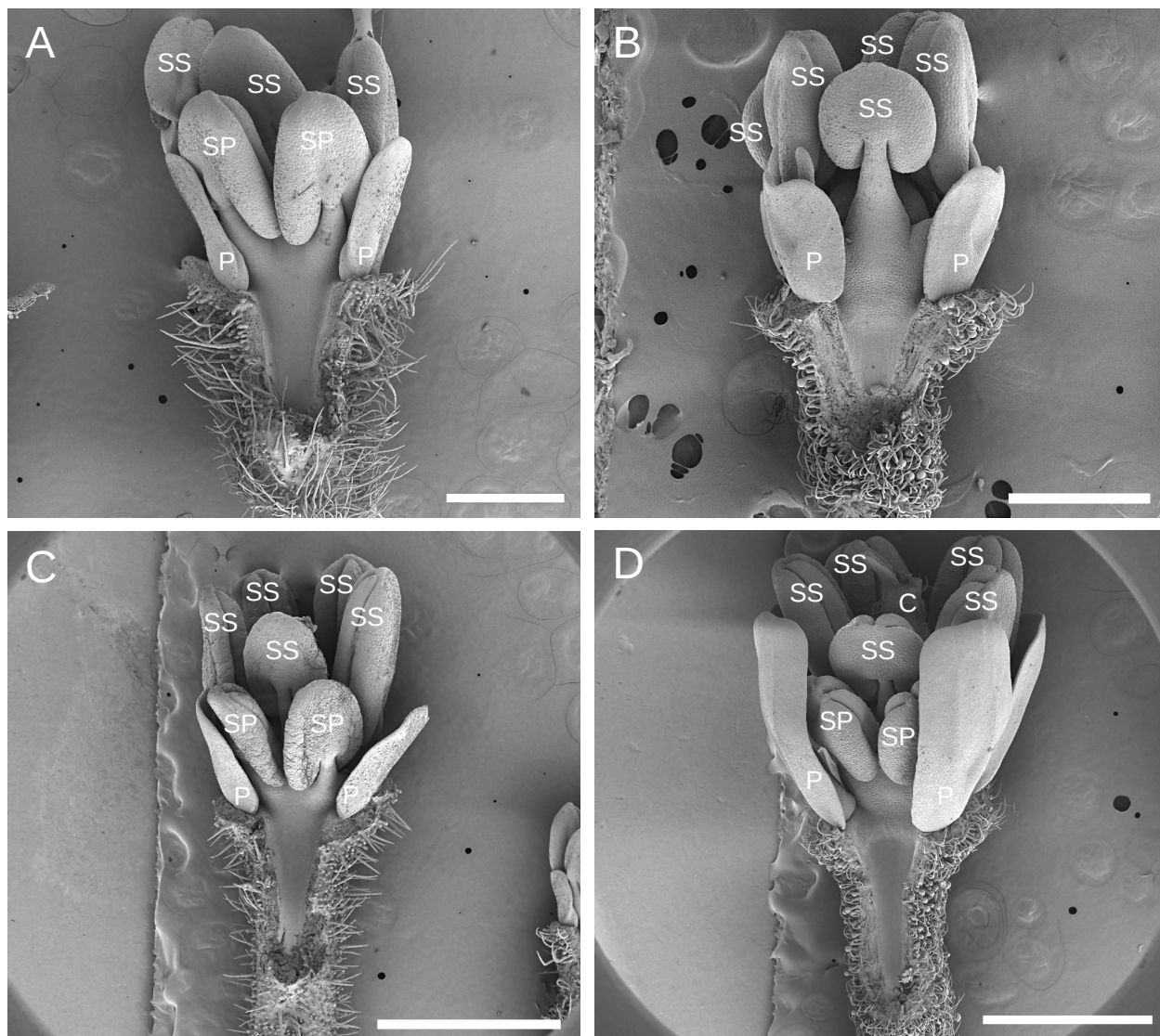


FIG. 9. *Pelargonium ionidiflorum* and *Pelargonium odoratissimum*. SEM micographs of flowers. (M,N) 3 mm long *P. ionidiflorum* and *P. odoratissimum* buds, respectively, showing the nectar tubes continuing to elongate. The antepetalous anthers in *P. odoratissimum* have aborted. The tube of this particular *P. ionidiflorum* bud is shown to be longer than that of *P. odoratissimum*. (O,P) 4 mm long *P. ionidiflorum* and *P. odoratissimum* buds, respectively, showing the nectar tubes having undergone noticeable growth. Unlike the previous stage, the tubes in both species are similar, suggesting for variation in tube lengths on flowers of similar stages. The petals in *P. odoratissimum* are significantly larger than the those of *P. ionidiflorum*, large enough to encase the antepetalous stamens. The carpel is also visible on *P. odoratissimum*. P, petal; SP, antepetalous stamen; SS, antepetalous stamen; C, carpel. Scale bars (A,B) = 500 μ m; (C,D) = 1 mm

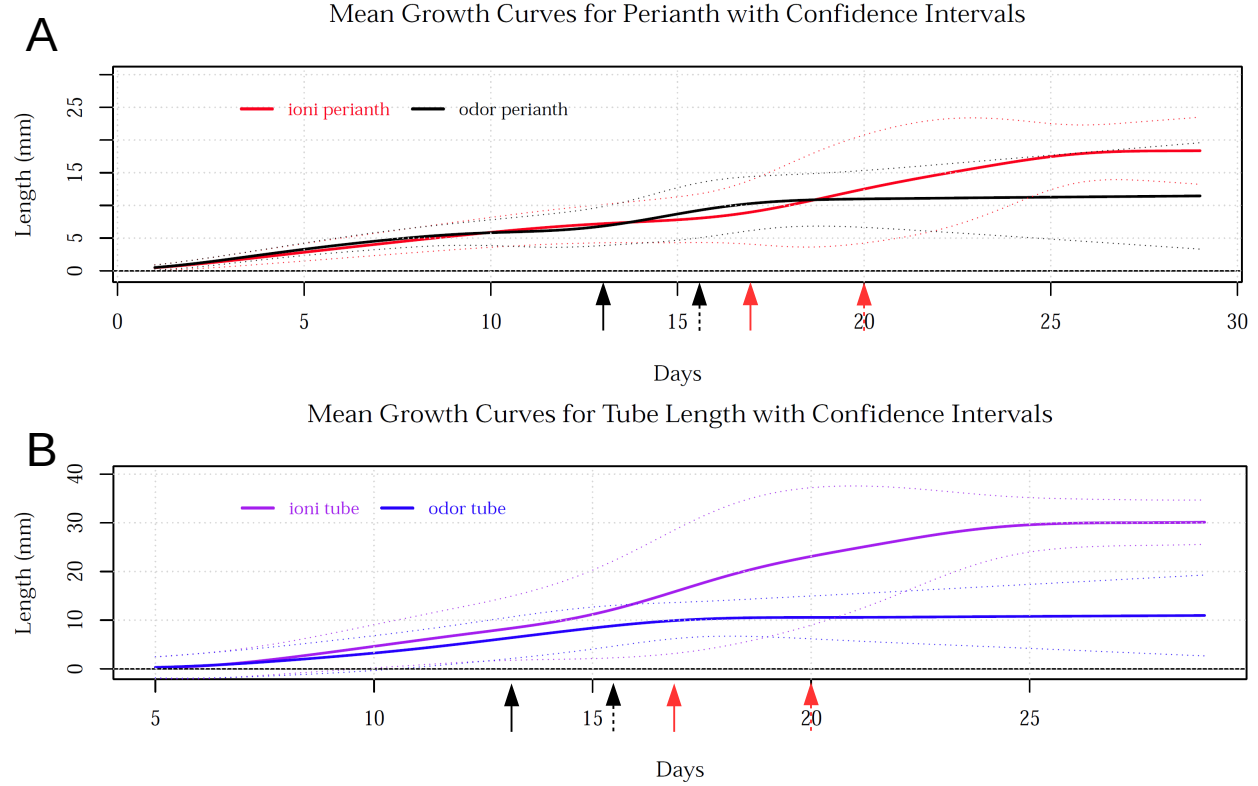


FIG. 10. (A) Mean growth curves for perianth length with 95% confidence intervals of *P. ionidiflorum* and *P. odoratissimum*. The overlapping between the lower confidence interval of *P. ionidiflorum* and upper confidence interval of *P. odoratissimum* indicates that there is no significant difference of perianth length between both species. (B) Mean growth curves of nectar tube length with 95% confidence intervals of *P. ionidiflorum* and *P. odoratissimum*. The lower confidence interval of *P. ionidiflorum* and upper confidence interval of *P. odoratissimum* overlap with each other until day 22, indicating significant divergence. Solid black arrow = *P. odoratissimum* petal emergence; Dotted black arrow = *P. odoratissimum* anthesis; Solid red arrow = *P. ionidiflorum* petal emergence; Dotted red arrow = *P. ionidiflorum* anthesis.

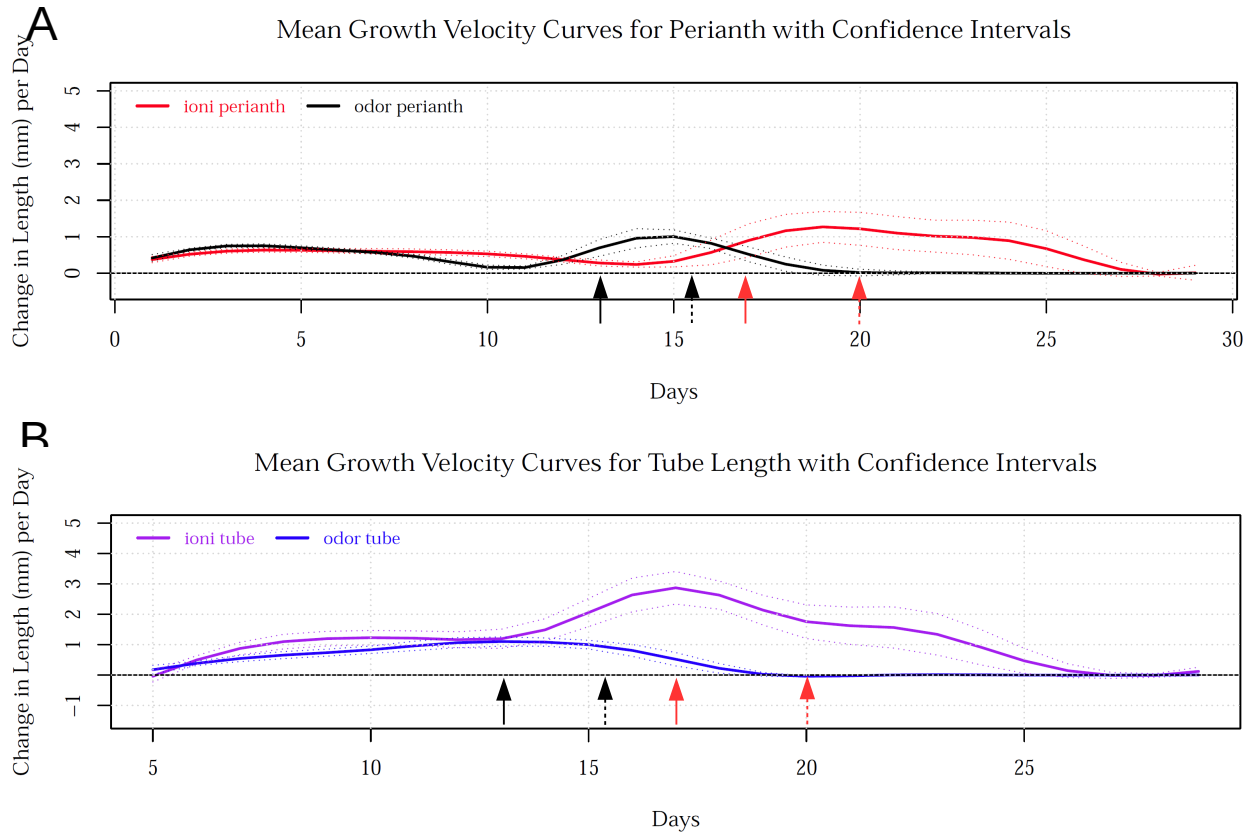


FIG. 11. (A) Mean velocity growth curves for perianth length with 95% confidence intervals of *P. ionidiflorum* and *P. odoratissimum*. The lower confidence interval of *P. ionidiflorum* and upper confidence interval of *P. odoratissimum* overlap with each other until day 28, indicating significant divergence. (B) Mean growth curves of nectar tube length with 95% confidence intervals of *P. ionidiflorum* and *P. odoratissimum*. The overlapping between the lower confidence interval of *P. ionidiflorum* and upper confidence interval of *P. odoratissimum* indicates that there is no significant difference of tube elongation rate between both species. Solid black arrow = *P. odoratissimum* petal emergence; Dotted black arrow = *P. odoratissimum* anthesis; Solid red arrow = *P. ionidiflorum* petal emergence; Dotted red arrow = *P. ionidiflorum* anthesis.

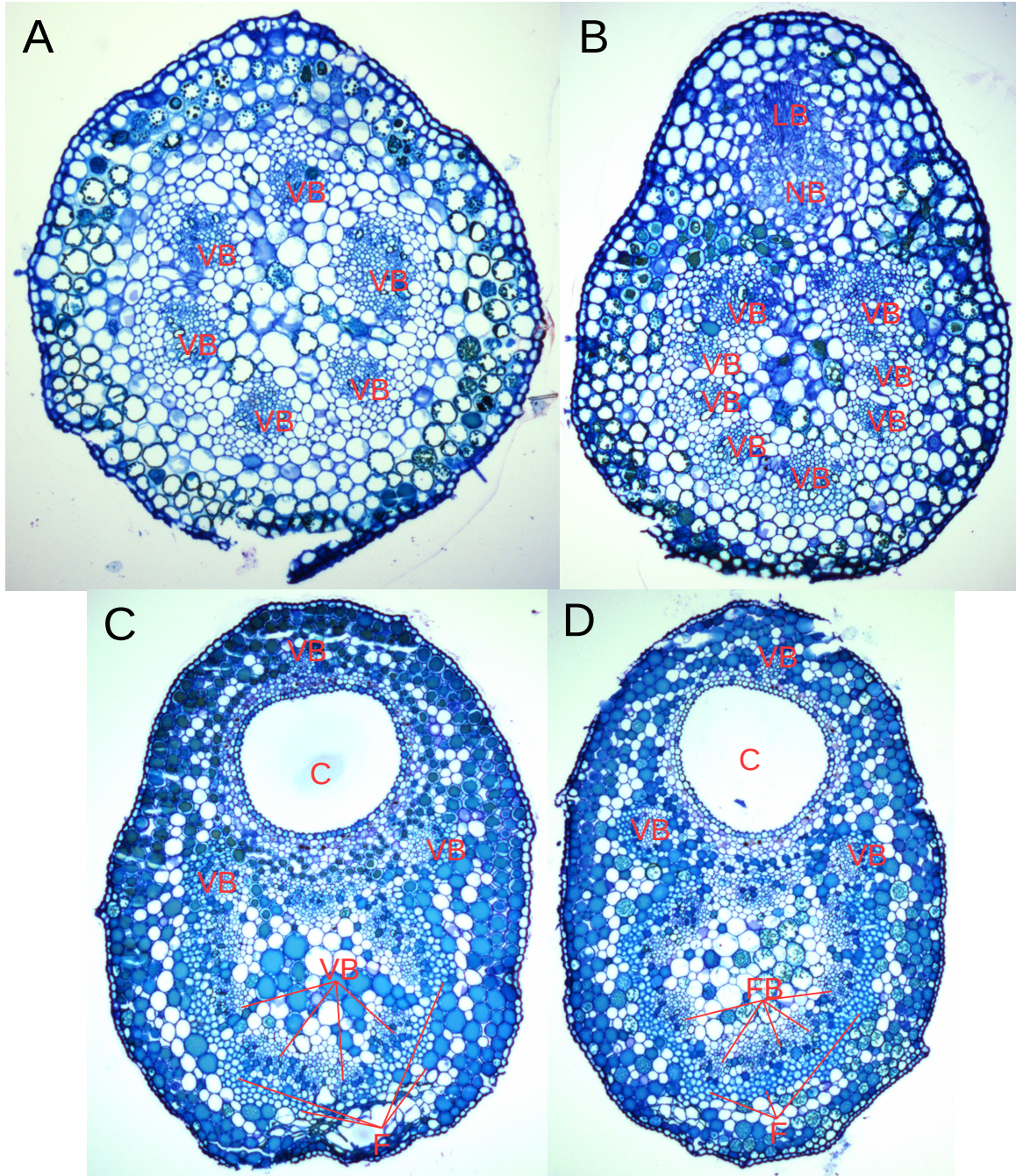


FIG. 12. Cross sections of the pedicel and nectar tube of a mature *P. ionidiflorum* flower. (A) Cross section through the pedicel showing six vascular bundles. The bump of the nectary is just beginning to appear. (B) Cross section through the base of the nectar tube showing nine vascular bundles with eight ventral to the nectary base and one longitudinal bundle dorsal to the nectary. (C) Cross section through the nectar tube 1 mm above the base, showing eleven vascular bundles, with ten ventral to the cavity and one dorsal to the cavity. Eight of the ventral bundles are organized in a ring while the two others are adjacent to the ventral side of the cavity. Fibers are present around the ventral vascular bundles (D) Cross section through the nectar tube 2 mm above the base. The ventral side of the ring of vascular bundles have fused and fibers are present around the ring. There are two vascular bundles adjacent to the left and right ventral sides of the cavity and one bundle adjacent to the dorsal side of the cavity. VB, vascular bundle; NB, nectary base; LB, longitudinal bundle; C, cavity; F, fibers; FB, fused bundle. Magnification = 50x.

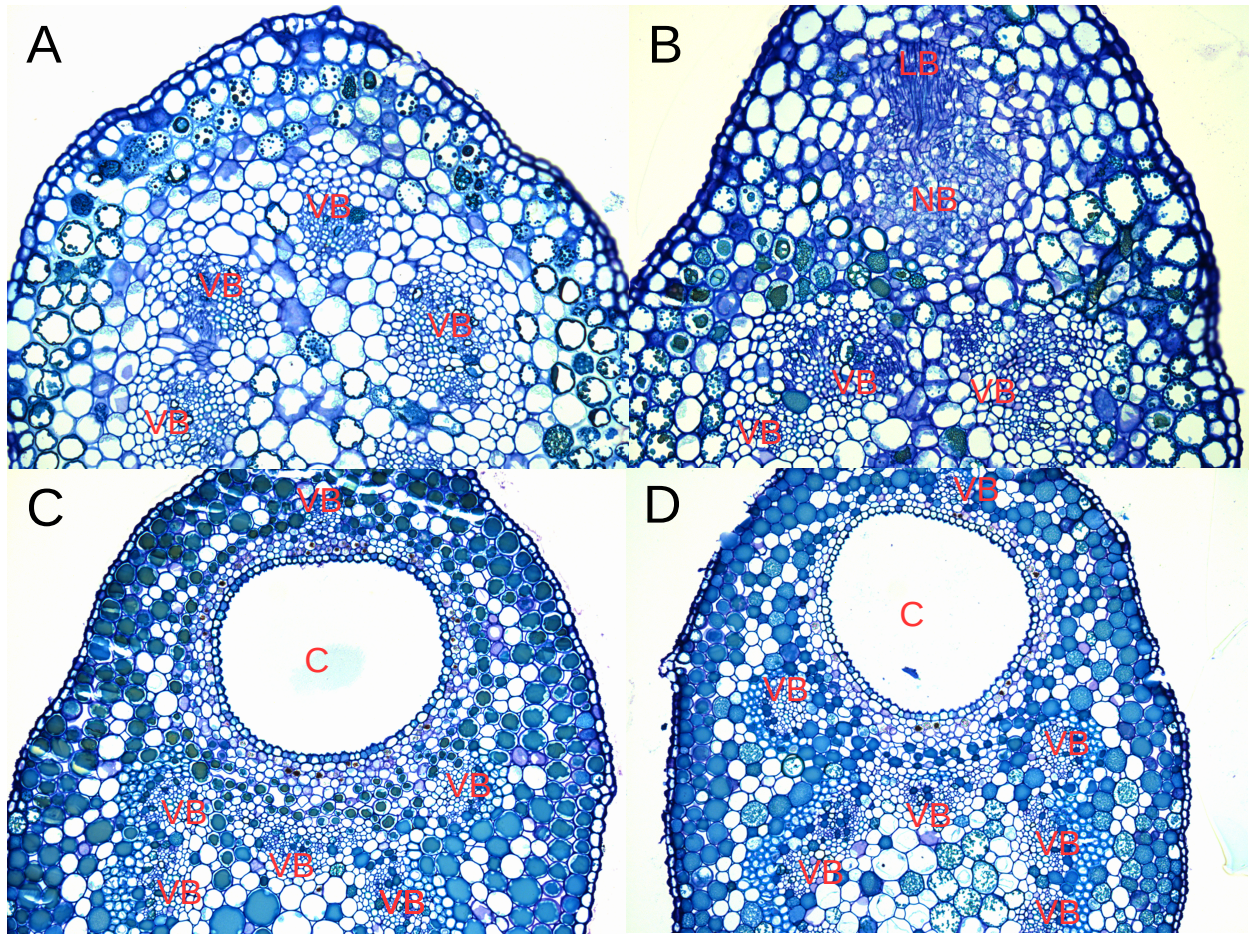


FIG. 13. Cross sections of the pedicel and nectar tube of a mature *P. ionidiflorum* flower at a higher magnification. (A) Cross section through the pedicel showing four of the six vascular bundles. (B) Cross section through the base of the nectar tube showing four of the nine vascular bundles with three ventral to the nectary base and one longitudinal bundle dorsal to the nectary. (C) Cross section through the nectar tube 1 mm above the base, showing six of the eleven vascular bundles, with five ventral to the cavity and one dorsal to the cavity. Three of the ventral bundles are organized in a ring while the two others are adjacent to the ventral side of the cavity. (D) Cross section through the nectar tube 2 mm above the base. This image shows the vascular bundles of the ring that have not fused. There are two vascular bundles adjacent to the left and right ventral sides of the cavity and one bundle adjacent to the dorsal side of the cavity. VB, vascular bundle; NB, nectary base; LB, longitudinal bundle; C, cavity; VR, vascular ring. Magnification = 100x.

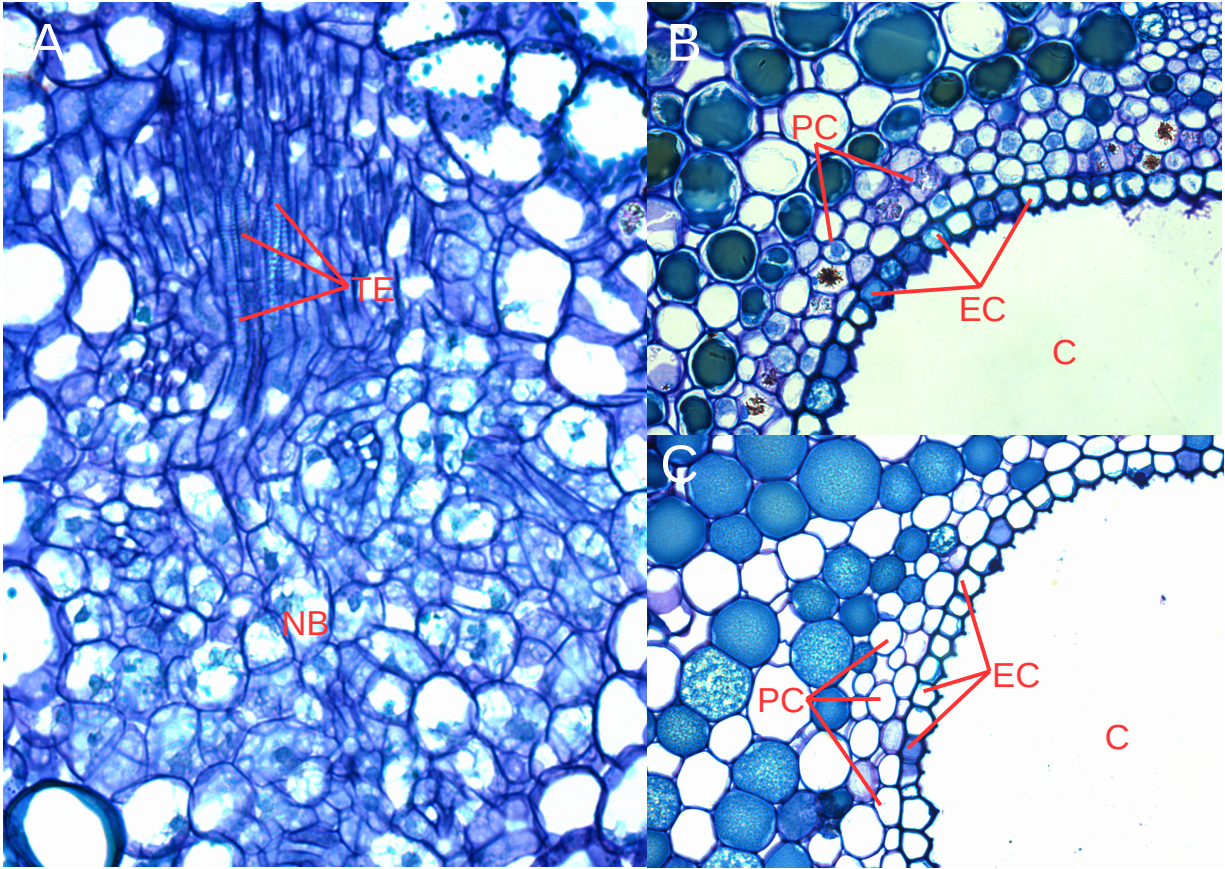


FIG. 14. Cross sections of the nectar tube of a mature *P. ionidiflorum* flower at a higher magnification. (A) Cross section through the base of the nectar tube showing the base of the nectary and longitudinally sectioned tracheary elements. (B) Cross section through the nectar tube 1 mm above the base, showing papillate epidermal cells lining the tube and stained parenchyma cells. (C) Cross section through the nectar tube 2 mm above the base, showing papillate epidermal cells and unstained parenchyma cells. NB, nectary base; TE, tracheary elements; C, cavity; EC, epidermal cells; PC, parenchyma cells. Magnification = 400x.

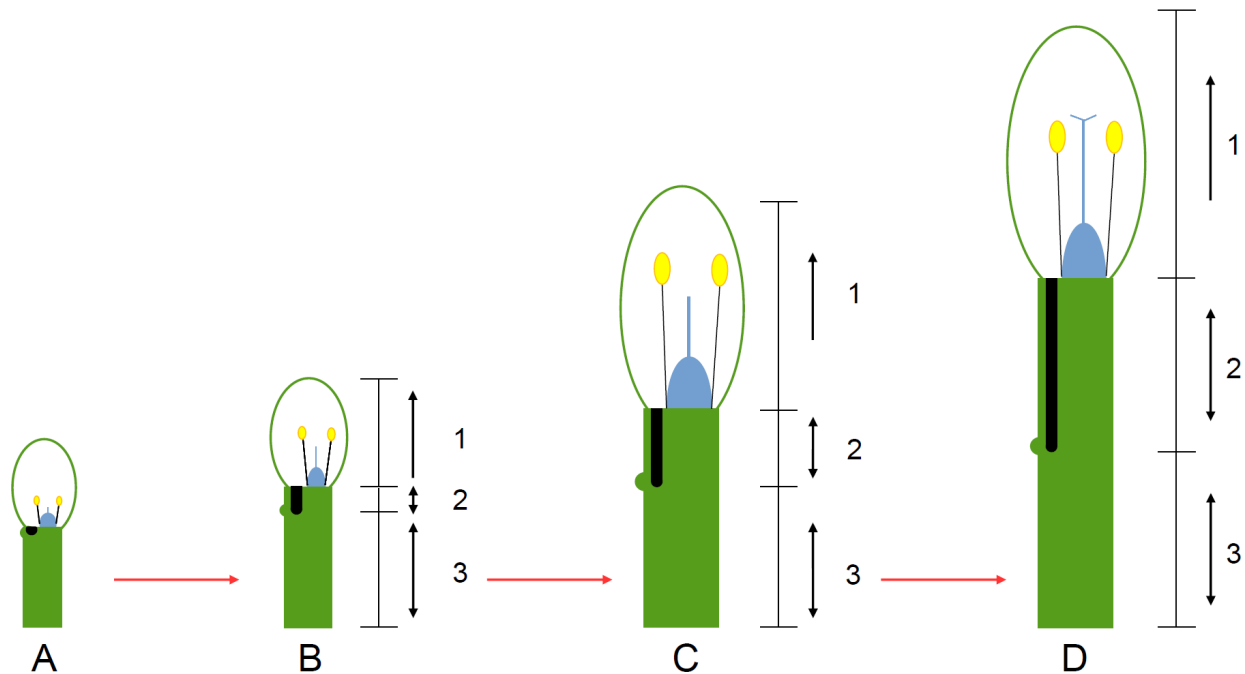


FIG. 15. A diagram of how the nectar tube of *Pelargonium* hypothetically develops. (A) An early stage where the receptacle establishes a concavity to form the base of the nectar tube. (B) A slightly later stage where the nectar tube has elongated via intercalary growth of the receptacle (Region 2). The calyx has also elongated (Region 1) as well as the pedicel (Region 3) (C) A later stage where the nectar tube has elongated from more intercalary growth of the receptacle in addition to further elongation of the calyx and pedicel. (D) A more advanced stage where the nectar tube, calyx, and pedicel have continued to elongate.

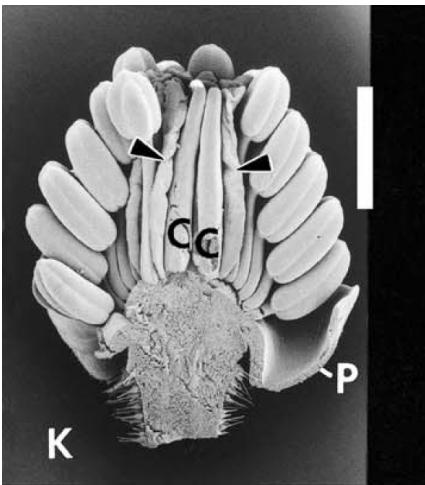
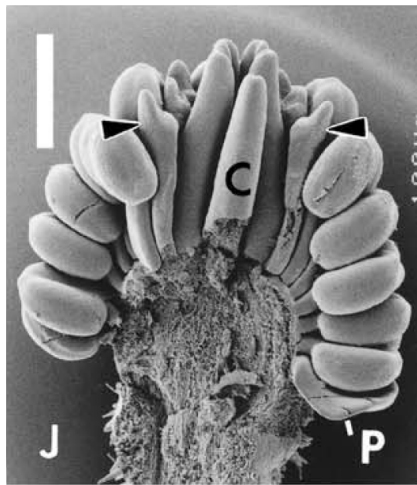


FIG. 16. Longitudinal sections of *Aquilegia olympica* flowers from Tucker and Hodges (2005). (J) The petal has not yet formed concavity. The carpels are elongated relative to the stamens (K) Concavity has been established in the petal and the spur is elongating downwards. C, carpel; P, petal. Scale bars (A) = 400 μ m; (B) = 1 mm.

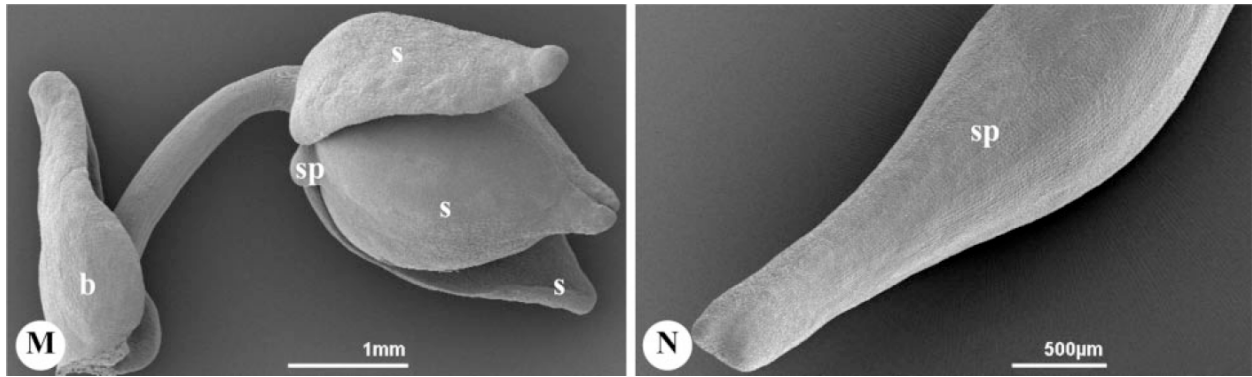


FIG. 17. SEM micrographs of *Impatiens columbaria* from Caris et al. (2006). (M) Lateral view of a young flower bud. The sepal spur is only a small bump. (N) Distal part of the sepal spur which has grown significantly larger.

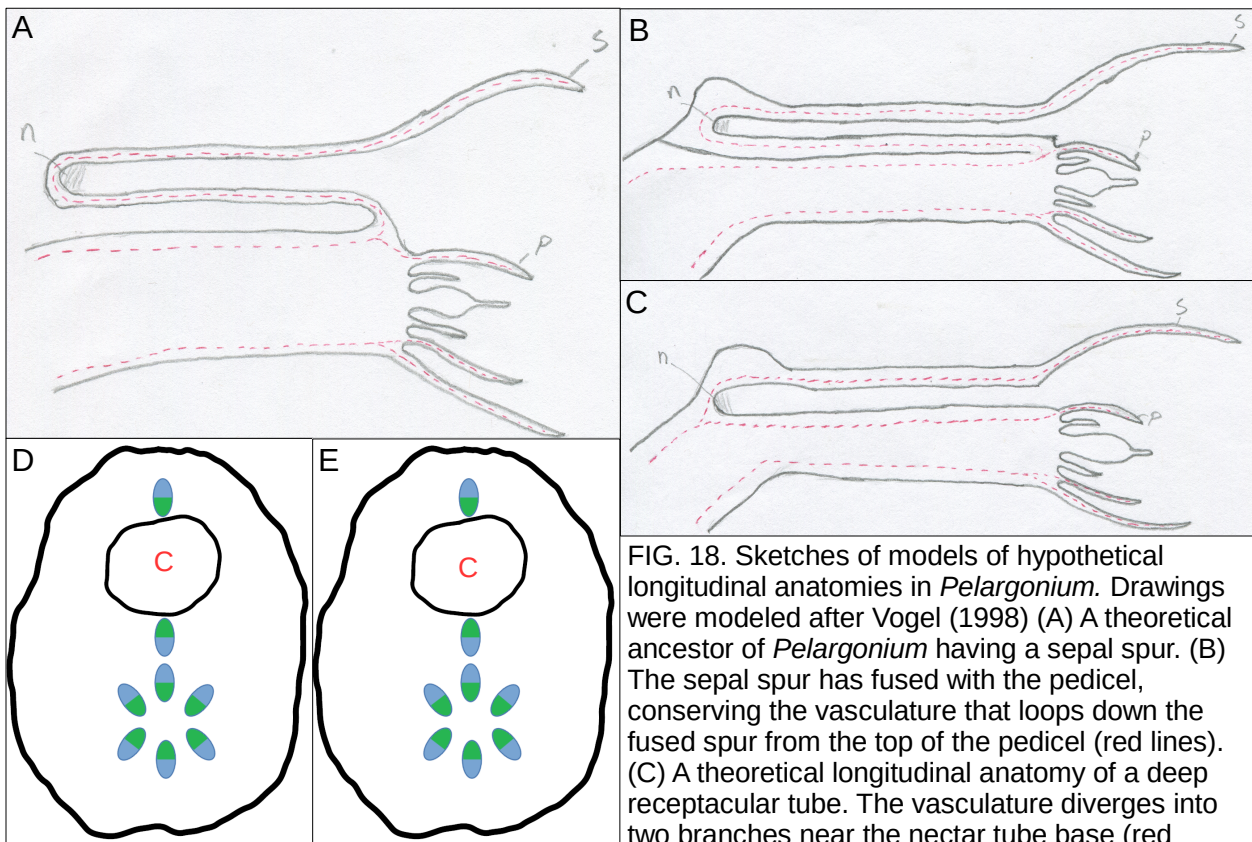


FIG. 18. Sketches of models of hypothetical longitudinal anatomies in *Pelargonium*. Drawings were modeled after Vogel (1998) (A) A theoretical ancestor of *Pelargonium* having a sepal spur. (B) The sepal spur has fused with the pedicel, conserving the vasculature that loops down the fused spur from the top of the pedicel (red lines). (C) A theoretical longitudinal anatomy of a deep receptacular tube. The vasculature diverges into two branches near the nectar tube base (red lines). (D) Cross section through the adnate spur (see arrow on B). One vascular bundle is dorsal to the cavity with the phloem (blue) outside the xylem (green). Another bundle ventral to the cavity has the xylem outside the phloem. (E) Cross section through the receptacular tube (see arrow on C). One vascular bundle is dorsal to the cavity with the phloem outside the xylem. However, it lacks the bundle on the ventral side of the cavity that has the xylem outside the phloem. S, sepal; P, petal; N, nectary; C, cavity.