

The Causes of Self-sterility in Natural Populations of the Relictual Angiosperm, *Illicium floridanum* (Illiciaceae)

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• **Background and Aims** *Illicium floridanum*, a species belonging to the basal extant angiosperm taxon Illiciaceae, reportedly exhibits self-incompatibility (SI). To date, the site and timing of SI within the carpel of this species remains unidentified. Thus, the objective of this research was to determine the cellular and temporal aspects of SI in *I. floridanum*.

• **Methods** Following controlled application of cross- and self-pollen in natural populations of *I. floridanum*, embryo sac development and temporal aspects of stigma receptivity, as well as pollen tube growth, fertilization, and embryo and endosperm development, were investigated with the aid of light and fluorescence microscopy.

• **Key Results** Flowers of *I. floridanum* exhibited complete dichogamy whereby stigmas only supported cross- and self-pollen tube growth prior to anther dehiscence. In contrast to earlier reports of SI in this species, a prezygotic SI resulting in rejection of self-pollen tube growth at the stigma was absent and there were no significant differences between cross- versus self-pollen germination and pollen tube growth within the style and ovary during the first 5 d after pollination. Structural development of the four-celled embryo sac was not differentially influenced by pollen type as noted to occur in other angiosperms with late-acting ovarian SI. The ovule micropyle and embryo sac were penetrated equally by cross- and self-pollen tubes. In addition, there were no statistically significant differences in cross- versus self-fertilization. A resting zygote and multicellular endosperm at a variety of developmental stages was present by 30 d after application of cross- or self-pollen.

• **Conclusions** In the clear absence of a prezygotic SI that was previously reported to result in differential self-pollen tube growth at the stigma, self-sterility in *I. floridanum* is likely due to early-acting inbreeding depression, although late-acting post-zygotic ovarian SI cannot be ruled out. © 2004 Annals of Botany Company

Key words: *Illicium floridanum*, self-incompatibility, self-sterility, complete dichogamy, early-acting inbreeding depression, Illiciaceae, Austrobaileyales.

INTRODUCTION

The Illiciaceae is comprised of a single genus of approx. 40 species distributed in eastern Asia and North and Central America (Smith, 1947; Saunders, 1995). As well as having a fossil record extending back to the Early Cretaceous (Friis *et al.*, 2000), the taxon is a member of the paraphyletic group of angiosperms comprising the first three branches of angiosperm phylogeny [Amborellaceae, Nymphaeaceae and Austrobaileyales (Austrobaileyaceae, Illiciaceae, Schisandraceae and Trimeniaceae); Angiosperm Phylogeny Group (APG), 1998; Mathews and Donoghue, 1999; Parkinson *et al.*, 1999; Qiu *et al.*, 1999, 2001; Barkman *et al.*, 2000; Graham and Olmstead, 2000; Soltis *et al.*, 2000; Zanis *et al.*, 2002].

Illicium floridanum, one of two North American species of the Illiciaceae (Hao *et al.*, 2000), is a small evergreen shrub restricted to the floodplains of small streams in the Gulf States of the south-eastern United States (Smith, 1947). The flowers of the species are bisexual and protogynous, producing on average 13 unfused uniovulate carpels

(Robertson and Tucker, 1979; Thien *et al.*, 1983). Each carpel has a stigmatic crest that lies on the same plane as undehisced stamens but which subsequently moves 90° to stand upright at the time of anther dehiscence (Thien *et al.*, 1983). An external compitum is present in *I. floridanum* whereby pollen tubes germinating on the stigmatic crest of one carpel can exit the carpel and grow to an adjacent carpel via growth around a centrally located tissue (Williams *et al.*, 1993) termed the apical residuum (Robertson and Tucker, 1979). Pollination in *I. floridanum* is affected by litter-dwelling insects to include Diptera and Coleoptera (Thien *et al.*, 1983).

Application of self-pollen to stigmas of *I. floridanum* reportedly results in the absence of fruit production leading to the conclusion that the species exhibits self-incompatibility (SI) (Thien *et al.*, 1983). SI has been noted to occur on the stigma or within the substigmatic transmitting cells of the stigmatic crest, since self-pollen tubes appear to stop growing after reaching a few millimetres in length (Thien *et al.*, 1983). Ovules from self-pollinated carpels appear to degenerate early in development (Thien *et al.*, 1983). Using traditional classification schemes

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(de Nettancourt, 1977, 1997), SI has been classified as gametophytic because *I. floridanum* has bicellular pollen and the stigma has been interpreted as wet (Thien *et al.*, 1983).

Recently, however, the stigma of *I. floridanum* has been demonstrated to be of the dry versus wet type (Koehl, 2002). These results are significant for two reasons. First of all, SI in combination with bicellular pollen and a dry-type stigma was once considered anomalous (de Nettancourt, 1977). If *I. floridanum* exhibits SI in association with these stigmatic cellular characteristics, it would join a growing number of species with such features reinforcing the view that these character traits are in fact quite common (Franklin *et al.*, 1995; Pontieri and Sage, 1999; Sage *et al.*, 2000, 2001). The cellular nature of pollen and stigmatic epidermal cells involved in SI will likely have important implications for the type of self-recognition and rejection mechanisms that are operating (Franklin *et al.*, 1995; Franklin-Tong and Franklin, 2003; Stone *et al.*, 2003). Secondly, a recent study has shown the presence of stigmatic SI in association with a dry-type stigma and bicellular pollen in *Trimenia moorei* (Trimeniaceae; Bernhardt *et al.*, 2003), another species rooted at the base of the angiosperm phylogeny and occupying the same branch as the Illiciaceae. As well, a potential stigmatic SI has been reported in the third member of the branch, *Austrobaileya scandens* (Austrobaileyaceae; Prakash and Alexander, 1984). If SI in *I. floridanum*, *T. moorei* and subsequently *A. scandens* can be demonstrated to share similar cellular, molecular and genetic mechanisms, then it may have important implications for the reconstruction of relictual forms of SI, a long-standing issue of interest (Whitehouse, 1950; Barrett, 1988; Olmstead, 1989; Bell, 1995; Read *et al.*, 1995; Weller *et al.*, 1995; Sage *et al.*, 2000; Igic and Kohn, 2001; Steinbachs and Holsinger, 2002). The purpose of the present study is to characterize the exact site and timing of self-pollen recognition and rejection by assessing cross- and self-pollen tube growth and early stages of ovule/seed development following self- and cross-pollen application in naturally occurring populations of *I. floridanum*.

MATERIALS AND METHODS

Study populations

Experimental material of *Illicium floridanum* Ellis used in this study was located in three populations in the southern United States. Population A was located adjacent to an oxbow of the Bowie River, in Forest County, Mississippi (31.37688, -89.35886). Population B was west of Rawls Springs, Forest County, near Hattiesburg (31.37286, -89.42064), and population C was located on Morgan's Bluff along the West Pearl River, near Slidell, Louisiana (30.32899, -89.71267). Cross- and self- (inter- and intra-population) pollinations were conducted in the field using randomly selected flowering shrubs. To exclude pollination by insects, floral buds were enclosed in light-weight pollination bags constructed from bridal veil (mesh gauge approx. 100 μ m) approx. 24 h prior to floral anthesis.

Flowers were cross- and self-pollinated by tapping freshly dehisced anthers just above receptive stigmas. Flowers were then re-bagged until harvest. Self-pollinations were conducted with pollen of the same plant. Intra-population crosses were conducted for the three populations in addition to inter-population crosses in all combinations.

Cross- versus self-pollen tube growth in I. floridanum

Prior to conducting cross- and self-pollinations, the timing of stigma receptivity in *I. floridanum* was assessed by applying pollen to stigmas when stigmas were (a) in the plane of undehisced anthers (female phase; Thien *et al.*, 1983; $n = 39$) and (b) when stigmas had moved 90° to an upright position coinciding with anther dehiscence (male phase; Thien *et al.*, 1983; $n = 39$). From these controlled pollinations, it was determined that stigmas were not receptive during the male phase as they did not support pollen germination. Therefore, all cross- and self-pollinations were subsequently conducted during the female phase of floral development.

Qualitative and quantitative comparisons of cross- and self-pollen tube growth prior to embryo sac penetration were characterized using aniline blue fluorescence microscopy (Martin, 1959; Sage *et al.*, 1999) and light microscopy (Sage *et al.*, 1999). Self- and intra-population cross-pollinations within populations A and B were conducted on five to ten plants per population and harvested at 48 h ($n = 6-15$ flowers/treatment/plant/population). Self- and intra-population cross-pollinations were performed on five to ten plants within population C and harvested at 1 h, 3 h, 6 h, 9 h, 15 h, 24 h, 72 h, 5 d and 10 d ($n = 6-15$ flowers/treatment/plant/harvest time). Inter-population cross-pollinations between the same plants used for intra-population pollinations were conducted between B \times C and A \times C. These samples were harvested at 6 h, 9 h, 15 h, 24 h, and 72 h ($n = 3$ flowers/treatment/plant/harvest time) and A \times B and B \times A were harvested at 48 h ($n = 7-10$ /flowers/treatment/plant). Quantitative characterization of self- and cross-pollen tube growth was performed by measuring the following parameters at each harvest time for each treatment: (1) the number of germinated pollen grains; (2) the number of pollen tubes in the substigmatic transmitting tissue (SST); (3) the number of pollen tubes entering the ovary locule; and (4) the number of pollen tubes within the ovule micropyle. The data obtained for each of the four parameters was converted into a percentage of the total number of grains that landed on the stigmatic crest for that particular gynoecial unit. Initially, ANOVA was performed to determine if there were differences between data from intra- and interpopulation with respect to each parameter at each harvest time for each pollination treatment. No differences were detected in these analyses. Therefore, data from all cross-pollination treatments were pooled as were data from all self-pollinations. Two-way ANOVAs (SigmaStat 2.0) were then used to examine differences over time between cross- and self-pollen tube growth for each of the four quantified parameters.

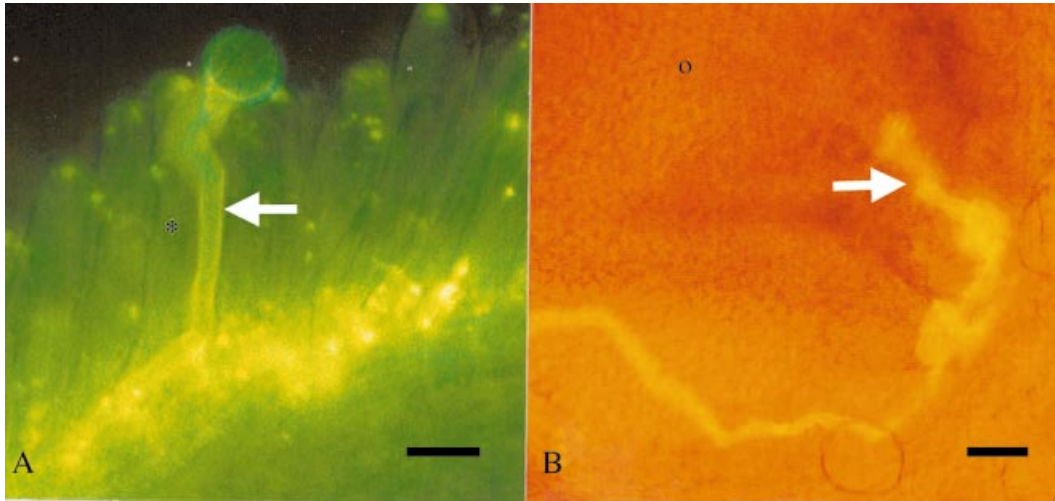


FIG. 1. Aniline blue fluorescence of self-pollen tube (arrow) growth on a stigmatic cell (*) at 6-h post-pollination (A) and within the ovule micropyle (arrow) at 72 h following pollination (B). Bars: A = 25 μm ; B = 50 μm . O, Ovule.

Embryo sac development, fertilization, and embryo and endosperm development following cross- versus self-pollination in I. floridanum

Embryo sac development was characterized prior to pollination to first assess whether or not an ovule contained a mature embryo sac at anthesis, and hence whether cross- versus self-pollen influenced embryo sac maturation as reported for other angiosperms (Waser and Price, 1991; Sage *et al.*, 1999). Ovules for characterization of embryo sac development were harvested from developing carpels and prepared using cryofixation/freeze substitution as described by Lam *et al.* (2001). To determine the success of fertilization, ovules/seeds from flowers in populations A, B and C were harvested 5 d, 10 d and 30 d following cross- and self-pollination ($n = 12\text{--}30$ flowers/harvest time/treatment), fixed in formalin–acetic acid–ethanol (FAA), acetic acid–ethanol (1 : 3) or glutaraldehyde and prepared for light microscopy as described by Sage and Williams (1995). Serial sections were scored for fertilization as described by Sage *et al.* (1998) and Sage and Sampson (2003). Fertilization was indicated by: (a) the absence of unfused sperm nuclei within embryo sacs penetrated by a pollen tube; (b) the presence of a resting zygote and/or embryo; and (c) the presence of one or more endosperm nuclei. Unpollinated ovules were also harvested at 5 d, 10 d and 30 d post-anthesis to compare embryo sac development with that from cross- and self-pollinated ovules. Initially, ANOVA was performed to determine if there were differences between data from intra- and/or inter-population with respect to presence or absence of fertilization at each harvest time for each pollination treatment. No differences were detected in these analyses. Therefore, data from all cross-pollination treatments were pooled as were data from all self-pollinations. Student's *t*-tests were then employed to determine whether significant differences existed between cross- and self-embryo sac penetration as well as cross- and self-fertilization.

RESULTS

Cross- and self-pollen tube growth in I. floridanum

There were no qualitative differences at the structural level between cross- and self-pollen tube growth from the time of pollen grain germination to ovule and embryo sac penetration. Cross- and self-pollen tubes germinated within 1 h of pollination and grew to the base of the stigma papillae by 3 h. Pollen tube entry into the SST occurred by 6 h (Fig. 1A) and pollen tubes subsequently reached the ovary locule by 24 h and entered the micropyle within 48–72 h post-pollination (Fig. 1B). Statistical analyses comparing cross- and self-pollen tube growth over time revealed few quantitative differences. A two-way ANOVA revealed no significant differences between cross- and self-pollen grain germination over time (Fig. 2A; d.f. = 1, $F = 0.217$, $P = 0.955$). Similarly, there were no significant differences between cross- and self-pollen tube growth over time within the SST (Fig. 2B; d.f. = 1, $F = 0.776$, $P = 0.38$), although pairwise comparisons showed significant differences within the SST at 15 h (d.f. = 1, $H = 4.563$, $P = 0.033$) and 24 h (d.f. = 1, $F = 11.846$, $P = 0.001$). No significant differences between cross- and self-pollen tube growth entry into the ovary (Fig. 2C; d.f. = 1, $F = 0.274$, $P = 0.601$) were noted, although pairwise comparisons again revealed significant differences between cross- and self-pollen tube growth in this instance at 24 h (d.f. = 1, $H = 5.185$, $P = 0.023$). Finally, there were no significant differences between cross- and self-pollen tube entry into the micropyle during the first 5 d after pollination (Fig. 2D; d.f. = 1, $F = 2.345$, $P = 0.126$).

Embryo sac development, embryo sac penetration, fertilization, and embryo/endosperm development following cross- versus self-pollination in I. floridanum

Embryo sac development in *I. floridanum* from a chalazal spore of a T-shaped tetrad of megaspores conformed to the

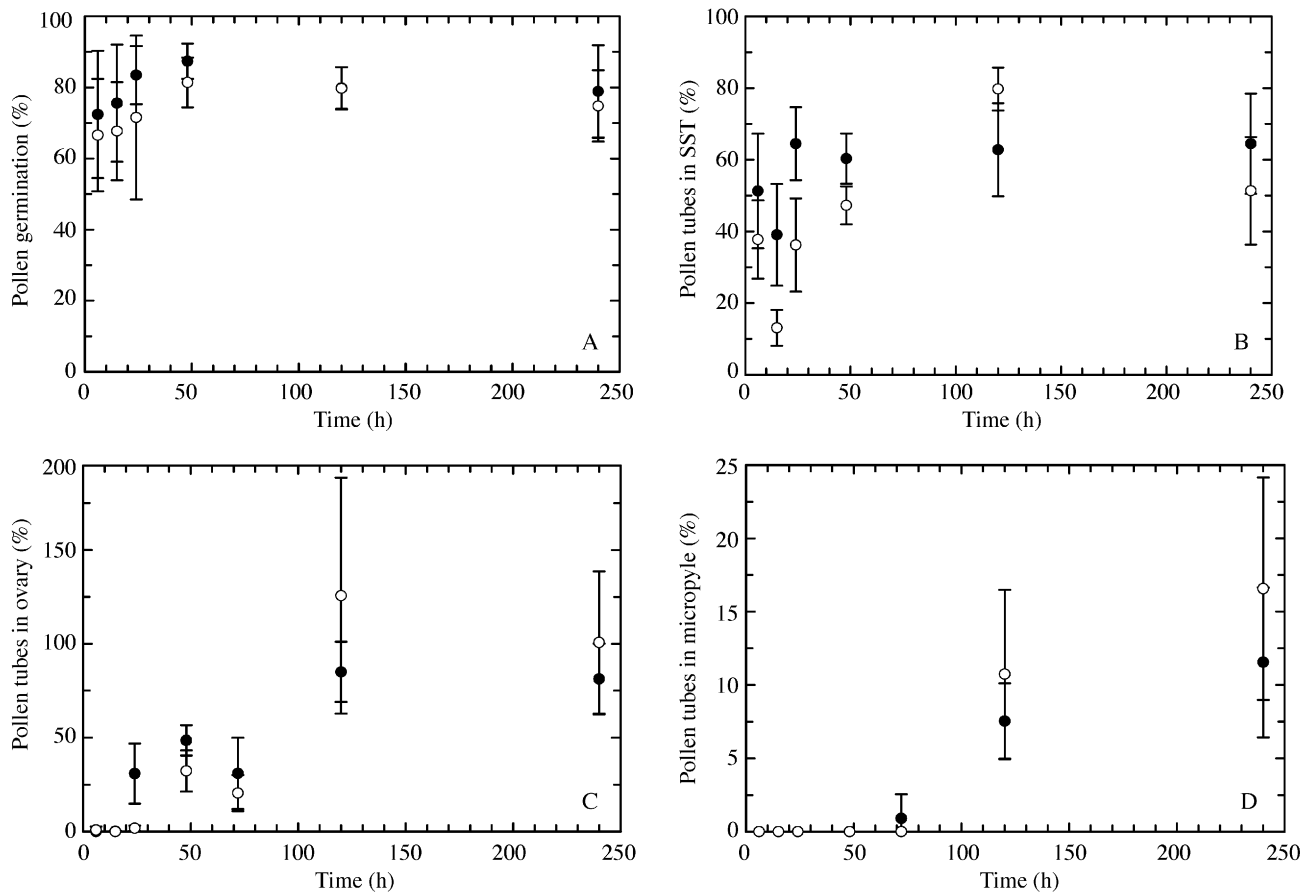


FIG. 2. Changes in cross- (open circles) and self- (filled circles) pollen germination (A) and cross- and self-pollen tube growth within transmitting tissue (B–D) of *Illicium floridanum* carpels. Data are mean values \pm 95 % confidence interval. See Results for details of statistics. SST, Substigmatic transmitting tissue.

Schizandra type (as defined by Battaglia, 1986) whereby the mature embryo sac of ovules from unpollinated ovaries contained a partially cellularized egg (Fig. 3A), two partially cellularized synergids and a single polar nucleus (Fig. 3B) as has been described for other members of the Austrobaileyales (Swamy, 1964; Friedman *et al.*, 2003) but which is in variance with some species of the Illiciaceae wherein the embryo sac development is reported to be of the *Polygonum* type (Hayashi, 1963). Unpollinated embryo sacs were still four-celled with intact synergids at 30 d post-anthesis. Initial embryo sac penetration (cross, \bar{x} = 2.4 % \pm 6.4; self, \bar{x} = 0.0 % \pm 0.0; d.f. = 1; P = 0.731) was observed at 5 d following cross- and self-pollination (Fig. 3C). A resting zygote and cellular endosperm was present by 30 d (Fig. 3D) following both pollination treatments. In each case, cellular endosperm development was variable ranging from four to 300 cells. The percentages of cross- and self-fertilization were statistically indistinguishable at 10 d (cross, \bar{x} = 62.33 % \pm 48.6 %; self, \bar{x} = 64.2 % \pm 41.6 %; d.f. = 9, P = 0.948) and 30 d (cross, \bar{x} = 88.8 % \pm 20.2 %; self, \bar{x} = 66.7 % \pm 57.7 %; d.f. = 7, P = 0.404).

DISCUSSION

Results from the present study examining cross- and self-pollen tube growth are at odds with a previous interpretation of data that concluded SI was present within the stigmatic crest of *I. floridanum* (Thien *et al.*, 1983). In contrast, the present study indicates that self-pollen tubes did not cease growth at the stigma and there were no significant differences over time between cross- and self-pollen tube germination and growth to the ovary. Furthermore, there was no differential cross- versus self-pollen tube entry of micropyles and embryo sacs. Development of the embryo sac did not appear to be influenced by pollen type and percentages of cross- and self-fertilization, although quite variable, were not significantly different such that a multicellular endosperm and a zygote were present 30 d following pollination. The absence of differences in ovule entry and fertilization between pollination treatments was observed in three distinct natural populations. In addition to these observations, novel information on one other feature of the reproductive biology of *I. floridanum*, that is important for intrafloral geitonogamy, is also provided. It

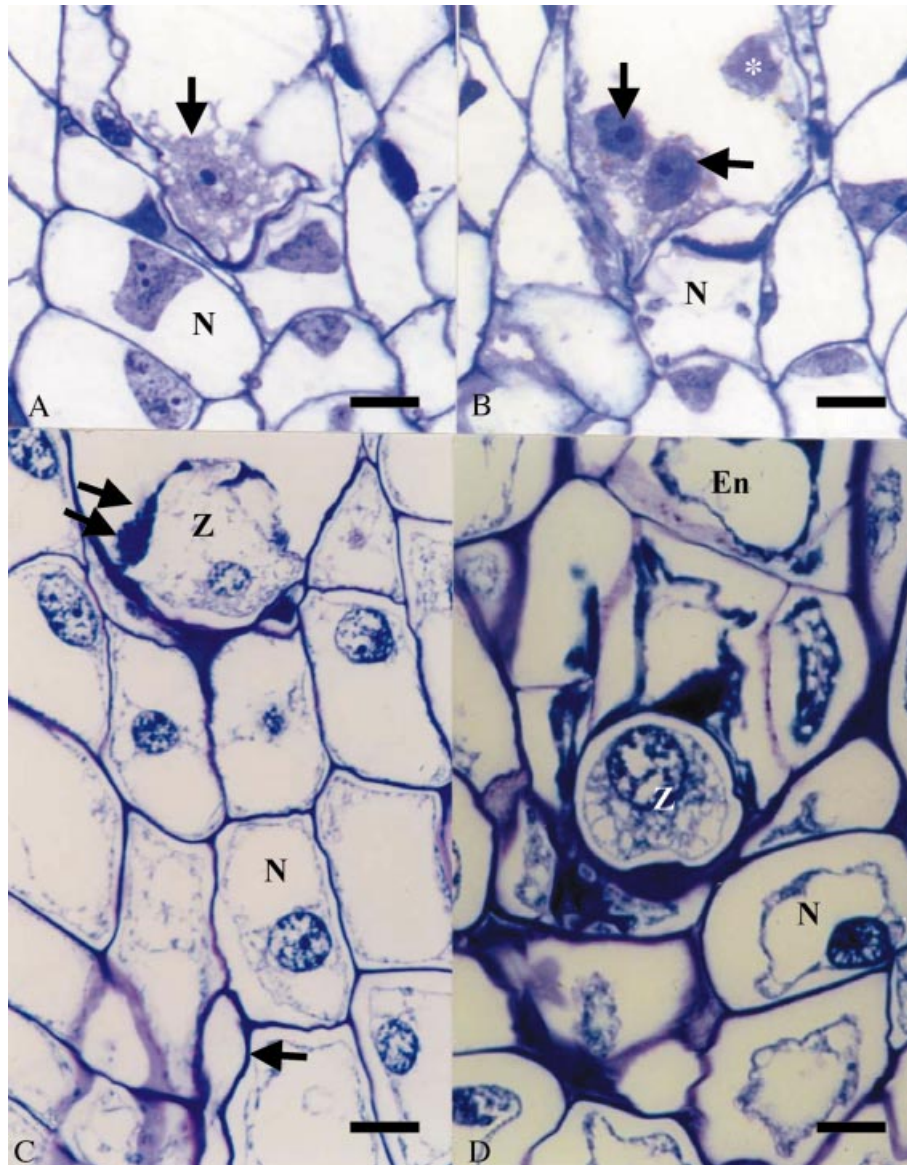


FIG. 3. Embryo sac contents at anthesis prior to pollination (A and B) and 10 d (C) and 30 d (D) post-pollination. (A) Arrow denotes partially cellularized egg cell; (B) arrows mark partially cellularized synergids. *, Polar nucleus. (C) Single arrow marks pollen tube in nucellus; double arrow delineates penetrated synergid. Bars: A–C = 100 μ m; D = 50 μ m. En, Cellular endosperm; N, nucellus; Z, zygote.

is reported that stigmas support pollen germination only during the female phase of floral ontogeny. The discussion starts by reviewing other work on self-sterility in angiosperms and then addresses potential mechanisms that could account for the previous report of a lack of self-seed set in *I. floridanum* in the absence of an apparent prezygotic SI, as observed in the present study. Since these studies on the timing of stigma receptivity have important implications for the success of self-pollen after anther dehiscence within a flower, this issue is then addressed. In conclusion, the results on stigma receptivity and compatibility status of *I. floridanum* are placed in the context of hypotheses on the roles of these two features in the reproduction of early angiosperms.

Mechanisms of self-sterility in angiosperms

Self-sterility in angiosperms results in reduced fruit/seed set following self-pollination as compared with cross-pollination and is principally achieved through two mechanisms: SI and early-acting inbreeding depression. Self-incompatibility is a genetically controlled mechanism functioning to enhance heterozygosity (Lundqvist, 1964; de Nettancourt, 1977, 1997). In some species, SI results in the prezygotic failure of self-pollen tube growth at the stigma, style or ovary (Kenrick *et al.*, 1986; Franklin *et al.*, 1995; de Nettancourt, 1997; Sage *et al.*, 2001). In other species, SI fails to result in differential prezygotic self-pollen tube growth. Rather, self-pollen tube growth in

the gynoeceium induces failed embryo sac development apparently via long-distance signalling resulting in the elimination of ovules available for double fertilization (Sage *et al.*, 1999). SI in such cases has been termed late-acting ovarian SI (OSI) or just late-acting SI. In addition, OSI in some species has also been posited to act after fertilization through the abortion of zygotes prior to division (Seavey and Bawa, 1986; Gibbs and Bianchi, 1993, 1999; Sage *et al.*, 1994; Gibbs *et al.*, 1999; Bittencourt *et al.*, 2003; Sage and Sampson, 2003). The presence of SI operating after gamete fusion remains controversial and SI in such instances may be due to as of yet undetermined mechanisms of pre-zygotic self-recognition (Sage and Sampson, 2003). In contrast to SI, early-acting inbreeding depression occurs only post-zygotically and results in abortion of offspring homozygous for deleterious recessive alleles (Charlesworth and Charlesworth, 1987; Husband and Schemske, 1996). Abortion due to inbreeding occurs at a variety of developmental stages as opposed to a single stage as observed in post-zygotic SI (Seavey and Bawa, 1986; Sage *et al.*, 1994; Seavey and Carter, 1994).

In the absence of differential ovule entry and fertilization of the four-nucleate embryo sac of *I. floridanum* following cross- versus self-pollination, self-sterility can be attributed to either early-acting inbreeding depression or post-zygotic OSI. Notably, results from the present study indicate that endosperm development is not uniform but is quite variable following both cross- and self-pollination. As well, fruits of *I. floridanum* abscise at various times after receiving cross- and self-pollen and fruit size can be quite variable after either pollination treatment (Thien *et al.*, 1983; T. L. Sage, pers. comm.). Although variation in endosperm and fruit development as well as the timing of fruit abscission during the reproductive phase is a common, well-documented phenomenon in angiosperms attributed to a variety of features, including plant hormonal and nutrient status (Stephenson, 1981; Sage and Webster, 1987, 1990; Korbecka *et al.*, 2002), such factors are also indicative of early-acting inbreeding depression (Charlesworth and Charlesworth, 1987; Husband and Schemske, 1996). Alternatively, the present study on *I. floridanum* notes that, 30 d after receiving cross- and self-pollen, seeds from both pollination treatments contained embryos that had not yet proceeded beyond the zygotic stage of development. Post-zygotic OSI following fertilization whereby the zygote fails to divide but endosperm development proceeds has been documented to occur in the relictual species, *Pseudowintera axillaris* (Sage and Sampson, 2003) and phylogenetically younger species of *Asclepias* (Sparrow and Pearson, 1948; Sage and Williams, 1991). Hence, in the absence of differential embryo development following cross- and self-pollination beyond the zygote stage of ontogeny, developmental data from the present study cannot entirely eliminate the possibility of some form of OSI. Long-term studies comparing seed development following the application of cross- and self-pollen will be required to determine whether early-acting inbreeding depression or post-zygotic SI is operating in *I. floridanum*.

Temporal aspects of stigma receptivity in I. floridanum

A significant finding of this study is the presence of complete dichogamy in *I. floridanum*. The timing of stigma receptivity coincided with the previously defined female phase of floral ontogeny (Thien *et al.*, 1983). Notably, the cessation of stigma receptivity is not only temporally associated with anther dehiscence and stigmatic movement, but also with changes in composition of esterified/unesterified pectins and arabinogalactan/arabinogalactan proteins in the extracellular matrix of the receptive region of the stigmatic epidermal cells (Koehl, 2002). While *I. floridanum* has previously been characterized as protogynous (Thien *et al.*, 1983), this report of complete dichogamy is novel for the species. These results are noteworthy because they indicate that, although a pre-zygotic SI is absent in *I. floridanum*, temporal features of stigma receptivity will prevent self-fertilization within a flower. However, successful geitonogamy likely occurs because flowers at different stages of development are present at any one time on a plant and pollinators appear to move pollen effectively between such flowers (Thien *et al.*, 1983). The extent to which geitonogamy is effected by pollinators and the subsequent effects on reproductive success and inbreeding within populations of *I. floridanum* remains to be determined.

CONCLUSIONS

The well-resolved picture of basal angiosperm relationships has been noted to provide new opportunities to examine a suite of vegetative and reproductive characters potentially present in early angiosperms through comparative work on extant basal taxa (Friis *et al.*, 2000; Endress, 2001; Feild *et al.*, 2001). When considering protogyny as a breeding system character trait, this feature is widespread within basal angiosperms (Bernhardt and Thien, 1987; Ervik *et al.*, 1995; Endress, 2001). And, amongst other members of core angiosperms at the base of the phylogeny, complete dichogamy at the floral level, as observed here for *I. floridanum*, occurs within Cabombaceae and Nymphaeaceae (Schneider and Jeter, 1982; Capperino and Schneider, 1985; Osborn and Schneider, 1988). The predominance of protogyny in basal angiosperms has led to the speculation that the first angiosperms displayed this temporal feature (Bernhardt and Thien, 1987), although such functional character traits have not been subject explicitly to phylogenetic analysis.

Along similar lines, SI, a phenomenon estimated to be present in over one-half of angiosperms (East, 1940; Brewbaker 1957; Arasu, 1968; Barrett, 1988) and unique to the group (de Nettancourt, 1977; although see Runions and Owens, 1998), has been posited by some to have played an important role in early angiosperm success (Whitehouse, 1950; Bell, 1995). In contrast, others have suggested that SI was not important in the early history of angiosperms noting that self-compatibility is more common than SI amongst basal extant taxa angiosperms (Weller *et al.*, 1995). Conclusions regarding the compatibility status of basal extant angiosperms are restricted due to the limited number

of studies examining cross- and self-pollen tube growth in these taxa at the microscopic level. Amongst the families at the base of the phylogeny, such studies are available only for *Trimenia moorei* (Bernhardt *et al.*, 2003), three of five species comprising the Saururaceae (Pontieri and Sage, 1997, 1999) and two species within the Winteraceae, *Drimys winteri* and *Pseudowintera axillaris* (Sage *et al.*, 1998; Sage and Sampson, 2003) whereby stigmatic SI has been demonstrated in the first two taxa and OSI in the latter. The remaining classification of the presence or absence of SI in other species are based on analysis of seed production (Prakash and Alexander, 1984; von Balthazar and Endress, 1999; Tosaki *et al.*, 2001). Significantly, self-seed set can occur in the presence of leaky SI systems (Stephenson *et al.*, 2000; Sage *et al.*, 2001), thereby leading to erroneous conclusions regarding the presence/absence of SI and necessitating the need to critically examine cross- and self-pollen tube growth as well as ovule/seed development on an individual species basis. Thus, inferences from seed-set data regarding the presence or absence of SI in basal angiosperms and its potential role in the early history of angiosperms are inconclusive. Through a critical analysis of cross- and self-pollen tube growth, the present study has confirmed the absence of a prezygotic SI in *I. floridanum*, a species once considered to exhibit SI at the stigma (Thien *et al.*, 1983).

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