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Anatomical Analysis of Double-flower Morphogenesis in a *Nicotiana alata* Mutant

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Abstract. **Anatomical analysis was performed using a double-flowered mutant of *Nicotiana alata* Link & Otto. Flower doubleness resulted from petaloid modification of the androecium. Vascularized petal-like outgrowths arose from the anther, connective, and filament of the stamen. The vasculature in petaloid outgrowths from the anther and upper part of the filament originated from and was continuous with the vascular bundle of the filament. In contrast, the vascular bundles formed in the outgrowths from the lower part of the filament developed independently of the vascular bundle of the filament and were not connected to it at any time. Emergences consisting of epidermal and ground parenchyma tissue and lacking vascularization arose from the filament.**

Nicotiana alata (Solanaceae), or Jasmine tobacco, is a flowering ornamental tobacco native to NE Argentina and S. Brazil (Griffiths, 1994). Plants are ≈1.5 m in height with sticky hairy stalks, ovate to lanceolate leaves, and long trumpet-shaped flowers. The flowers are 5 to 8 cm long, pale purple or white within, and yellowish at the outside surface. Crosses of *N. alata* × *N. forgetiana* hort. Sander ex Hemsl. (syn. *N.* ×*sanderae* hort. Sander ex Wild. Wats.) have produced white, greenish, pink, and red-flowered hybrids used commonly as bedding plants (Griffiths, 1994).

A white double-flowering form of *N. alata* was found growing among a cultivated F_2 population of otherwise single white-flowered plants. The extra petal-like outgrowths make the flowers more showy and therefore increase the horticultural value of the plant. Genetic analysis of the double-flower form of *N. alata* showed the phenotype was heritable, under nuclear control, and controlled by a single recessive gene (Zainol and Stimart, 1998). A dominant gene expressed in either the homozygous or heterozygous state controlled nondouble-flower development.

Flower doubleness has been reported in several *Solanaceae* taxa including *N. langsdorfii* J. A. Weinm. hybrids (White, 1914), *N. tabacum* L. (Hitier, 1950, Komari; 1990) and *Petunia* ×*hybrida* Hort. (Saunders, 1910; Scott, 1937). No reports were found of flower doubleness in *N. alata*. Anatomical analysis was initiated to characterize the double flowering trait of *N. alata*.

Materials and Methods

A double-flowered mutant of *N. alata* 'White' Link & Otto (Fig. 1) was found by D.P. Stimart in an F_2 population. This plant was self pollinated and outcrossed to nondouble *N. alata* 'Domino Salmon' or 'Metro Lime' to stabilize the trait (Zainol and Stimart, 1998). Seeds obtained from the above matings were used to generate flowering plants for anatomical evaluation.

Seeds were germinated in an incubator at 25 °C under cool-white fluorescent light (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) from 0800 to 2400 HR in cell packs in a 1:1:1 (by volume) mix of soil, sand and sphagnum peat. Seedlings were transferred to the greenhouse after 6 d and transplanted to cell packs after the first pair of true leaves expanded. They were transplanted later to 10×10-cm plastic pots and grown in the greenhouse. Plants were fertilized with 200 $mg \cdot L^{-1}$ of (in percent) 20N–8.7P–16.5K Peter's (Scott's-Sierra Horticultural Products Co. Marysville, Ohio) fertilizer every 2 weeks. Flowering plants were scored phenotypically for nondouble and double flowers. A plant was scored as double when petal-like outgrowths appeared (Zainol and Stimart, 1998).

Microtechnique. Flower buds of nondouble and double flowers were harvested to study stages of flower development. Buds from 0.4 to 10 mm in length were placed in 5% glutaraldehyde, vacuum infiltrated, and refrigerated overnight. The next day, the buds were washed twice with a cold potassium phosphate buffer and rinsed three times with cold deionized water. Samples were dehydrated on the first day with a series of nine ethanol solutions: 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, and 70%, with 30 min/change. Dehydration continued the next day with four additional treatments: 80%, 95% and two changes of 100% ethanol with 30 min/change. Samples were infiltrated with L.R. White embedding medium, polymerized by stacking two flat aluminum weighing

Fig. 1. Double flower of *Nicotiana alata* 'White'.

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boats as an embedding mold, sandwiched, and left in an oven at 60 °C for 24 h. The next day polymerized buds were removed from the molds and mounted on plexiglass pegs and sectioned at 1 to 2 mm using a microtome. Sections were transferred to a slide with a drop of deionized water and water was evaporated subsequently on a hot plate at ≈40 °C. Tissues were stained with 0.05% toludine blue for a few seconds on the hot plate, rinsed with deionized water and dried. The tissues were covered with a coverslip using immersion oil as the mounting medium.

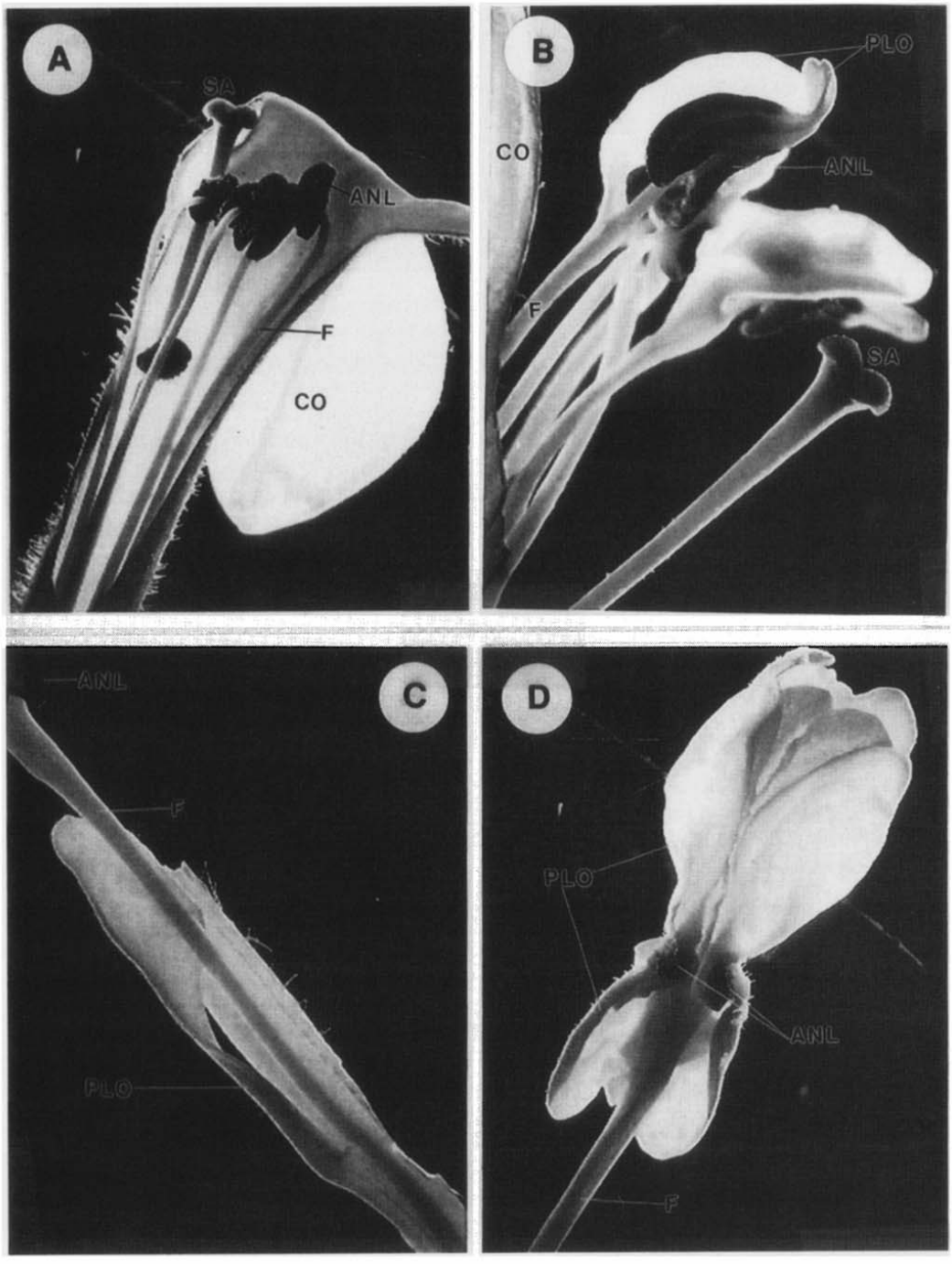
Clearing technique. Stamens and petals of nondouble and double flowers were submerged in 75% ethanol and stored in a refrigerator for 3 d. Materials were obtained from open flowers and flower buds 5 to 15 mm long. Cleared tissues were transferred to a slide, mounted with immersion oil, covered with a cover slip, and examined with a microscope.

Results

Flower description

Nondouble genotype. The single flower of *N. alata* is hypogynous where sepals, petals and stamens are free from the calyx and attached to the receptacle at the base of a superior ovary. The normal flower consists of five sepals, a corolla represented by five petals, five stamens which are adnate partially to the corolla from the base, and a gynoecium. Stamens are basifixed and consist of a filament with two anther lobes (Fig. 2A).

Double genotype. In the double flower, petal-like outgrowths developed out of the filament and anther of each flower. On the filament, the petal-like outgrowths arose from the upper and lower parts of the filament. In general, the size and occurrence of the upper outgrowths were similar and constant in all five stamens (Fig. 2B). The outgrowths formed on the lower part of the filaments (Fig. 2C) varied in size among stamens within the same flower. The petal-like outgrowths that formed at the anther appeared as an excrescense on the tip of the fertile anther lobes and connective, filling the corolla with frilled structures. The formation of these outgrowths was stable in all anthers in terms of size and occurrence. In the stronger expression, this outgrowth had a distinct shape of a petal-like structure (Fig. 2D). The pollen-producing areas or anther lobes became more distorted and no pollen was produced. In all forms and degrees of doubleness, the gynoecium and other floral parts developed normally, except in the self-pollinated plants in which the petals were deformed slightly. Therefore, formation of these petal-like outgrowths, which developed on both the filament and anther of the stamen, were caused by petalodization of the stamen.



Floral development and vascular system of nondouble and double flowers

In general, the development in the early meristematic stage of floral initiation of the double-flower genotype was similar to the nondouble genotype (Fig. 3 A–C). Differences appeared when the anther became distinct from the filament.

Fig. 2. Androecium of nondouble (**A**) and double (**B–D**) flowers of *Nicotiana alata*. Magnification: (**A**) = 5.5×, (**B**) = 7.5×, (**C**) = 6.75×, (**D**) = 5.5×. ANL = anther lobe, CO = corolla, F = filament, PLO = petal-like outgrowth, and SA = stigma.

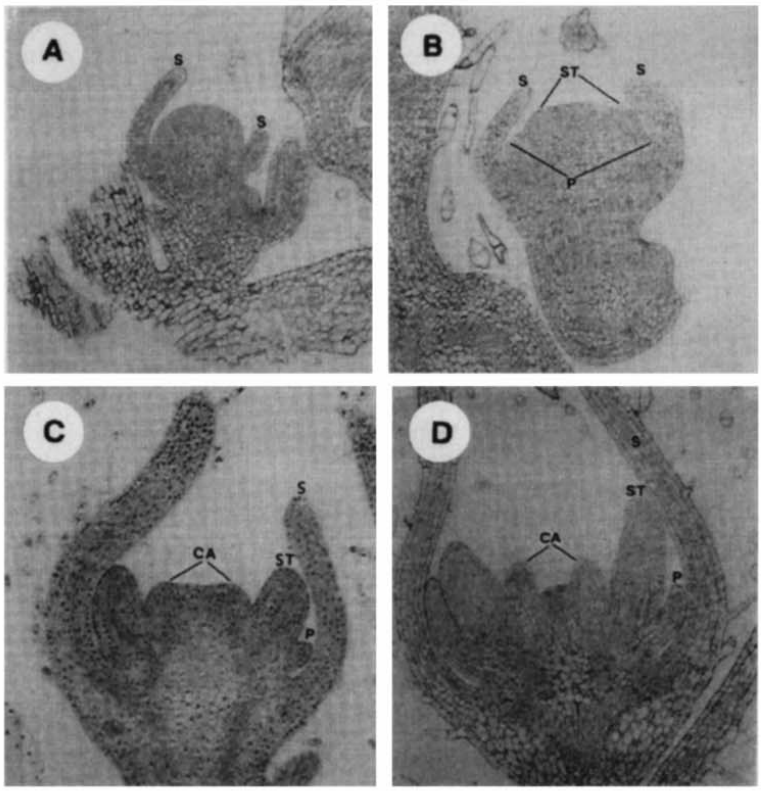


Fig. 3 (**above**). Longitudinal sections of nondouble flower buds of *Nicotiana alata*. Bud lengths = 0.4 mm (**A**), 0.45 mm (**B**), 0.75 mm (**C**), and 1 mm (**D**). Magnification: 94.5×. CA = carpel, P = petal, S = sepal, and ST = stamen.

Initiation of sepals, petals, stamens, and carpel primordia occurred sequentially. The development of these floral organs began with formation of a dome-shaped meristematic structure (Fig. 3A). Then, one of the sepal primordia was initiated and arose in advance of the others. As sepal primordia enclosed the bud, petal primordia appeared and developed as marginal protuberances (Fig. 3B). The petal primordia were followed by stamen primordia (Fig. 3B). Next, two carpel primordia arose on the floral apex (Fig. 3C). Stamen primordia differentiated into anther lobes on a short filament. The two carpel initials continued to grow both vertically and laterally.

Morphological differences between nondouble and double flowers became apparent after appearance of the anther lobes and before differentiation of a style and stigma (Fig. 4). Shown are an early stage (Fig. 4A) and a later stage (Fig. 4B) of petal-like outgrowth development arising from tips of anthers.

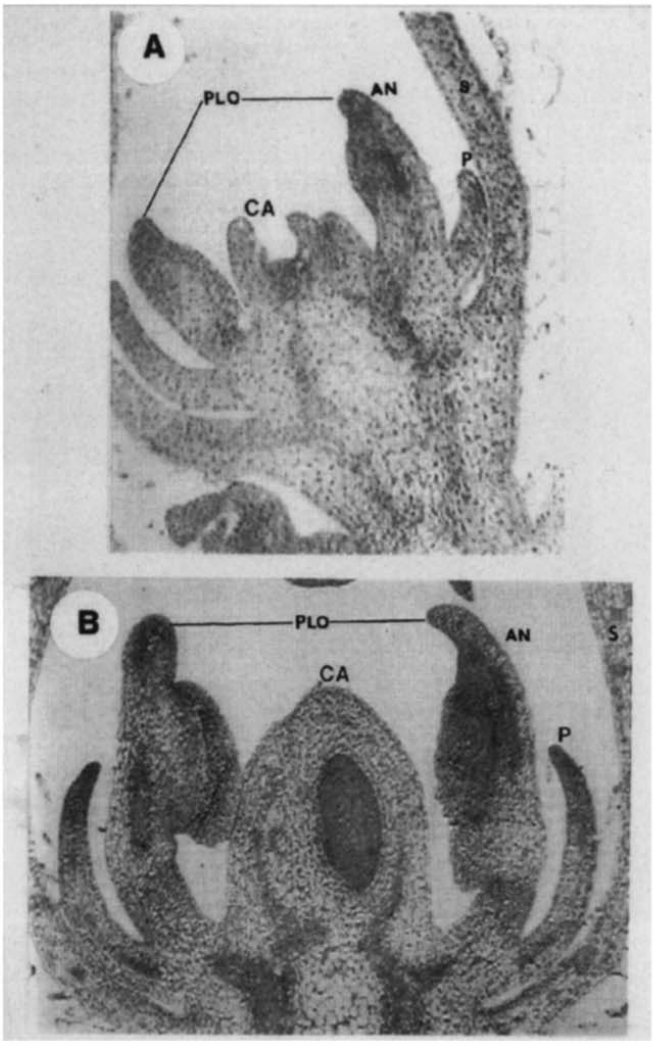
Generally, development of the vascular system was similar in nondouble and double flowers except in the area where petalodization occurred. Vascular bundles extended from pedicel to receptacle and diverged at the level of attachment of the sepals (Fig. 3C). Above this level, other vascular bundles extended to petals, stamens and carpels. Each stamen had one vascular bundle, which extended from the filament into the anther and terminated at the end of the anther (Fig. 5). In a double-flower genotype, the vascular bundle continued to grow into the petaloid outgrowth, which arose at the anther and formed an extensive vascular system (Fig. 6B). In fact, in extreme doubleness an extensive vascular system was formed, but not as extensive as that in ordinary petals (corolla) (Fig. 6A).

The vascular systems of the petal-like outgrowths that developed on the upper and lower parts of the filaments differed in their development. The vascular system of the petal-like outgrowths that arose from the upper part of the filament developed as an extension of the vascular bundle in the filament (Fig. 7). Two or three bundles extended into the petal-like outgrowth, forming a simple venation system. By contrast, the vascular system of the petal-like outgrowth that arose from the lower portion of the filament was not connected to the vascular bundle of the filament (Fig. 8 A and B). Examination of earlier stages of development revealed the vascular system of such petal-like outgrowths was never connected to the vascular bundle of the filament (Fig. 9). A very small outgrowth or 'emergence' occasionally developed from the filament on double-flowered plants. This outgrowth lacked vascular tissue (Fig. 8 A and C), consisting only of an epidermis and ground parenchyma.

Discussion

Anatomical analysis of *N. alata* double flowers showed additional petal-like outgrowths originated on stamens from anthers and filaments within otherwise normal flowers. The petal-like outgrowths from the anthers and upper portion of the filament were vascularized continuous with the vascular system of the filament. Outgrowths derived

Fig. 4 (**below**). Longitudinal sections of double flower buds of *Nicotiana alata*. Bud lengths = 1 mm (**A**) and 3 mm (**B**). Magnification: (**A**) = 94.5× and (**B**) = 45×. AN = anther, CA = carpel, P = petal, PLO = petal-like outgrowth, and S = sepal.



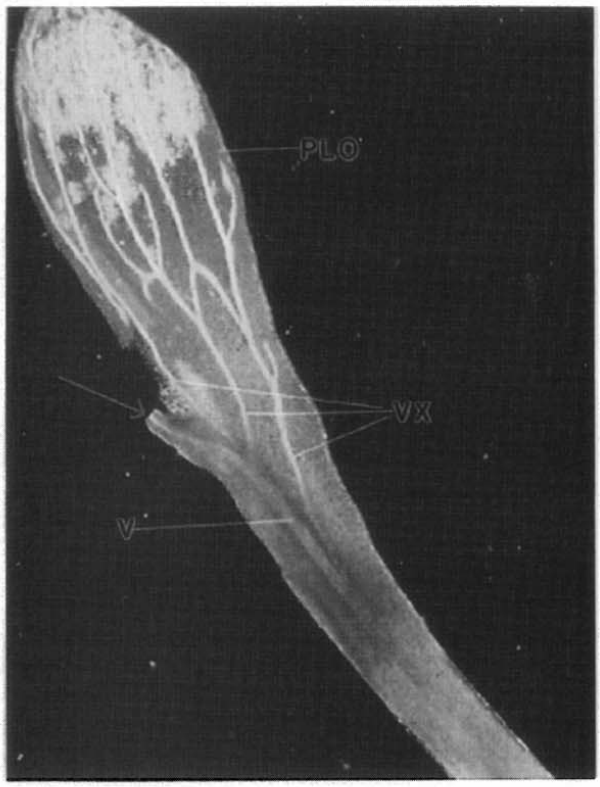


Fig. 7. Vascular bundles of petal-like outgrowth from top of filament in a double flower of *Nicotiana alata*. Arrow shows anther has been removed to enhance viewing. Magnification: 20×. PLO = petal-like outgrowth, V = vascular bundle of the filament, and VX = vascular bundle of PLO.

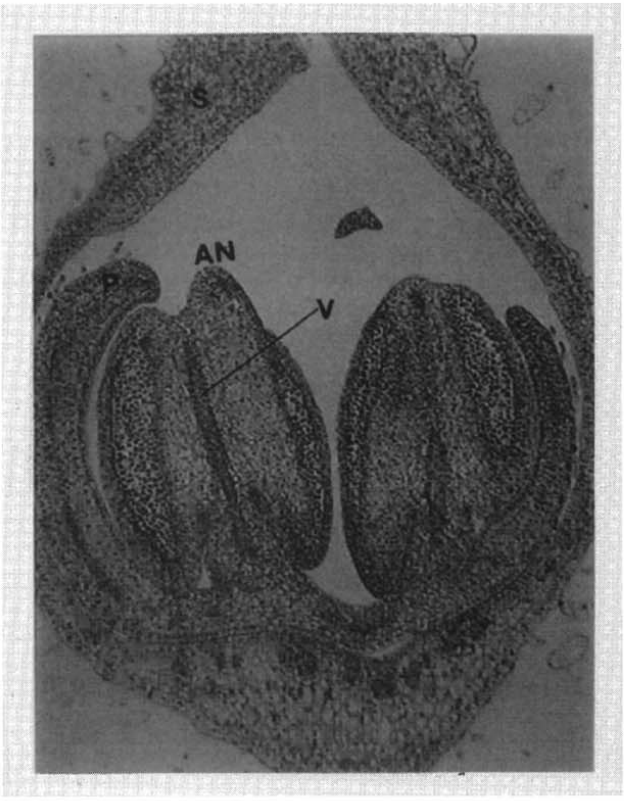


Fig. 5. Longitudinal section through the anthers of a nondouble flower bud of *Nicotiana alata* showing vascular bundles. Magnification: 60×. AN = anther, P = petal, S = sepal, and V = vascular bundle.

from the lower part of the filament were not vascularized or were vascularized but not connected to the filament vascular system. The unvascularized tissues consisted of epidermal and ground parenchyma cells.

Two types of flower doubleness, petalody and catacorolla, were observed in *N. langsdorffii* hybrids (White, 1914). Under petalody, the formation of petal-like outgrowths from the filament were observed in progeny of self pollinated *N. langsdorffii grandiflora* and in the F_2 generation of *N. langsdorffii* x *N. forgetiana* (White, 1914). In catacorolla doubleness, in the F_2 generation of *N. langsdorffii* x *N. alata* it was observed that petaloid segments

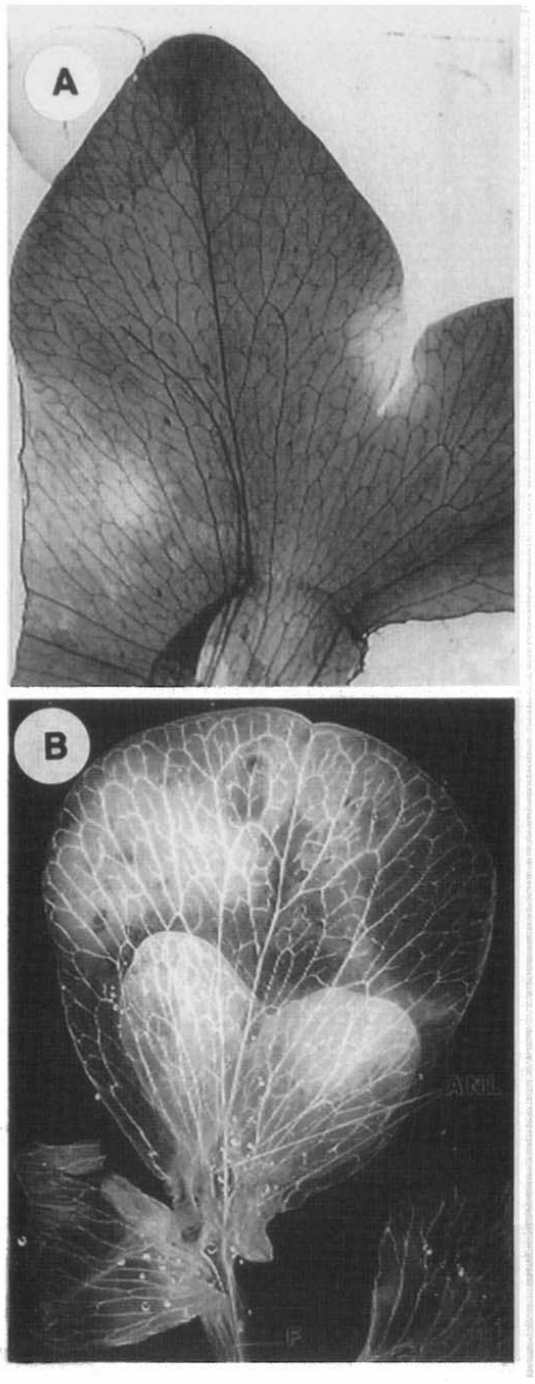


Fig. 6. Venation of ordinary petal in a nondouble flower (**A**) and petal-like outgrowths of the anther of a double flower (**B**) of *Nicotiana alata*. ANL = anther lobe and F = filament.

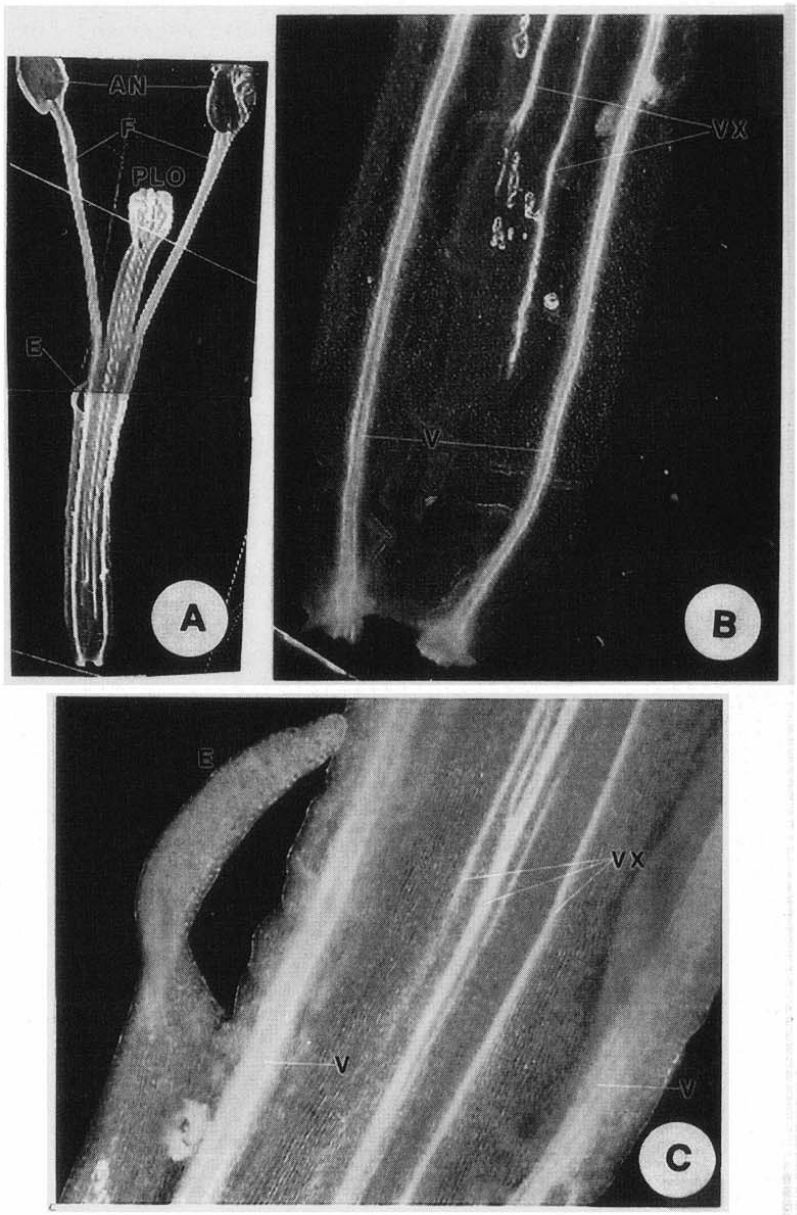


Fig. 8. Vasculature in double flowers of *Nicotiana alata* with petal-like outgrowths (**A** and **B**) and emergences (**C**). Magnification: (**A**) = 6×, (**B**) = 20×, and (**C**) = 31.5×. AN = anther, E = emergence, F = filament, PLO = petal-like outgrowth, V = vascular bundle of the filament, and VX = vascular bundle of PLO.

formed outside the normal corolla and partially adhered to the normal corolla. White (1914) suggested that, during ontogeny, the five segments that normally form the corolla were separated initially. Therefore, when fusion took place to form the normal corolla, tissue remained at the point of union of the two segments forming the catacorolla. It was observed that catacorolla size varied from slender to the normal width of true corolla lobes. White concluded the catacorolla originated from discontinuous variation. In our study with *N. alata,* the petal-like outgrowths that produced flower doubleness were most like those characterizing petalody (White, 1914). Variation of petal-like outgrowths was observed, with those formed from anther tips being largest when compared to those formed on filaments. Catacorolla doubleness was not observed on double-flowered *N. alata*.

Petalodization can occur early or late in stamen development. Henslow (1888) stated petaloid development from the stamen could occur at the anther lobe, connective or filament. In *Narcissus* 'Cheerfullness', Reynolds and Tampion (1983) reported the petaloid outgrowth of the anther. The development of the gynoecium was normal and no additional initial was produced on the floral apex. It was stated the gene for doubleness affected a later stage of stamen development. Also, they found formation of petal tissues occurred in the stamen zone after corolla development. In our study, petal-like outgrowths on the stamen were observed forming early on anthers and not on filaments (Fig. 4). At a later stage of anther development, filaments developed petal-like outgrowths (Fig. 8). Thus, it appears that petal-like outgrowths that formed on stamens of *N. alata* developed at different stages of stamen development depending on their location on the stamen.

The vascular anatomy of the flower is used often to clarify flower morphology. Considering evolutionary specialization, it is believed that vascular structure is slowest to change (Esau, 1977; Puri, 1951). In a double flower of *Ranunculaceae,* the pattern of vascular structure was used to determine the origin and type of doubleness (Smith, 1928). Smith concluded that petals (including extra petals) in *Ranunculus* species (*R. abortivus* L., *R. acris* L., *R. recurvatus* Poir., and *R. septentrionalis* Poir.), *Caltha palustris* L., and *Delphinium* L. originated from modified stamens. One anatomical feature consistently supporting this conclusion was the single vascular trace character for both petals and stamens. In most angiosperms, a specialized stamen contains a single vascular bundle that transverses the filament and ends blindly either at the base of the anther or in the connective (Esau, 1977). The formation of a branched vascular system in a stamen is a rare phenomenon (Puri, 1951). In our study of the double flowered *N. alata,* a branched vascular system was observed (Fig. 7). This branching and prolongation

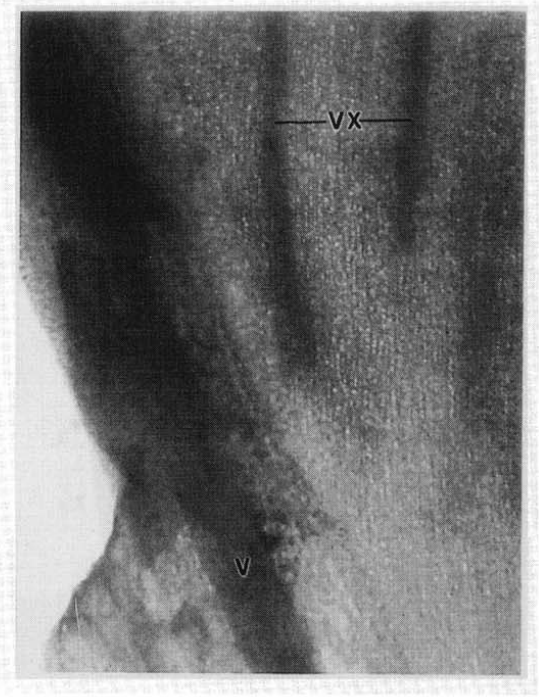


Fig. 9. Vasculature of petal-like outgrowth of a immature double-flower bud of *Nicotiana alata*. Bud length = 5 mm. Magnification: 60×. V = vascular bundle of the filament and VX = vascular bundle of PLO.

of the vascular bundle of the filament into the petal-like outgrowth, not only strengthens the fact that it is an extension of the stamen, but clarifies that the extra petals are organs. However, the petal-like outgrowth with unconnected vasculature to the filament raises the question whether the outgrowth was once connected to the terminal bundle of the filament and later was disconnected as elongation of the tissue occurred, or whether it was never connected to the terminal vascular bundle. To clarify this situation, younger, immature stamens obtained from a smaller flower bud were observed with unconnected vasculature (Fig. 9). Therefore, it appears the vascular bundle, which formed at the lower part of the filament, was never connected to the vascular bundle of the stamen.

The 'emergence', which occasionally arose on double-flowered plants on the filament lacked a vascular bundle and was not considered an organ. This condition was similar to that termed 'enation' in the leaf of *N. tabacum* 'Enation 701' and *N. tabacum* 'Corolla Double X' (Hitier, 1950). Here, a thin parenchyma developed along the vein, which appeared usually on the abaxial side of the leaf. An enation includes a supplementary lobe or excrescence arising from excess or development (but not growth or duplication) of any organ (Masters, 1869). Therefore, the emergence observed in double-flowered *N. alata* is an enation arising from excess tissue of the filament formed during development of the filament. The cause of this emergence is unknown. However, according to Hitier (1950), the enation could originate by accident (without exact cause), viral infection, or genetic mutation.

Future investigation into flower doubleness of *N. alata* should focus on genetic and physiological factors. The chronological separation of petal-like-outgrowth formation on anthers and filaments suggests timing differences of gene expression for controlling development of this trait. This offers potential for use of molecular approaches to investigate this process. Formation of nonattached vascular bundles in the petal-like outgrowths of the filament needs further study also. Investigations may involve site of initiation, what cells are involved in the formation of the vascular bundle of the outgrowth and whether the bundle has similar function to that of the vascular bundle in the filament proper. The functionality of this incompletely formed vasculature remains unknown.

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