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DIFFERENTIAL OVULE DEVELOPMENT FOLLOWING SELF- AND CROSS-POLLINATION: THE BASIS OF SELF-STERILITY IN *NARCISSUS TRIANDRUS* (AMARYLLIDACEAE)[1]

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Self-pollination results in significantly lower seed set than cross-pollination in tristylous *Narcissus triandrus.* We investigated structural and functional aspects of pollen–pistil interactions and ovule–seed development following cross- and self-pollination to assess the timing and mechanism of self-sterility. Ovule development within an ovary was asynchronous at anthesis. There were no significant differences in pollen tube behavior following cross- vs. self-pollination during the first 6 d of growth, regardless of style morph type. Double fertilization was significantly higher following cross- vs. self-pollination. Aborted embryo development was not detected following either pollination type up to seed maturity. Prior to pollen tube entry, a significantly greater number of ovules ceased to develop following self- vs. cross-pollination. These results indicate that self-sterility in *N. triandrus* operates prezygotically but does not involve differential pollen tube growth typical of many self-incompatibility (SI) systems. Instead, low seed set following self-pollination is caused by a reduction in ovule availability resulting from embryo sac degeneration. We hypothesize that this is due to the absence of a required stimulus for normal ovule development. If this is correct, current concepts of SI may need to be broadened to include a wider range of pollen–pistil interactions.

Key words: Amaryllidaceae; late-acting self-incompatibility; *Narcissus*; ovule development; pollen tube growth; self-sterility; tristyly.

Self-sterility in plants is manifested by a significant reduction in seed set following self-pollination in comparison with cross-pollination. The two major causes of self-sterility that have been identified are physiological self-incompatibility (SI), whereby a genetically based self-recognition system reduces the frequency of self-fertilization (de Nettancourt, 1977; Barrett, 1988; Matton et al., 1994), and early-acting inbreeding depression in which selfed zygotes homozygous for deleterious recessive alleles abort (Charlesworth and Charlesworth, 1987; Husband and Schemske, 1996). While it is conceptually straightforward to distinguish between these phenomena, in practice it can be difficult, especially in cases where rejection of self occurs in the ovary (late-acting or ovarian self-incompatibility; Seavey and Bawa, 1986; Sage, Bertin, and Williams, 1994). Most workers agree that a critical issue in understanding the basis of self-sterility in plants is to determine whether self-rejection occurs pre- or postzygotically. This distinction is important because of the widespread view that SI is unlikely to operate postzygotically since self-rejection of developing embryos is more likely to result from inbreeding effects. If, however, postzygotic SI occurs, it may be distinguished from inbreeding depression by characterizing the timing of abortion of selfed zygotes. Postzygotic SI is more likely to result in developmental failure at a single critical stage, whereas abortion due to inbreeding depression occurs at a variety of stages following double fertilization (Charlesworth, 1985; Seavey and Bawa, 1986).

Narcissus (Amaryllidaceae) comprises ~40 species of perennial geophytes native to the Mediterranean region. Species are either monomorphic, dimorphic, or trimorphic for style length (Barrett, Lloyd, and Arroyo, 1996). Trimorphic heterostyly is restricted to a single species complex, *Narcissus triandrus,* native to Spain and Portugal. *Narcissus triandrus* is unique among tristylous plants in possessing a self-sterility system in which floral trimorphism is uncoupled from mating type (Bateman, 1952; Barrett et al., 1997). In contrast to species with conventional heteromorphic incompatibility, all cross-pollinations are equally fertile regardless of morph type. This unusual association also occurs in *Narcissus* species with stigma–height dimorphisms (Dulberger, 1964; A. M. Baker and S. C. H. Barrett, unpublished data, University of Toronto). While self-sterility is widespread in *Narcissus,* having been reported from 12 of 14 species that have been investigated experimentally (Bateman, 1954; Barrett, Lloyd, and Arroyo, 1996), it is unclear whether low seed set from self-pollination results primarily from an SI system or is largely a manifestation of early-acting inbreeding depression (Bateman, 1954; Dulberger, 1964; Barrett et al., 1997). Qualitative observations of pollen tube growth following self-pollination of *N. triandrus* and *N. tazetta* by Bateman (1954) and Dulberger (1964), respectively, indicated that self-rejection occurred in the ovary. Bateman (1954) concluded that ''incompatibility

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must be late-acting, somewhere in the ovary, and perhaps even after fertilization.'' Dulberger (1964) also suggested that inhibition of self-pollen occurred in the ovules after fertilization. The suggestion that self-sterility in *Narcissus* represents some form of postzygotic SI with inhibition occurring in the ovary is tentative, however, since there are no detailed developmental studies at the structural level from the time of pollination to seed maturity.

The purpose of the present investigation was to determine whether self-sterility in *N. triandrus* can be attributed to pre- or postzygotic SI and/or inbreeding depression. To address this issue, we characterized structural and functional aspects of pollen–pistil interactions and ovule–seed development following cross- and self-pollination. Three basic questions were addressed: (1) Do cross- and self-pollen tubes exhibit differential growth characteristics within the pistil? (2) Do self-pollen tubes effect double fertilization to the same extent as cross-pollen tubes? (3) Are there differences in ovule and seed development following cross- and self-pollinations, and, if so, what is the timing of such events? Since our results for *N. triandrus* indicate a potentially novel prezygotic self-recognition mechanism, we therefore conclude by placing our findings in a broader ecological and evolutionary context that emphasizes the heterogeneous nature of SI mechanisms in plants.

MATERIALS AND METHODS

Study organism—*Narcissus triandrus* L. is a bee-pollinated geophyte widespread and common in central and northern regions of the Iberian Peninsula. Experimental material used in our studies was sampled from natural populations, with the vast majority of plants originating from either population 1 (Calera de León, Badajoz province, Spain) or population 79 (Hoya de Pinares-1, Avila Province, Spain, see Barrett et al. [1997] for further details of sampling). Bulbs were collected from the field in early spring and subsequently cultivated under glasshouse conditions at the University of Toronto at 15°–25°C.

Pollen–pistil interactions following cross- and self-pollination—Structural features of cross- and self-pollen tube growth and transmitting tissues pollen tubes encounter were characterized using fluorescence, light, and scanning and transmission electron microscopy as described by Williams, Sage, and Thien (1993) and Sage and Williams (1995). Flowers were sampled at anthesis and 3 and 6 d following pollinations.

To compare the growth of cross- and self-pollen tubes under field conditions, controlled hand-pollinations were conducted at population 1 from 5 to 12 March 1995. Pollinations were conducted on the first day of flowering following inspection of the stigma to ensure no prior deposition of pollen. Hand-pollinated flowers were collected at 8, 16, 24, 32, 43, 50, 56, 67, and 76 h postpollination, fixed, and prepared for fluorescence microscopy as described by Williams and Knox (1982), except autoclaving of flowers was omitted. Deletion of autoclaving permitted use of ovules for clearing to investigate ovule–seed development during the first 76 h postpollination (see below). For cross-pollinations, individual plants were crossed to a single pollen donor and each style morph was crossed to both other morph types (intermorph). In addition, self-pollinations were conducted on each style morph. Previous studies of the compatibility of different pollen–pistil combinations in *N. triandrus* indicated no significant differences in seed set between the two anther levels within a flower (Barrett et al., 1997). Hence, in both cross- and self-pollinations, pollen from either anther level was employed. A total of 567 plants (seven plants per pollination treatment per harvest time) were used in this field study.

Parameters measured at each harvest time included: (1) the number of germinated pollen grains on the stigma, (2) the number of pollen tubes at 0.2-mm intervals along the length of the style and ovary (pollen tube density), and (3) the number of ovules penetrated by pollen tubes. Mean pollen tube length at each time interval was determined as described in Cruzan (1986). To determine whether there were differences between cross- and self-pollen germination and pollen tube length over time, linear regression analysis was performed. Regression parameters for cross- and self-pollinations were then compared using one-way ANOVA. To determine whether there were differences in pollen tube density within the style and ovary, mean pollen tube density/number of pollen grains germinated $\times$ 100 was plotted as a function of distance grown in the style at each time interval. Data were fitted to a nonlinear regression model of the relation between pollen tube density and log distance and differences between self- and cross-pollination compared using ANOVA. Mean percentage of ovules penetrated during the first 76 h following cross- and self-pollination were contrasted using one-way ANOVA. Initially, a Tukey's test was performed to determine whether there was an effect of style morph on mean pollen tube length or pollen tube density over time. No effect of style morph was revealed in these analyses and therefore data from all cross- vs. self-pollinations were pooled.

Double fertilization and ovule–seed development following cross- and self-pollination—Two separate sets of controlled cross- and self-pollinations were conducted on plants grown in glasshouses at the University of Toronto to determine whether differences existed in rates of double fertilization and ovule–seed development. In experiment 1 (1992), plants originated from several sites but with the majority from the El Hoyo de Pinares-1 population, whereas in experiment 2 (1995), all plants originated from the Calera de León population. Pistils from experiments 1 and 2 were harvested 6–40 and 6–18 d, respectively, postpollination. Pistils from all experimental hand-pollinations were cleared as described by Stelly et al. (1984). Ovules/seeds from cleared pistils were dissected, viewed with a Zeis axiophot compound light microscope and scored for the presence or absence of double fertilization. Double fertilization was indicated by: (1) the absence of unfused sperm within embryo sacs penetrated by a pollen tube; (2) the presence of a resting zygote or embryo; and (3) the presence of one or more endosperm nuclei. The number of double fertilization events following cross- and self-pollination in experiments 1 and 2 was determined in 187 pistils (8100 ovules) and 81 pistils (5500 ovules), respectively. Cross- and self-pollinations and statistical analyses were conducted as described above.

Cleared ovules and seeds were examined to determine the timing of development following cross- and self-pollination. In addition, ovules and/or seeds from pollinated and unpollinated flowers used in quantitative studies of cross- and self-pollen tube growth were cleared for characterization of ovule and seed development during the first 76 h following anthesis. Serial sections of ovaries prepared for light microscopy were also used to determine temporal aspects of ovule and seed development.

Frequencies of cross- and self-fertilization following mixed pollinations—To assess the siring ability of cross- and self-pollen, mixtures of the two pollen types of approximately equal proportions, were made on microscope slides and applied to stigmas. Plants used for this experiment originated from population 79 (Hoya de Pinares) and were polymorphic at the isozyme locus *Pgi-2*. Pollinations were conducted under glasshouse conditions with a mixture of equivalent amounts of pollen from the maternal and paternal genotype. The two genotypes were homozygous for alternative alleles at *Pgi-2*. A total of 28 plants were used and the 18 fruit obtained from the mixed pollinations yielded a total of 260 seeds. All seeds were assayed electrophoretically (see Barrett et al., 1997, for methods). Levels of cross- and self-fertilization were determined for each progeny array. Seeds heterozygous in a given

family were the result of cross-fertilization; those that were homozygous resulted from self-fertilization.

RESULTS

Structural features of the pollen tube pathway—The stigma of *N. triandrus* is trilobed (Fig. 1) and composed of papillate cells with a highly rugose cuticle (Figs. 2, 3). The cuticle of unpollinated stigmas is subtended by a flocculent electron-dense secretion, which overlays a loosely graded cell wall (Fig. 3). The plasmalemma of papillate cells is highly invaginate and surrounds a cytoplasm rich in profiles of rough endoplasmic reticulum (RER) and Golgi with inflated cisternae, endomembrane vesicles, and mitochondria (Fig. 4). The stylar canal is initially radial in shape and becomes trilobed towards the base. Cytoplasmic ultrastructural characteristics of epidermal transmitting cells lining the hollow style prior to pollination are similar to those of papillate stigmatic cells, although undifferentiated plastids are frequently present. Secretions arise from a loosely graded cell wall and fill the hollow canal (Figs. 5, 6).

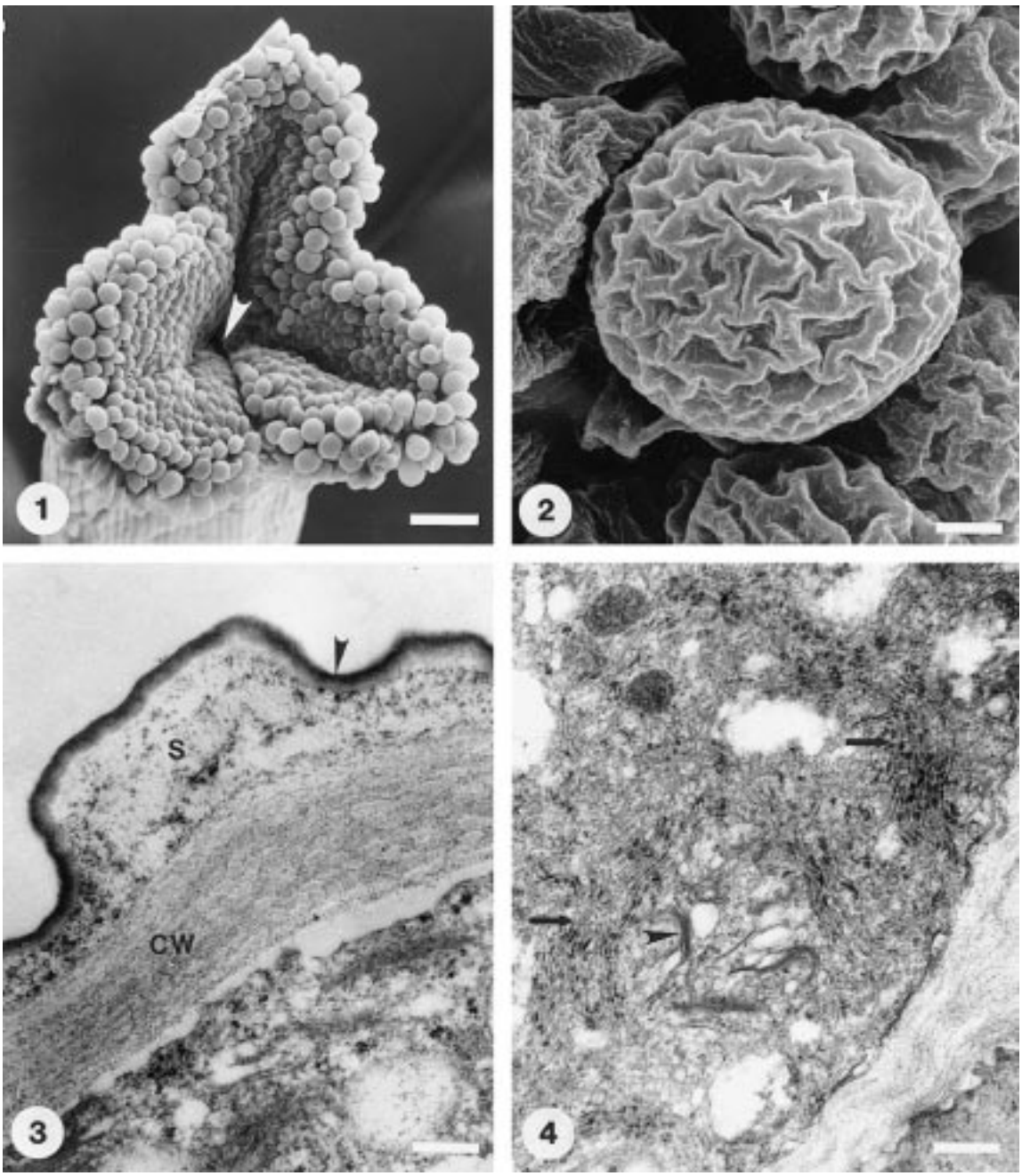
The anatropous ovule of *N. triandrus* is bitegmic with the inner integument adjacent to the placental epithelium (Fig. 7). Exudates are present adjacent to the placental (Figs. 8, 9) and funicular epithelium in association with a thin, discontinuous cuticle. Cytoplasmic ultrastructural characteristics of placental and funicular epithelium prior to pollination are the same as transmitting cells of the stigma and style, except RER profiles were more extensively located throughout the cytoplasm (Fig. 10). Early stages of transfer cell wall development are also apparent in placental and funicular epithelial cells (Fig. 10).

Pollen–pistil interactions following cross- and self-pollinations—No significant differences were observed in interactions between cross- and self-pollen tubes and transmitting tissue during the first 6 d following pollination. Pollen tubes germinated on the stigmatic surface and grew between papillate stigmatic cells without penetrating the cuticle (Fig. 11). Within the style, pollen tubes developed a thin callosic wall and produced callosic plugs at regular intervals. Pollen tubes grew adjacent or nearby to epidermal transmitting cells and were surrounded by an electron-dense postpollination exudate, which appeared to arise from epidermal and subepidermal cells lining the canal (Figs. 12–14). Within the ovary, pollen tubes traversed the surface of the placental and funicular epithelium and subsequently entered the micropyle of ovules (Fig. 15). Micropyles contained a prominent secretion (Fig. 16) not apparent at anthesis or 76 h following anthesis in unpollinated flowers. Three days following pollination, placental and funicular epithelial cells had prominent transfer cell walls and a cytoplasm similar in ultrastructure to that observed at anthesis. Six days following pollination, placental and funicular epithelial cells exhibited thick cell walls, cytoplasm containing numerous profiles of RER, free ribosomes, lipids, and vacuoles (Fig. 17). Pollen tubes in the ovary had thick callosic walls (Fig. 18).

Pollen grains germinated within 8–16 h following pollination. There was no significant difference between the germination of cross- vs. self-pollen on stigmas of *N. triandrus* under field conditions during the first 76 h following pollination ($df = 1,96$, $F = 0.21$, $P = 0.810$) . Similarly, comparisons of pollen tube length at each harvest time during the first 76 h also failed to reveal any significant differences ($df = 1,96$, $F = 0.90$, $P = 0.346$). Measurements of mean cross- vs. self-pollen tube density at different locations in the style and ovary showed no significant differences at each of four time intervals (Fig. 19). No significant differences were observed in the mean percentage of ovules penetrated by cross- and self-pollen tubes by 76 h following pollination (cross = 12.97 ± 3.88; self = 13.46 ± 5.49; $df = 1,96$, $F = 0.005$, $P = 0.943$).

Ovule structure at anthesis—Ovule development within an ovary of *N. triandrus* at anthesis was heterogeneous. Ovaries contained, on average, 60 bitegmic ovules. Ovules contained either a dyad-derived megaspore (24.2 ± 4.5%) or a seven-celled embryo sac (75.8 ± 4.5%). There was no relation between the stage of development of an ovule and its position on a placenta. The megaspore was surrounded by a thick callose wall with cytoplasm containing numerous starch grains, vacuoles, and a prominent nucleus (Fig. 20). The seven-celled embryo sac was composed of three antipodal cells with dense cytoplasm and prominent nuclei, a central cell with a single polar fusion nucleus (Fig. 21), and an egg apparatus. The egg apparatus consisted of two synergids with an extensive filiform apparatus (Figs. 22, 23), prominent chalazal nuclei, dense cytoplasm with no vacuole, and an egg cell with a chalazally located nucleus surrounded by cytoplasm containing prominent starch grains (Fig. 23). Embryo sac development (megagametogenesis) from the megaspore was of the *Allium* type (Stiffler, 1925; Davis, 1966) and occurred during the first 76 h after cross- and self-pollination. In the absence of pollination, the proportion of ovules containing megaspores was similar to that at anthesis (26.1 ± 4.7%).

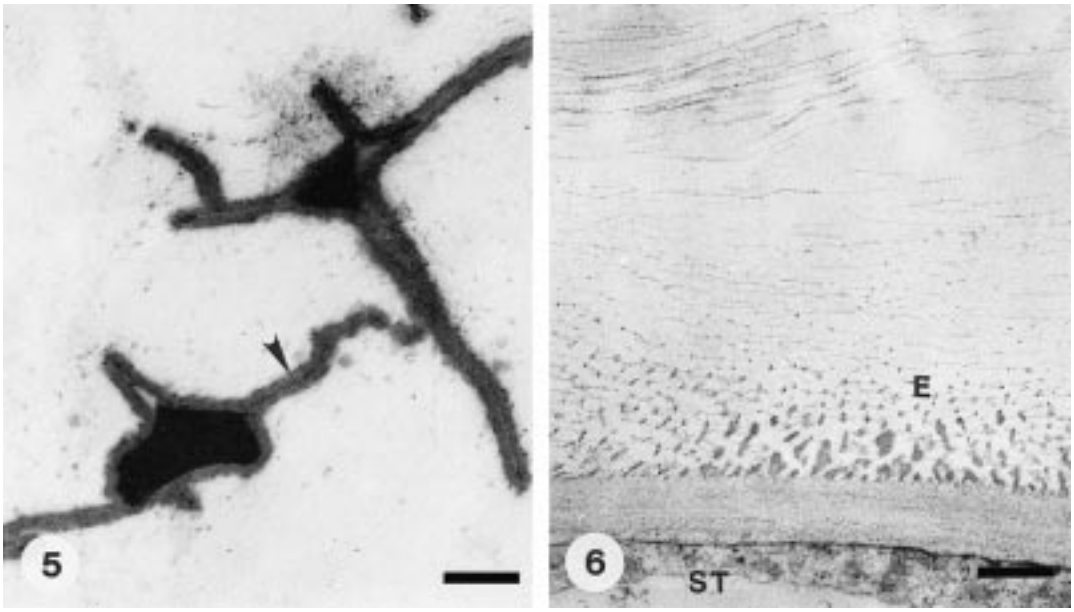
Ovule development following cross- and self-pollination—Field pollinations of *N. triandrus* revealed significant differences in ovule development between cross- vs. self-pollinations. A significantly greater proportion of ovules contained megaspores that failed to undergo megagametogenesis following self- vs. cross-pollination during the 76 h following pollination (self = 12.2 ± 3.23%; cross = 1.39 ± 0.6%; $df = 1$, $H = 11.56$, $P = 0.001$). These differences were first apparent by 32 h following pollination when pollen tubes in the long- and mid-styled morph had not entered ovaries and less than a third of pollen tubes had entered ovaries in the short-styled morph (Fig. 19). Ovules containing undifferentiated megaspores were observed to degenerate by 76 h, rendering them unreceptive or sterile. In addition, seven-celled embryo sacs were also observed to degenerate, and this process occurred more frequently following self- vs. cross-pollination (cross = 0.61 ± 0.33%; self = 13.8 ± 4.76%; $df = 1$, $H = 9.99$, $P = 0.002$). Embryo sac degeneration occurred by 43 and 56 h following self- and cross-pollination, respectively. Similar differences in ovule degeneration following cross- vs. self-pollination were also observed under glasshouse conditions in experiments 1 and 2 described above. When ovules con-



Figs. 1–4. Unpollinated stigma of *N. triandrus* at anthesis. **1.** Trilobed stigma and hollow canal (arrowhead). Bar = 41 μm. **2.** Rugose cuticle (arrowheads) of papillate stigmatic cell. Bar = 3 μm. **3.** Transmission electron micrograph of a papillate stigmatic cell illustrating exudate which overlies a loosely graded cell wall and subtends the rugose cuticle (arrowhead). Bar = 4.5 μm. **4.** Fine structure of papillate stigmatic cells. Note Golgi (arrowhead), associated endomembrane-derived vesicles and aggregate profiles of RER (arrows). Bar = 0.74 μm. *Figure Abbreviations*: CW, cell wall; S, secretion.

taining either degenerated megaspores or embryo sacs were classified as sterile, their cumulative proportion was significantly greater following self-pollination compared with cross-pollinations (Fig. 24).

Integumentary and nucellar cells of degenerating ovules containing megaspores lacked cytoplasm and appeared ''translucent'' following clearing (Fig. 25); nucellar cells surrounding the megaspore had callosic walls (Fig. 26). The megaspore in translucent ovules was frequently enucleate. Degenerated seven-celled embryo sacs were characterized by: (1) enucleate, vacuolate egg cells (Fig. 27); (2) frequent enucleate synergids (Fig. 27); (3) shrunken, vacuolate antipodals with reduced nuclei (Fig. 28); (4) the absence of the polar fusion nucleus in the central cell; and (5) an absence of micropylar secretions. Cells comprising the outer and inner integument and the single layer of nucellus surrounding degenerating embryo sacs were hypertrophied and vacuolate (Figs. 29, 30) in



Figs. 5, 6. Transmission electron micrographs of unpollinated style of *N. triandrus* at anthesis. **5.** Osmiophilic exudate (arrowhead) in central core of hollow style. Bar = 4.5 μm. **6.** Fine structure of exudate arising from stylar epidermal cell lining hollow canal. Bar = 1.0 μm. *Figure Abbreviations*: E, exudate; ST, stylar epidermal cell.

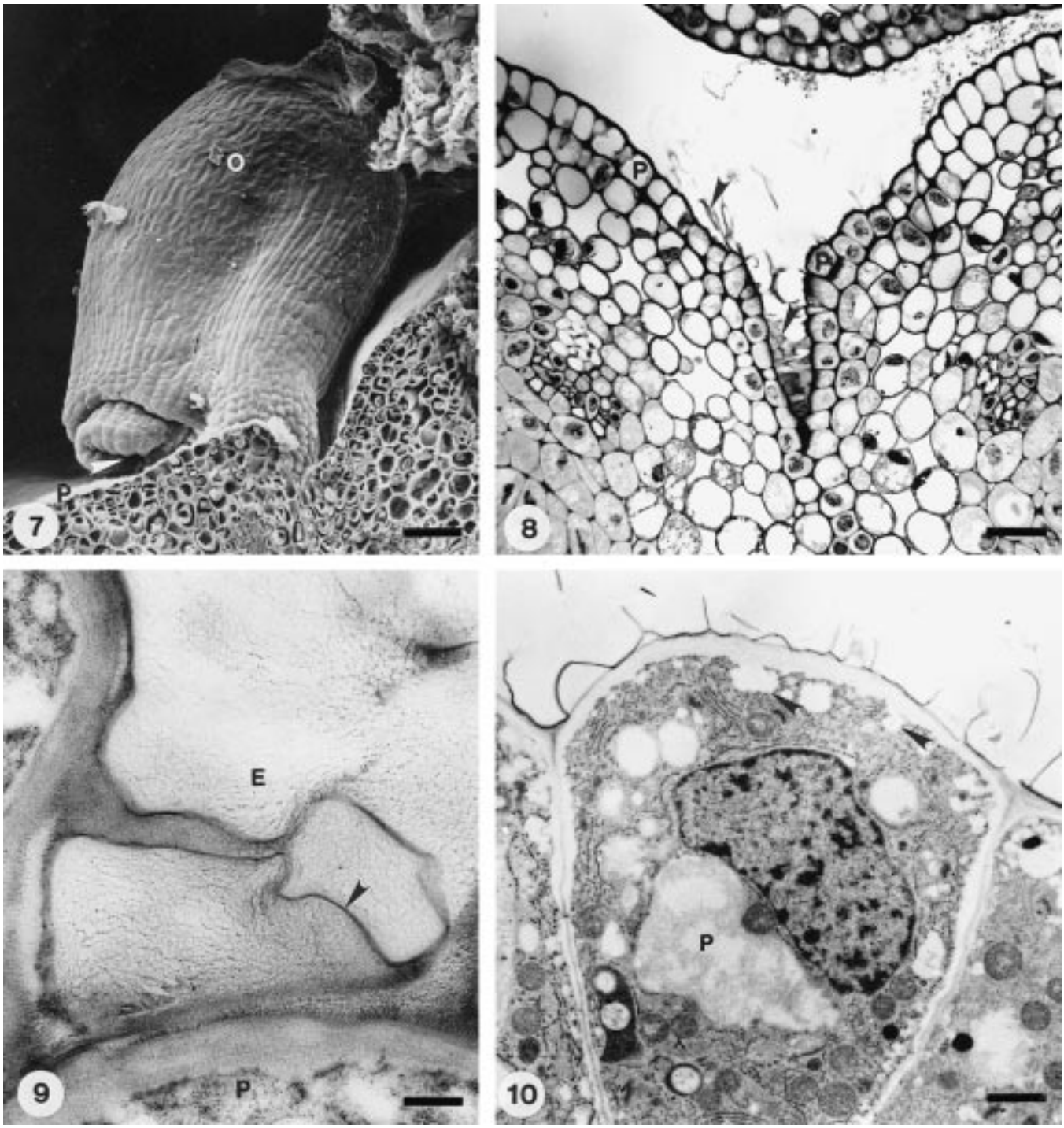
comparison with corresponding cells in nondegenerate ovules (Fig. 31). Degenerated ovules with megaspores remain small, whereas those with failed embryo sacs were of similar size to seeds in early stages of development. Degenerated embryo sacs were in some instances observed with embryos arising from nucellar cells (Fig. 32; adventitious embryony; Koltunow, 1993). Percentage adventitious embryony was significantly greater following self-pollination compared with cross-pollination (experiment 1; cross $M = 3.41 \pm 1.42\%$, self $M = 11.63 \pm 0.86\%$; $df = 1,184$, $F = 24.29$, $P \leq 0.001$; experiment 2; cross $M = 3.28 \pm 1.80\%$, self $M = 14.05 \pm 1.63\%$: $df = 1,78$, $F = 19.71$, $P < 0.001$).

Comparison of double fertilization following cross- and self-pollinations—Double fertilization in mature, nondegenerate, seven-celled embryo sacs was apparent 76 h following both cross- and self-pollination. Mean percentage double fertilization was significantly higher following cross- vs. self-pollination in both experiments 1 and 2 (Fig. 24). Embryo and endosperm development proceeded similarly following both pollination treatments. Division of the primary endosperm nucleus precedes zygotic division and is initially coenocytic; endosperm cellularization was apparent by 15 d following pollination. Embryo development conforms to the *Asterad* type (Davis, 1966). A two-celled proembryo was apparent by 6–8 d following pollination (Fig. 33). The proembryo basal cell divided transversely to give rise to a tiered suspensor by 12–15 d, and the apical proembryo cell divided to give rise to a multicellular embryo proper (Figs. 34, 35). The cotyledon was well developed by 21 d (Fig. 36). Postzygotic abortion of developing embryos was not detected following either cross- or self-pollination.

Cross- and self-fertilization following mixed pollinations—Analysis of progeny arrays arising from mixed pollinations clearly indicated that cross-pollen had a siring advantage over self-pollen. On average, only 12% of seeds arising from mixed pollinations were self-fertilized with the remainder resulting from cross-fertilization ($G_p = 175.84$, $df = 1$, $P < 0.005$ based on an hypothesis of equal siring ability). There was significant heterogeneity among maternal parents in the fraction of seeds sired by self-pollen ($G_{het} = 41.70$, $df = 13$, $P < 0.005$). There was no relation between the frequency of self-fertilization and the number of seeds per fruit ($df = 1,16$, $F = 0.26$, $P = 0.620$). Mean seed set per fruit from mixed pollinations was 14.4 compared with 36.6 from cross-pollinated controls.

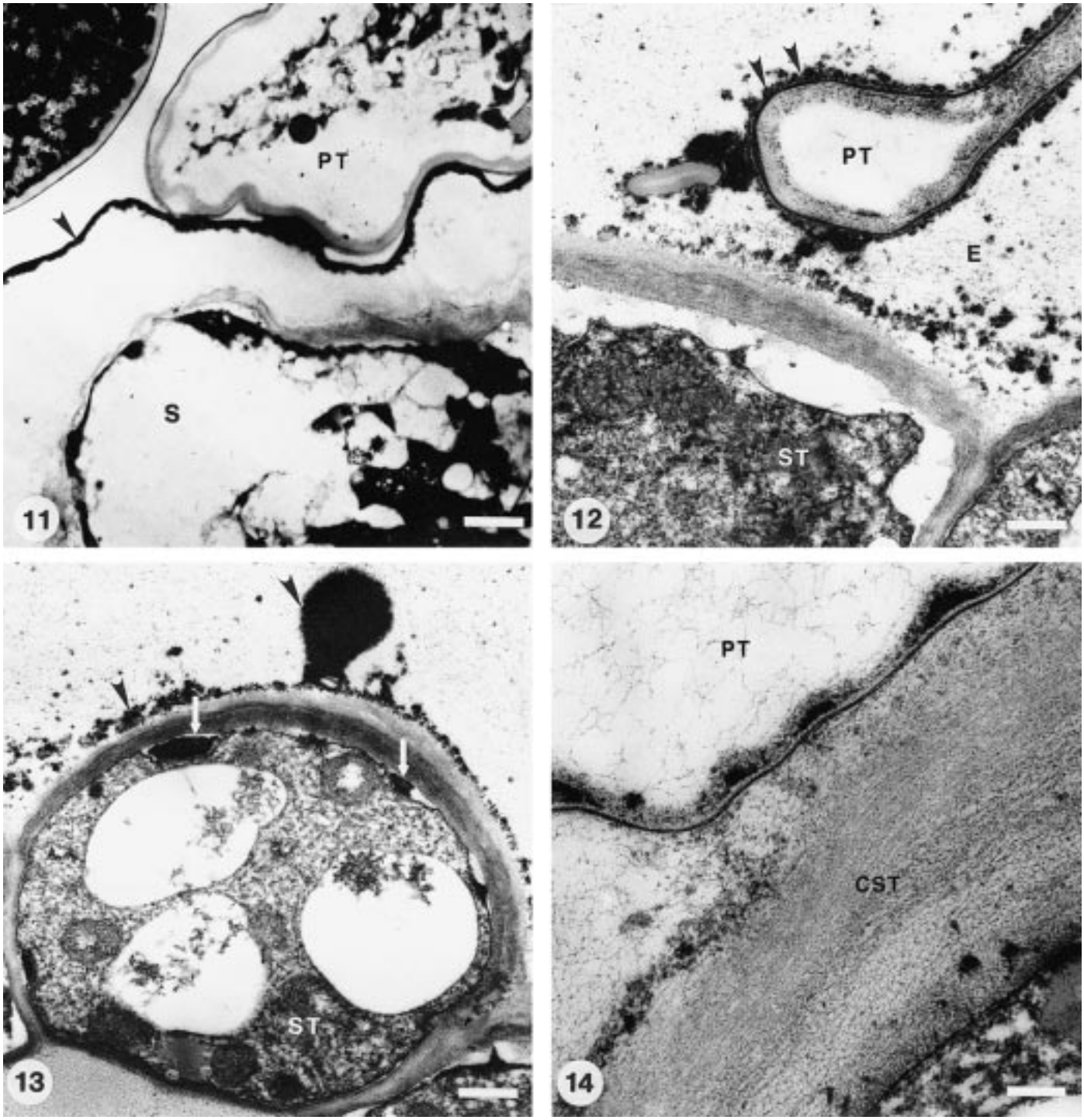
DISCUSSION

The major finding of this study was that self-sterility in *N. triandrus* is prezygotic and involves a reduction in the availability of fertile ovules resulting from embryo sac degeneration following self-pollination. Our results provided no evidence that early-acting inbreeding depression contributes to self-sterility as indicated by the absence of embryo abortion following double fertilization. We begin the discussion by reviewing other work on ovarian self-incompatibility (OSI) and review potential mechanisms that could account for our results in *N. triandrus.* Since our studies implicate a novel prezygotic self-recognition mechanism, we discuss its functional consequences and evolutionary implications. We conclude by challenging traditional views of SI and propose instead that incompatibility phenomena should be expanded to account for a broader range of pollen–pistil interactions.



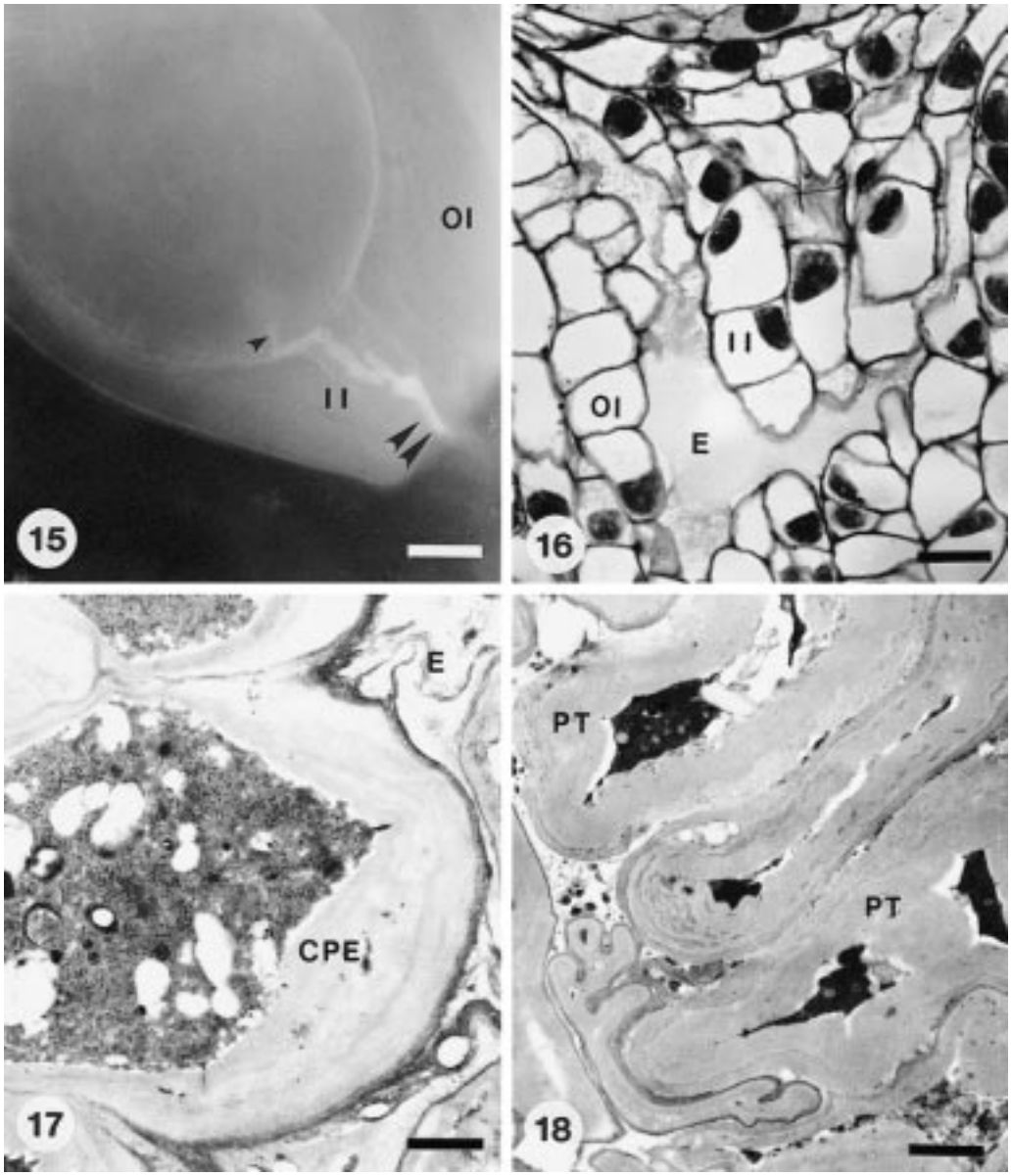
Figs. 7–10. Placental transmitting tissues of *N. triandrus* at anthesis. **7.** Scanning transmission electron micrograph of anatropous ovule illustrating proximity of micropyle (arrowhead) to placental epidermis. Bar = 40 μm. **8.** Light micrograph of placental exudate (arrowheads). Bar = 80 μm. **9.** Fine structure of exudate as viewed with the transmission electron microscope. Note distended cuticle of placental epidermis (arrowhead). Bar = 0.37 μm. **10.** Transmission electron micrograph illustrating extensive profiles of rough endoplasmic reticulum, abundant mitochondria, and early stages of transfer cell wall synthesis (arrowheads) typical of placental epidermal cells. Bar = 1.28 μm. *Figure Abbreviations*: E, exudate; O, ovule; P, placental epidermis.

Mechanisms of ovarian self-incompatibility—Historically, most studies on SI have focused on the dynamics of male gametophyte interactions within the sterile tissues of the stigma and style (de Nettancourt, 1977, 1997; Elleman and Dickinson, 1994) with little attention given to events occurring in the ovary. However, investigations of OSI mechanisms require particular scrutiny of female gametophyte development and processes leading to double fertilization (reviewed in Seavey and Bawa, 1986; Sage, Bertin, and Williams, 1994). An important issue is to de-



Figs. 11–14. Transmission electron micrographs illustrating pollen tube growth on the stigma and in the style of *N. triandrus* 3 d following pollination. **11.** Pollen tube adjacent rugose cuticle (arrowhead) of senescent papillate stigmatic cell. Bar = 1.54 μm. **12.** Pollen tube growing within stylar exudate (arrowheads). Bar = 1.61 μm. **13.** Exudate arrising from epidermal cell lining the stylar canal. Note periplasmic (arrows) and extracellular (arrowheads) locations of exudate. Bar = 0.87 μm. **14.** Close interaction between pollen tube and cell wall of stylar epidermal cell. Bar = 0.35 μm. *Figure Abbreviations*: CST, cell wall of stylar epidermal cell; E, exudate; PT, pollen tube; S, papillate stigmatic cell; ST, stylar epidermal cell.

termine temporal aspects of the self-recognition mechanism and whether rejection is delayed relative to recognition. Rejection of self can occur in the ovary prior to pollen tube penetration of ovules (Beardsell, 1991) or embryo sacs (Kenrick, Kaul, and Williams, 1986), by lack of gamete fusion following embryo sac penetration (Cope, 1962) or after gamete fusion (Sage and Williams, 1991; Gibbs and Bianchi, 1993). As discussed above,



Figs. 15–18. Fluorescent, light, and transmission electron micrographs illustrating ovule and placenta structure and pollen tube growth within the ovary of *N. triandrus*. **15.** Self-pollen tube entering a synergid (single arrowhead). Double arrowhead marks entrance of pollen tube into micropyle. Bar = 160 μm. **16.** Micropylar exudate present 3 d postpollination in an ovule with a 7-celled embryo sac. Bar = 25 μm. **17, 18.** Thick cell walls of a placental epidermal cell and pollen tubes 6 d following cross-pollination. Bar = 0.86 μm. *Figure Abbreviations*: CPE, cell wall of placental epidermal cell; E, exudate; II, inner integument; OI, outer integument; PT, pollen tube.

previous studies of *Narcissus* spp. implicated OSI without identifying whether self-rejection was pre- or postzygotic. Our results clearly demonstrate that both self-recognition and rejection operate prezygotically in *N. triandrus* and function in the absence of differential pollen tube growth.

It has been suggested for several species exhibiting OSI that self-pollen tubes do not provide the appropriate signals for stimulation of ovule and seed development. In *Gasteria verrucosa* (Sears, 1937), *Theobroma cacao* (Cope, 1962), and *Asclepias exaltata* (Sage and Williams, 1991), integumentary growth fails to proceed normally after entry of a self-pollen tube into the ovule. Sears (1937) proposed that failure of integumentary growth after selfing was an indication that SI reactions involve pollen tube–integument interactions. He also suggested that interaction of compatible pollen tubes with integuments might be important for stimulation of normal seed development. Pimienta and Polito (1983) found that embryo sac development in *Prunus dulcis* was strongly affected by pollen tube activity in the pistil. Cross-pollen tubes had a greater stimulatory effect than self-pollen tubes and irregularities in embryo sac development were more frequent following self-pollination. It should be noted, however, that *P. dulcis* does not possess OSI and self-pollen tubes grow at a significantly slower rate than cross-pollen tubes. Our results for *N. triandrus* are the first to demonstrate differences in ovule development fol-

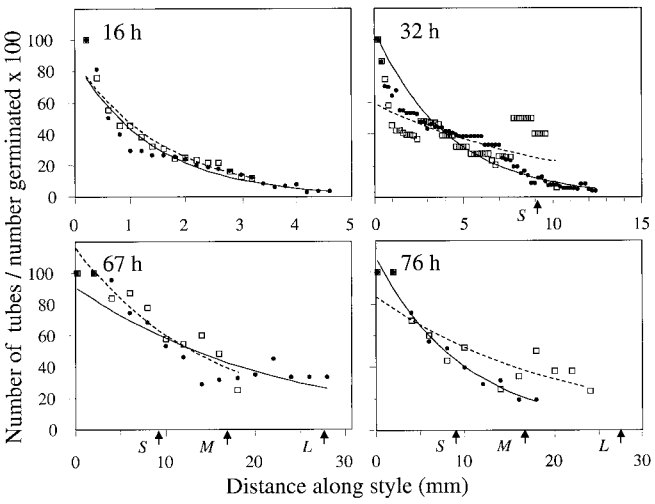


Fig. 19. The relation between pollen tube density (number of pollen tubes/number of germinated pollen grains on stigma) and distance along the style at four time intervals (16, 32, 67, and 76 h) in self- (open squares, dashed line) and cross- (closed circles, solid line) pollinations of *N. triandrus.* Fitted lines were generated using nonlinear regression analysis; R^2 values varied between 0.68 and 0.97. No significant differences in pollen tube growth were evident between cross- and self-pollinations (16 h: df = 1,37, $F = 2.15$, $P = 0.151$; 32 h: df = 1,112, $F = 3.92$, $P = 0.058$; 67 h: df = 1,23, $F = 2.16$, $P = 0.155$; 76 h: df = 1,20, $F = 0.005$, $P = 0.947$). Arrows indicate position of the stylar–ovary junction in the long- (L), mid- (M), and short-styled (S) morphs.

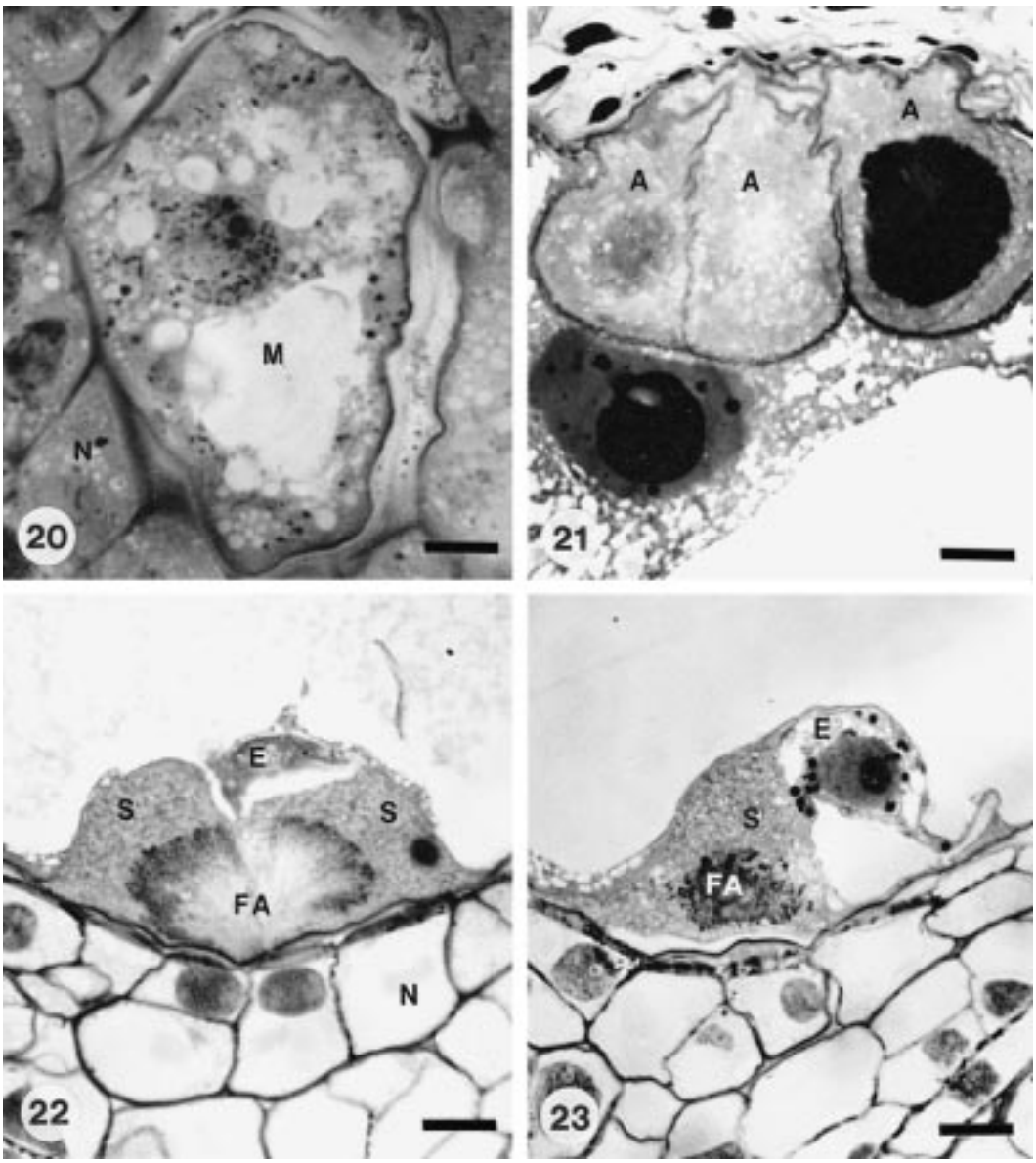
lowing cross- vs. self-pollination in a species with OSI. This result is significant because cross- and self-pollen tube growth rates in this species are similar, and hence ovule degeneration is unlikely to be associated with delayed arrival of self-pollen tubes, as likely occurs in *P. dulcis.*

Reduced female receptivity associated with embryo sac degeneration following self-pollination in *N. triandrus* may result from the absence of a required stimulus for normal ovule development. Differential responses of ovules following self- vs. cross-pollination were apparent well before pollen tube entry into ovaries, whereas the appearance of ovules in unpollinated flowers remained unchanged. These observations suggest the occurrence of ''long-distance'' signalling events between pollen tubes and ovarian tissue. Data from several studies indicate that pollen tubes provide essential stimuli necessary for a number of reproductive processes including ovule development (O'Neill, 1997), initiation of the secretory phase of transmitting tissue (Herrero and Arbeloa, 1989), induction of prolonged embryo sac viability (Herrero and Gascon, 1987), and stimulation of carpellary wall development (Fuller and Leopold, 1975). Signalling events between pollen tubes and maternal tissues are evident in many seed plants (e.g., Doyle, 1945; Nitsch, 1956), and it has been suggested that these interactions may have played a role in the evolutionary success and persistence of the seed habit (Sage, Bertin, and Williams, 1994). Indoleacetic acid, gibberellic acid, ethylene, and ethylene precursors have been posited to play a role in postpollination stimulation events by pollen tubes (for review, see O'Neill, 1997). As well, ovules appear to provide signals controlling pollen tube growth and guidance (for review, see Cheung, 1996). Signalling events are likely to be stage and site specific, occurring over short and long distances. Transmitting tissues and their extracellular matrix components are thought to play an integral role in signalling events. While our study does not provide information on the biochemical or molecular aspects of prezygotic self-sterility in *N. triandrus,* we do provide basic structural information essential for future identification of transmitting and ovular tissues likely involved in signalling events.

Temporal aspects of ovule development—Our studies in *N. triandrus* revealed an unexpected asynchrony in ovule development at anthesis within a single ovary. This pattern of staggered female receptivity may be associated with the duration of floral longevity, which in *N. triandrus* is ~7–10 d (Barrett et al., 1997). With the infrequent pollinator visitation that characterizes this species (Barrett, Lloyd, and Arroyo, 1996), staggered ovule development may provide reproductive assurance, particularly if periods of ovule receptivity are short. A similar suggestion has also been made for several other species with OSI (e.g., *Rhododendron*—Palser, Rouse, and Williams, 1989; *Lotus corniculatus*—Bubar, 1958) in which investigations have revealed ovules at different developmental stages during anthesis (and see also Franssen-Verheijen and Willemse, 1993). In autogamous species of *Lotus,* ovule development is synchronous, presumably reflecting the assured reproduction that selfing entails. In the extreme case of certain orchids, ovules at anthesis remain undeveloped and require a pollination stimulus for normal maturation (O'Neill, 1997). This may also be associated with extended floral longevities and uncertain pollinator visitation. Future comparative studies could profitably explore the potential functional associations among strategies of ovule deployment, floral longevity, and the pollination systems of flowering plants.

Our structural observations of ovule development revealed a conspicuous absence of micropylar secretions at anthesis in ovules with seven-celled embryo sacs. Micropylar secretions have been documented in a number of species (Chao, 1971; Tilton, 1980; Heslop-Harrison, Heslop-Harrison, and Reger, 1985; Kaul, Rouse, and Williams, 1986; Bruun and Olesen, 1989; Reger, Chaubal, and Pressey, 1992; Franssen-Verheijen and Willemse, 1993; Sage and Williams, 1995; Sage et al., 1999) and are involved in guidance and attraction of pollen tubes into the embryo sac (Knox, 1984). Secretions in *N. triandrus* were evident three days postpollination suggesting that ovules were not physiologicaly mature at anthesis and required a pollination stimulus to become receptive. This phenomenom has been reported in *Gasteria,* with differences in the degree of micropylar exudate evident following cross- vs. self-pollination (Willemse, Plyushch, and Reinders, 1995). Since micropylar secretions are one indicator of ovule receptivity, they could be profitably used as a tool to examine strategies of ovule deployment.

A second unexpected finding of our structural studies was the discovery of a low frequency of adventitious embryos following controlled pollinations. These embryos ceased normal development prior to cotyledon initiation and hence did not contribute to final seed set. Apomixis has been reported elsewhere in the Amaryllidaceae



Figs. 20–23. Light micrographs of the megaspore and seven-celled embryo sac at anthesis in *N. triandrus*. **20.** Functional haploid megaspore. Bar = 18 μm. **21.** Three antipodal cells and polar fusion nucleus of central cell. Bar = 24 μm. **22.** Synergids of egg apparatus. Note prominent filiform apparatus. Bar = 27 μm. **23.** Egg cell with prominent chalazally located nucleus. Amyloplasts surround the nucleus. Bar = 27 μm. *Figure Abbreviations*: A, antipodals; E, egg cell; FA, filiform apparatus; M, functional megaspore; N, nucellus; S, synergid.

(Davis, 1966), but we are unaware of previous reports indicating differences in the frequency of adventitious embryony following self- and outcross-pollination treatments. These results further support the hypothesis that signalling events important for ovule development and receptivity may differ between self- and outcross-pollen tubes. However, with adventitious embryony, the responses involve whether nongametophytic (somatic) tissue develops to form embryos. It is well known in species with pseudogamous apomixis that a pollination stimulus is required before asexual embryos can develop (reviewed in Richards, 1997), but whether this response is influenced by the source of pollen involved (e.g., outcross vs. self) appears not to have been investigated in detail.

Functional consequences of self-pollination—Our pollination experiments revealed significantly lower levels of double fertilization as a result of embryo sac degeneration following self- vs. cross-pollination. This results raises several issues concerning the functional consequences of OSI and how self-pollination might influence the reproductive biology of *N. triandrus*. Earlier studies proposed that self-pollination may involve significant reproductive costs because of the late-acting self-sterility system found in the species (Barrett, Lloyd, and Arroyo, 1996; Barrett et al., 1997). Because pollen tube growth rates of outcross- and self-pollen are similar, seed set can be reduced significantly if self-pollen is deposited on stigmas before outcross-pollen arrives. The loss of fe-

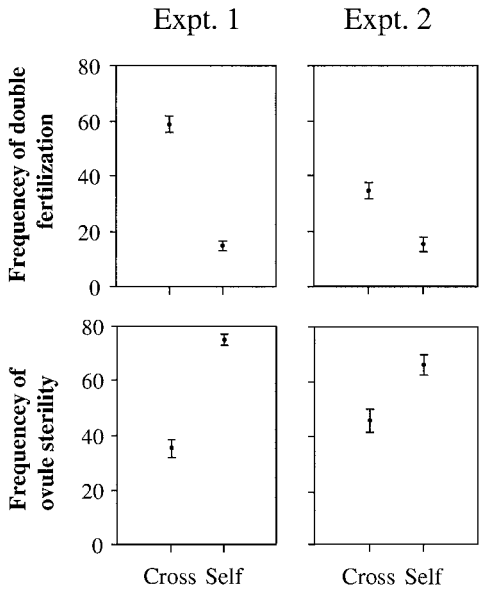


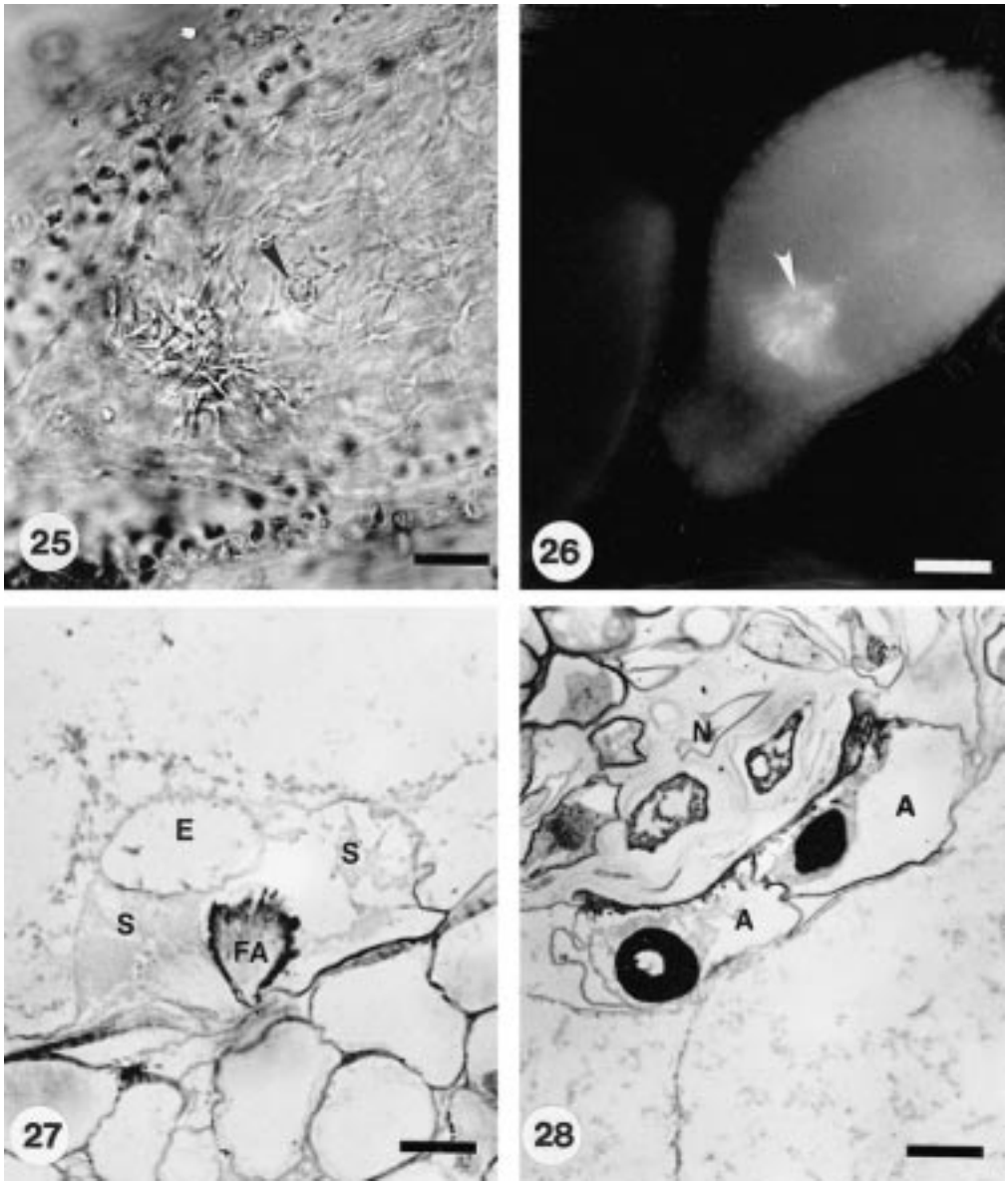
Fig. 24. The mean frequency (and standard error) of double fertilization and ovule sterility following cross- and self-pollinations of *N. triandrus* under glasshouse conditions. Experiments 1 and 2 were conducted in 1992 and 1995, respectively. All comparisons between cross- and self-pollination in each experiment were significantly different (experiment 1: double fertilization, df = 1,184, $F = 147.21$, $P < 0.001$; ovule sterility, df = 1,181, $F = 53.20$, $P < 0.001$; experiment 2: double fertilization, df = 1,78, $F = 24.72$, $P < 0.001$; ovule sterility, df = 1,78, $F = 13.86$, $P < 0.001$)

male fertility owing to self-pollen tubes rendering some ovules nonfunctional is termed ovule discounting (Barrett, Lloyd, and Arroyo, 1996). This effect has been demonstrated experimentally in *N. triandrus* by a 75% reduction in seed set in comparison with outcrossed controls when self-pollen is applied to stigmas 24 h before outcross-pollen (Barrett et al., 1997). Similar findings were reported by Dulberger (1964) in her work on *N. tazetta*. Structural observations in the present study showing increased ovule sterility following self-pollination provide a mechanistic basis for these results. Moreover, the significantly lower mean seed set when mixtures of cross- and self-pollen are deposited on stigmas, compared with cross-pollinations, suggest that these sterility responses are not restricted to pollinations using only self-pollen. This raises the question of whether the phenomenon of ovule discounting occurs frequently under field conditions. Studies of the schedule and amount of cross- and self-pollen deposition on stigmas and structural observations of ovule and seed development would be required to address this problem.

If the proposed signalling events from cross-pollen resulting in normal ovule development were independent of the presence of self-pollen on the stigma, it might have been predicted that in mixed pollinations the siring success of the two pollen types would be equivalent because of their similar pollen tube growth rates. However, results from mixed pollinations clearly refute this hypothesis since cross-pollen exhibited a significant siring advantage. This suggests that the proposed signal is not qualitative in action but instead is responsive to the composition of the pollen load. It is also possible that additional undetected mechanisms operate at the site of ovule or embryo sac entry favoring fertilizations by cross-pollen tubes. More than two pollen tubes were commonly observed in micropyles of *N. triandrus* following cross- and self-pollinations, although only one pollen tube was ever observed entering the embryo sac.

The ovule wastage that can arise from self-pollination in *N. triandrus* may commonly occur in other species with OSI. There are several reports of the negative influences of prior self-pollination on seed set in pollen chase experiments (Cope, 1962; Dulberger, 1964; Crowe, 1971; Waser and Price, 1991; Lloyd and Wells, 1992; Broyles and Wyatt, 1993; Seavey and Carter, 1994). Such effects have often been ascribed to ''stylar or ovular clogging'' by self-pollen tubes preventing ovules from being fertilized by later growing cross-pollen tubes. Our results, however, raise the possibility that reduced fertility may result from the absence of appropriate hormonal interactions for ovule development and receptivity rather than through physical blockage of the pollen tube pathway by self-pollen tubes. Ovule wastage through self-pollination has been viewed as maladaptive because of the loss in reproductive potential that it is assumed to entail. By minimizing investment of maternal resources in selfed seed during any one reproductive episode, however, plants with OSI may ultimately save resources, compared with those that invest in selfed seeds that make little contribution to fitness because of inbreeding depression. In perennial plants, resources saved in years in which self-pollination might be frequent can contribute to future reproductive effort hence increasing lifetime fitness (see Morgan and Schoen, 1997). Many species with OSI are relatively long-lived so limiting resource commitments by pollen tube–ovule signalling may provide one solution to reducing the reproductive costs associated with self-pollination.

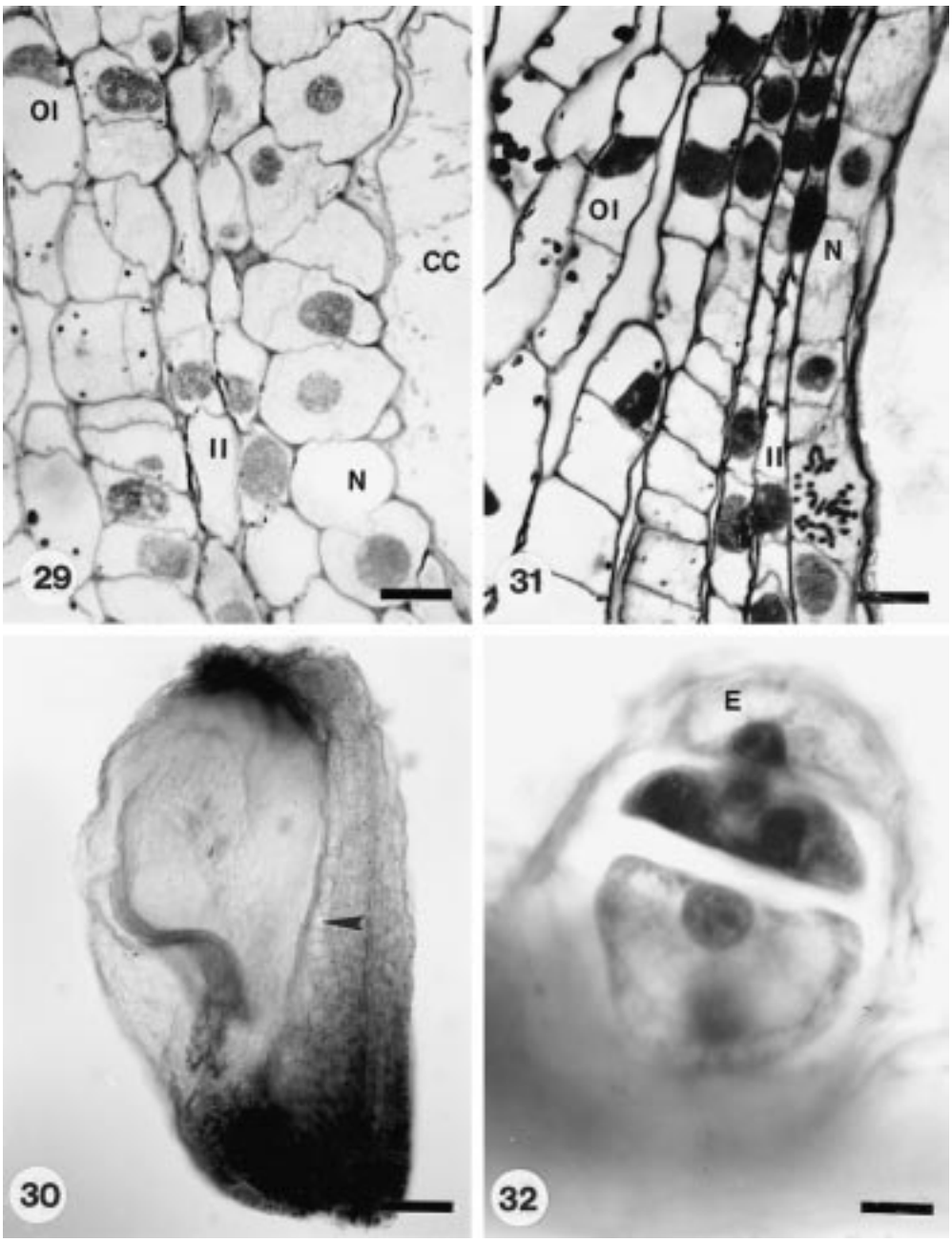
It is important to note that the self-sterility mechanism in *N. triandrus* does not preclude opportunities for self-fertilization to occur. While mixed pollinations showed a clear advantage to outcross-pollen, 12% of the seeds produced were the result of self-fertilization. Previous studies of *N. triandrus* demonstrated that mean seed set from hand self-pollination averages 16% of that obtained from cross-pollination (cross-pollination—36.0 seeds per fruit, $N = 91$ flowers; self-pollination—5.9 seeds per fruit, $N = 96$ flowers; Barrett et al., 1997) implying that a potential for some selfing to occur under field conditions exists. Indeed this has been verified by marker-gene estimates of the frequency of self-fertilization in four Spanish populations, which indicated modest amounts of selfing in each one (mean selfing rate = 0.23, range 0.13–0.32). Despite the largely outcrossed mating system of *N. triandrus* there is no evidence that selfed seeds experience greater selective abortion than outcrossed seed as a result of inbreeding depression. Embryo abortion was not detected in this study and in earlier work discussed above there were no significant differences in the small numbers of shrunken seeds obtained from self- vs. cross-pollination. Inbreeding depression seems probable in *N. triandrus* since parental F values in the four populations in which selfing rates were estimated were close to zero (S. C. H. Barrett, unpublished data) implying strong selection against selfed progeny. Fitness comparisons at other life cycle stages (see Husband and Schemske, 1996) would



Figs. 25–28. Light and fluoresence micrographs of degenerated megaspore and seven-celled embryo sacs 3 d following self-pollination in *N. triandrus*. **25.** Ovule clearing. Megaspore nucleus (arrowhead) embedded within the nucellus. Note translucent appearance of the degenerated ovule. Bar = 50 μm. **26.** Aniline blue fluorescence depicting callosic nature of nucellar cell walls (arrowhead) surrounding megaspore. Bar = 143 μm. **27.** Enucleate condition of egg cell and synergids. Bar = 23 μm. **28.** Degenerating antipodal cells. Bar = 29 μm. *Figure Abbreviations*: A, antipodals; E, egg cell; FA, filiform apparatus; S, synergid.

be required to detect inbreeding depression at later stages in the life cycle of *N. triandrus.*

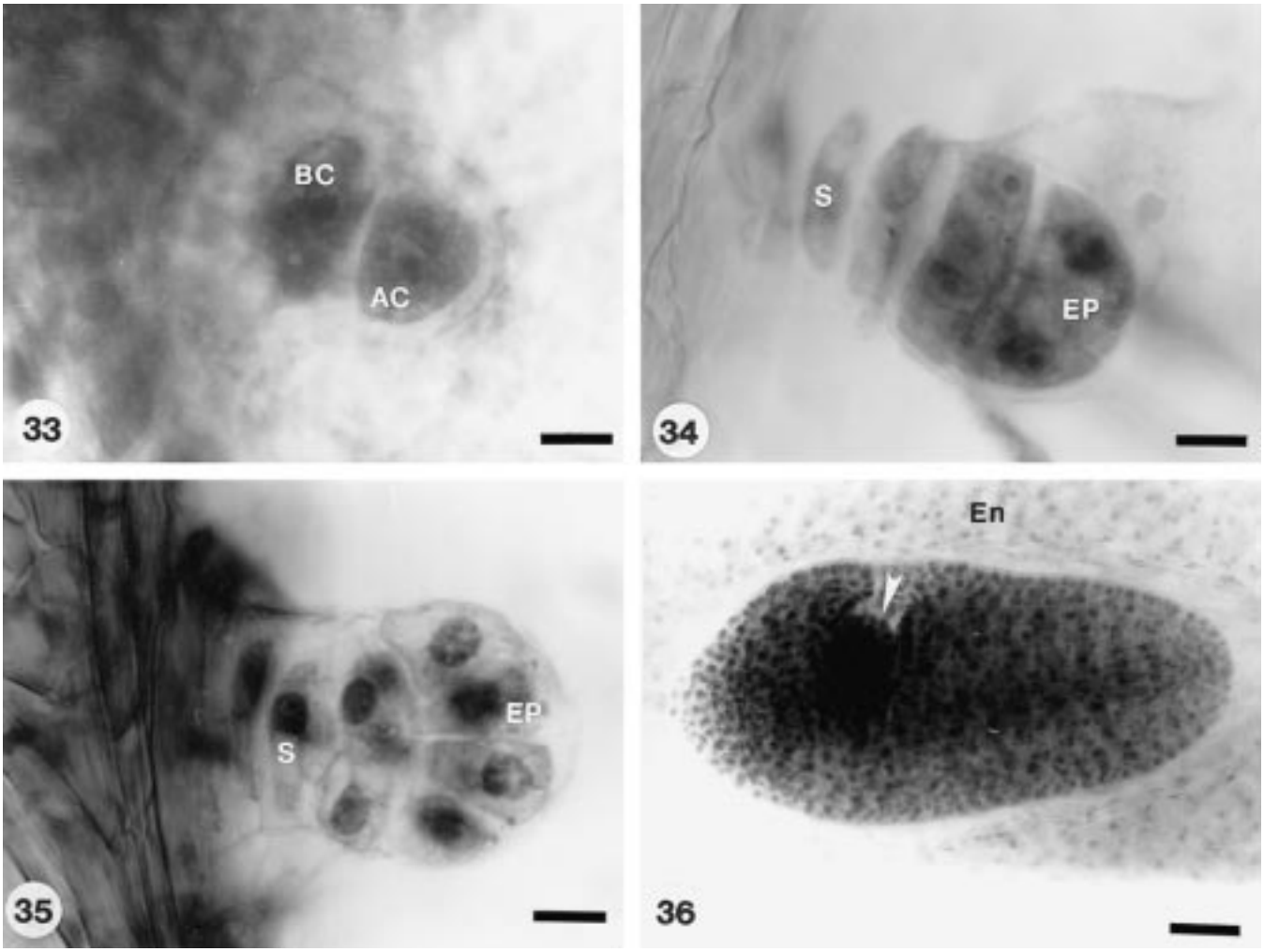
Self-sterility and floral trimorphism—*Narcissus triandrus* is the only member of its genus that is truly heterostylous with most populations morphologically tristylous (Barrett, Lloyd, and Arroyo, 1996). Our studies demonstrate an unusual association between the morphological components of tristyly and OSI. Elsewhere among heterostylous plants, ovarian incompatibility has only been reported in the short-styled morph of trimorphic *Pontederia* spp. (Anderson and Barrett, 1986; Scribailo and Barrett, 1991) and in dimorphic *Anchusa officinalis* (Schou and Philipp, 1983), a species with intramorph compatibility and several other features of its reproductive system that resemble those found in *Narcissus* spp. (Barrett et al., 1997). Most heterostylous plants exhibit a physiological incompatibility system that prevents self- and intramorph fertilizations (reviewed in Barrett, 1992). Only intermorph crosses between anthers and stigmas of equivalent height result in seed set. Heteromorphic incompatibility is virtually always associated with ancillary polymorphisms involving pollen size and stigmatic papillae length (Dulberger, 1992), but in *N. triandrus* these polymorphisms are absent and both intermorph and intramorph pollinations are equally fertile (Barrett et al.,



Figs. 29–32. Light micrographs of degenerated and nondegenerated ovules in *N. triandrus*. **29.** Hypertrophied nucellar and integument cells of degenerated ovule at 3 d following self-pollination. Bar = 29 μm. **30.** Degenerated cleared ovule 21 d following self-pollination. Note ghost-like appearance of embryo sac of translucent ovule. Arrowhead denotes hypertrophied cells of maternal tissue. Bar = 253 μm. **31.** Nonhypertrophied nucellar and integument cells of receptive ovule at 3 d following cross-pollination. Bar = 28 μm **32.** Adventitious embryo in degenerated ovule at 13 d following self-pollination. Bar = 40 μm. *Figure Abbreviations*: CC, central cell; E, embryo; II, inner integument; N, nucellus; OI, outer integument.

1997). The association between these features and OSI makes the tristylous syndrome of *N. triandrus* unique among flowering plants.

Our studies of pollen germination and pollen tube growth failed to reveal significant morph-specific effects. This is in contrast to most heterostylous species where pollen-tube growth rates and sites of inhibition usually differ between the floral morphs (reviewed in Dulberger, 1992; Barrett and Cruzan, 1994). The absence of such effects in *N. triandrus* is undoubtedly associated with the species' unique self-sterility system and lack of pollen–stigma polymorphisms. In our pollination experiments, the only significant difference among the morphs that was revealed involved the average time taken for pollen tubes to enter the ovary. Pollen tubes reached the ovary of short-styled flowers first and long-styled flowers last. These differences presumably reflect the fact that the floral morphs have uniform pollen sizes, implying similar



Figs. 33–36. Light micrographs of cleared ovules illustrating embryo development in *N. triandrus*. **33.** Two-celled embryo at 6 d following cross-pollination. Bar = 38 μm. **34, 35.** Globular embryos at 12 and 15 d, respectively, following cross-pollination. Bars = 54 and 47 μm, respectively. **36.** Cotyledon stage of embryo development at 21 d following cross-pollination. Arrow marks shoot apical meristem. Bar = 284 μm. *Figure Abbreviations*: AC, apical cell; BC, basal cell; En, endosperm; EP, embryo proper; S, suspensor.

growth rates, but styles that differ in length. Whether these timing differences have any functional significance is unclear, but the association of monomorphic pollen and stylar trimorphism indicates that differences in pollen size are not always a prerequisite for successful pollen–pistil interactions in heterostylous plants.

Ovarian self-sterility and concepts of self-incompatibility—Self-incompatibility has been known for several centuries, but concepts of what it constitutes have varied greatly depending on the perspectives of the investigator. At its most rudimentary, the term is used to refer to the inability of a fertile hermaphrodite plant to produce viable seed following self-pollination. Traditional classifications of SI usually involved placing species into either one of two major categories (homomorphic vs. heteromorphic or sporophytic vs. gametophytic) reflecting differences in their morphology, physiology, and genetics (de Nettancourt, 1977; Lewis, 1979; Heslop-Harrison, 1983; Barrett, 1988; Newbigin, Anderson, and Clarke, 1993). Despite early work on *Gasteria* (Sears, 1937) and *Theobroma* (Knight and Rogers, 1955; Cope, 1962), OSI was often ignored or treated as an anomaly of limited importance by workers studying the ''major'' types of SI. This situation has recently changed as numerous examples of apparent OSI have come to light, although in most instances the critical structural work required to distinguish whether self-sterility results primarily from a true incompatibility system based on self-recognition or is due to postzygotic abortion of embryos due to inbreeding depression has not been determined (reviewed in Seavey and Bawa, 1986; Sage, Bertin, and Williams, 1994; Sage et al., 1998).

Our studies of *N. triandrus* clearly indicate that self-sterility is not a simple manifestation of inbreeding depression. However, the form of self-recognition that occurs in this species does not fit easily into any existing category of SI. First, despite being heterostylous, *N. triandrus* exhibits a self-sterility system that is uncoupled to morphology and shares features common to all homomorphic SI systems; namely, individual plants are largely self-sterile, but the majority of cross-pollinations

are fertile. Second, the mechanism responsible for low seed set upon self-pollination does not involve reduced growth or active inhibition of self-pollen tubes as occurs in both homomorphic and heteromorphic incompatibility systems. Instead, self-pollen tube recognition somewhere in the pistil appears to elicit responses that prevent normal ovule development, thus reducing levels of pollen tube penetration and double fertilization. While the precise timing and mechanism(s) responsible for these responses are not known, it seems likely that, as discussed above, they result from contrasting signalling phenomena associated with cross- vs. self-pollen tube growth. If this is true, then self-sterilty in *N. triandrus* differs from other well-characterized SI mechanisms in which recognition and rejection of self are not as spatially and temporally separated.

Pollen–pistil interactions in *N. triandrus* may involve long-distance signals and a developmental component neither of which have usually been considered within the framework of SI mechanisms. Moreover, the interactions leading to self-sterility are not absolute in expression but are instead quantitative showing graded responses, perhaps controlled by the quantity and type of pollen deposited on stigmas. Clearly more detailed information on the physiological and genetic basis of self-sterilty in *N. triandrus* is required. However, at the present time we are inclined to include these phenomena within a broadened functional view of SI that recognizes prezygotic maternal recognition of self as the fundamental feature of all incompatibilty systems regardless of the underlying molecular and physiological mechanisms involved.

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