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Author(s): John H. Geiger, Paul D. Pratt, and Suzanne Koptur
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Breeding System of *Ruellia succulenta* Small (Acanthaceae)

John H. Geiger,[1]* Paul D. Pratt,[1] and Suzanne Koptur[2]
[1]United States Department of Agriculture, Agricultural Research Service, Invasive Plant Research Lab, Fort Lauderdale, Florida 33314
[2]Department of Biological Sciences, Florida International University, Miami, Florida 33199

ABSTRACT This study examines the breeding system of *Ruellia succulenta* (Acanthaceae), an herbaceous perennial found in the pine rockland habitat of southern Florida. Hand pollination treatments were performed on 75 plants, 25 from each of three sites. Treatments applied to test plants included: 1) control (no manipulation), 2) anthers-removed, 3) self-pollinated, and 4) cross-pollinated. The pollination protocol investigated facultative autogamy, apomixis, and self-compatibility. Fruit set and seed number per fruit were recorded. In addition to determining breeding system, the data were used to evaluate inbreeding depression at the earliest life history stages (i.e., fruit and seed set), and to identify the mechanism of self-pollination. Results showed *R. succulenta* to be fully self-compatible and facultatively autogamous. Plants were unable to set fruit without pollen deposition, indicating the lack of apomixis. There is no evidence of inbreeding depression in fruit set or seed set for the self- vs. cross-pollinated treatments. The mechanism of autofertility appeared to be delayed self-pollination as the corolla abscised and the anthers were dragged past the persistent stigma.

INTRODUCTION A breeding system includes all facets of sex expression in a species, setting the pattern for the transmission of genes from one generation to the next among individuals within a population (Wyatt 1983). In general, self-compatibility (selfing) restricts gene flow and may lead to inbreeding, which results in reduced genetic variation within populations and increased genetic variation among populations. In contrast, outcrossing (self-incompatibility) enhances gene flow and may lead to the reduction of microhabitat divergence and genetic substructuring of populations (Hamrick and Godt 1990). The five basic types of plant mating systems are: 1) predominantly selfing, 2) predominantly outcrossing, 3) mixed mating (both selfing and outcrossing occurring), 4) partial apomixis, and 5) partial selfing of gametophytes as in ferns (Brown 1990).

The relationship between pollination and mating systems has become an area of active research (see review, Barrett and Harder 1996). Previously, many pollination studies focused on plant ecology whereas studies of mating systems emphasized theoretical consequences to population genetics (Barrett and Harder 1996). A growing body of literature, in contrast, focuses on the interaction between ecological factors of plant mating and the fitness of different mating patterns (Gregorius et al. 1987, Holsinger 1991, Lloyd 1992, Kohn and Barrett 1994). For pollination studies, experimental evidence of breeding system type is necessary to evaluate the link between pollination and seed production (Wyatt 1983, Barrett and Eckert 1990).

Herein, we investigate the breeding system of *Ruellia succulenta* Small, testing for self-compatibility vs. self-incompatibility. Data are also presented on this species' ability to self-pollinate. This report is part of a larger study evaluating the effects of habitat fragmentation on the reproduction of *R. succulenta*. Knowledge of the species' breeding system is essential in looking at habitat fragmentation effects (Spears 1987, Jennersten 1988, Lamont et al. 1993, Aizen and Feinsinger 1994, Oostermeijer et al. 1998, Morgan 1999, Cunningham 2000).

*email address: John.Geiger@ars.usda.gov
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METHODS

Study Species

The pineland petunia, *Ruellia succulenta,* is an herbaceous, long-lived perennial endemic to southern Florida (Avery and Loope 1980). The plant most commonly occurs in pine rockland (Snyder et al. 1990) but also is found in ecotones between short hydroperiod sawgrass prairies and muhly grass marshes. Plants are trailing to ascending, usually less than 50 cm tall with one to several (most often) to more than 30 (rare) stems. The showy, five-petaled, lavender (rarely white or pink) flowers have a salverform corolla and are produced in clusters in the leaf axils. Vouchers of representative individuals studied herein were deposited at the Fairchild Tropical Botanic Garden herbarium in Coral Gables, Florida.

Floral Components

We observed tagged buds on plants in the field and in the greenhouse to determine the sequence of maturation, flower opening time, and floral longevity. The following measurements were made on greenhouse plant flowers: corolla tube length, flower face diameter, stigma exsertion, nectar concentration, nectar volume, number of pollen grains per flower, and number of ovules per ovary. Floral measurements were recorded in the greenhouse from 07:00 to 10:00. The corolla tube length was measured from the point of corolla tube constriction to the nectar (Barrow and Pickard 1985). Stigma exsertion was calculated as the distance from the flower face to the lowest point of the bilobed stigma. Nectar volume was measured with calibrated microcapillary pipettes and the percent sugar (weight/weight) nectar concentration with a hand-held refractometer (Bellingham and Stanley, Kent, United Kingdom). The number of pollen grains per flower was estimated using the protocol outlined by Kearns and Inouye (1993). All anthers from a flower were removed and added to a vial of alcohol. An aliquot from this vial was added to a hemacytometer and the number of pollen grains was counted. The total number of pollen grains per flower was estimated from this sample. The number of ovules per ovary was obtained by dissecting ovaries from newly opened flowers and counting the number of ovules under a dissecting microscope.

Breeding System

Plants for the breeding system experiment were obtained from stem tip cuttings of adult plants. Stem tip cuttings (ca. 10 cm in length) were collected on 15 July 1999 from plants at three sites in Long Pine Key, Everglades National Park. The three sites were separated by several kilometers. One stem tip cutting was removed from 25 individual plants at each of the three sites and brought to the Florida International University greenhouse where they were planted in 6-celled plastic pots (15 $\times$ 10 cm) with potting mix (Pro-Mix, Quebec, Canada). No rooting compound was used. Initially, all cuttings were kept under a shade cloth covered misting table for 2 wks to maintain a high moisture level. All 75 cuttings rooted within two weeks and were transferred to 20 cm diameter plastic pots using the same potting mix. The plants were grown under ambient light in the greenhouse and were given half strength liquid fertilizer (Miracle-Gro, 30% N-15% P-30% K, Marysville, Ohio) additions on 22 October 1999 and 29 November 1999 to stimulate growth and flowering.

Flowers of *Ruellia succulenta* generally opened just after sunrise on clear days, but on cloudy/overcast days, anthesis was delayed several hours. While still in bud, the stigma was already fully extended above the plane of the anthers and so, upon anthesis, the stigma was pollen free. Anthers tended to split open longitudinally toward the center of the corolla tube ca. one hour after the petals unfurled. Pollen was rather sticky and formed clumps containing many individual pollen grains. Hand pollinations were performed from 08:00–10:00. By 13:00, most of the corollas had abscised from the base of the ovary. The abscission of the corolla resulted in the anthers being dragged past the persistent stigma atop the long style; stigma and style often remained attached to the ovary for over a week. To consider the amount of pollen deposited on the stigma in this manner, a sample of stigmas from two untreated flower groups was collected. The first group contained stigmas removed shortly before the corollas abscised and the second group contained stigmas from flowers that had already shed their corollas, referred to as ''corolla on'' and ''corolla off'' groups, respectively. Stig-

mas from both groups were mounted in fuchsin gel (Kearns and Inouye 1993), examined under a light microscope, and the number of pollen grains per stigma was counted.

The hand pollination treatments were performed on plants in the pollinator free (i.e., no pollinating insects present) Florida International University greenhouse from 7 December 1999 through 29 February 2000. The four hand pollination treatments were: no treatment (control), anthers-removed, self, and cross (Dafni 1992, Kearns and Inouye 1993). The four treatments tested for facultative autogamy, apomixis, and self-compatibility. The control procedure involved no floral manipulation. For the anthers removed treatment, a pair of tweezers was used to remove all four anthers from the flower with no additional floral manipulation. Clean, wooden toothpicks were used to collect and deposit a large quantity of self and cross pollen on the flower's stigma for the self and cross treatments, respectively. The number of pollen grains placed on the stigma greatly exceeded the number of ovules per ovary. To avoid crossing possible siblings or even same genotypes within each population, cross pollen was obtained from one of the other populations. For identification of all the treatments, a small, threaded, jeweler's tag was attached around the pedicel of each treated flower. Data on fruit set and seed set were recorded for all the hand pollinations.

Data Analysis

Logistic regression was used to investigate the influence of pollination treatments on the incidence (presence or absence) of fruit on test plants (PROC GENMOD, Littell et al. 2002). Orthogonal contrasts were used to compare fruit incidence among pollination treatments. Two way ANOVAs were used to test for differences in mean number of seeds per fruit among the four hand pollination treatments and the three sites (PROC GLM, SAS Institute 1999). To meet assumptions of normality and homogeneity of variances, seed data were transformed by taking the reciprocal (1/x) of each value (Sokal and Rohlf 1981). Analyses were conducted using Bonferroni procedure. A t-test was performed on the data for the two flower groups, ''corolla on'' and ''corolla off.'' Data are presented as means (±1 standard error).

RESULTS

Floral Components

Flowers have been described as subsessile glomerules found in the leaf axils (Wunderlin 1998). Our measurements revealed the five petaled lavender flowers to have a salverform corolla ca. 4 cm long; the tube 2.49 ± 0.29 cm long with a flower face diameter 4.79 ± 0.44 cm. The flowers are herkogamous, with the bilobed stigma exserted 3.8 ± 1.9 mm above the flower face. The filaments of the four individual stamens are adnate halfway down the corolla tube and the anthers occur just below the rim of the corolla tube. In general, flowers last one day; flowers open at sunrise and the corollas abscise in the early afternoon, so they are visited by diurnal insects only. Floral rewards are pollen and nectar that is secreted at the proximal end of the corolla tube surrounding the base of the ovary. The sugar concentration of the nectar measured on a weight per total weight basis was 19.6% ± 0.2%. The nectar volume was 1.09 ± 0.06 μl. There were no significant differences among flowers from the three sites in either percent sugar concentration or volume of nectar ($p > 0.05$). The insect pollinators of *R. succulenta* include: butterflies, skippers, bombyliid flies, wasps, honeybees, and solitary bees (J. Geiger, pers. obs.). The number of pollen grains per flower was 4,255 ± 343. The number of ovules per ovary was 10.77 ± 0.03 and there were most often an equal number of ovules in each of the two locules of the ovary. Carpels mature into glabrous capsules containing up to 13 seeds within one to three weeks after pollination (J. Geiger, pers. obs.). The fruit is explosively dehiscent, and the seeds are dispersed several meters by the aid of hook shaped structures within the capsules.

Breeding System

The incidence of fruit set per plant did not differ among sites ($\chi^2 = 2.08$ df = 2, $p = 0.353$) but was influenced by floral treatments ($\chi^2 = 169.63$ df = 3, $p < 0.0001$). The self and cross treatments resulted in proportionally similar levels of fruit set, but were significantly higher when compared to the control and anthers-removed treatments (Figure 1). Sta-

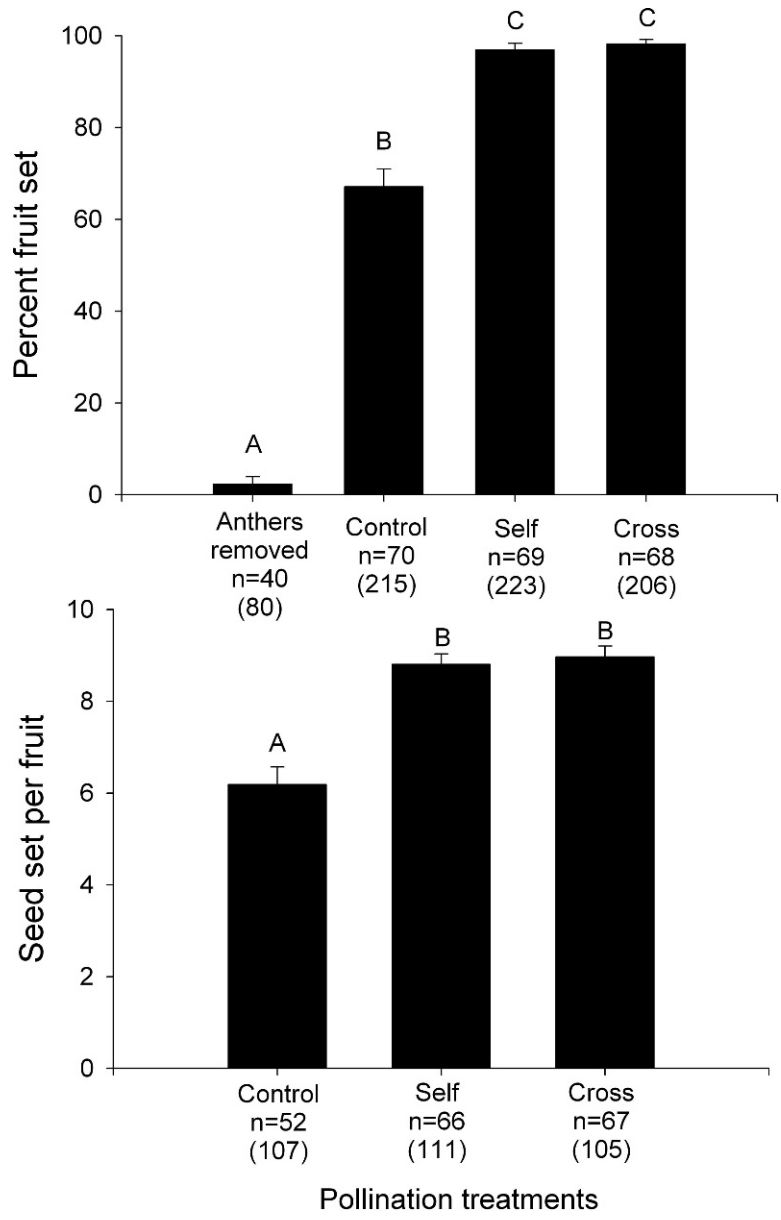

Figure 1. The influence of pollination treatments on percentage of flowers producing fruits (top) and number of seeds per fruit (bottom) following four controlled pollination treatments. Treatments with different uppercase letters are significantly different at $p < 0.05$ using the Bonferroni procedure. Error bars are standard errors of the mean; n = the total number of plants and (total number of flowers) used for each pollination treatment.

tistical testing failed to find a significant interaction between floral treatment and site on fruit set.

The anthers-removed treatment was excluded from the ANOVA testing for differences in seed set per fruit because only two of the 80 flowers with excised anthers produced fruit. Pollination treatments influenced the mean seed set per fruit ($F = 11.34$ df $= 2$, $p < 0.001$). The mean number of seeds per fruit produced from the self and cross treatments was similar although both treatments were significantly greater than the control treatment (Figure 1). Site influenced the number of seeds per fruit

($F = 3.17$ df = 2, $p = 0.044$). Seed numbers ranged from 8.6 ± 0.2 at site 1 to 7.8 ± 0.3 at site 3, with site 2 intermediate (8.0 ± 0.3 seeds per fruit). The disparity in seed set between no manipulation (control) and cross treatment was slightly greater at site 2 as compared to 1 and 3, which may explain the significant site by treatment interaction ($F = 2.56$ df = 4, $p = 0.040$).

The mean number of pollen grains was significantly greater on flowers without corollas (26.8 ± 3.6) vs. those with corollas (0.42 ± 0.35; $t = 7.48$, $p < 0.001$).

DISCUSSION All *Ruellia succulenta* plants observed were monostylous hermaphrodites, with no evidence of the distyly reported by Long and Uttal (1962) in their study of *Ruellia caroliniensis* (J.F. Gmel.) Steud., a close relative of *R. succulenta*. In addition, all plants had only chasmogamous flowers, with no cleistogamy as Long and Uttal (1962) described for *R. caroliniensis*. *R. succulenta* is self-compatible by the criteria of Bawa (1974) and highly self-compatible by those of Dafni (1992). The percent fruit set following self pollination was 97%, perhaps not surprising for a monostylous, hermaphroditic herb, as they tend to show a high degree of self compatibility (Bullock 1985). There was no significant difference in fruit set between the self and cross treatments, while both were significantly different from the control treatment. The percent fruit set of the anthers-removed treatment, even at just 2.1%, may be too high. Excluded in the analysis were a few fruit derived from the anthers-removed treatment that were performed very early during the breeding system experiment. It is impossible to determine if this fruit set was the product of inexperience in removing the anthers, which may have resulted in pollen deposition on the stigma, or actual cases of apomixis. We consider the former is the more likely explanation as beyond the first week of hand pollination there were no more instances of individuals in the anthers-removed treatment setting fruit. The percent fruit set following the control treatment, with no floral manipulation, was 67%. As these tests were performed in a pollinator free greenhouse, the results show that *R. succulenta* is facultatively autogamous, able to set fruit in the absence of pollinators. The method of pollination seems to be the deposition of self pollen as the corolla abscises and drags the adnate anthers past the stigma, i.e., delayed self pollination (Dole 1990).

Comparisons of seed set following the pollination treatments show differences between self and control and cross and control. There was no difference between self and cross; these two treatments had near identical means for number of seeds per fruit, 8.8 and 8.9, respectively. These findings further support the fruit set results that *Ruellia succulenta* is fully self-compatible. Additionally, these data provide evidence for the lack of inbreeding depression at these initial life history stages (i.e., fruit set and seed set) for the populations sampled.

Fruits from the control treatment had on average ca. 30% less seeds than from the self and cross treatments. This reduced seed set may be due to one facet of pollination intensity, namely, the relationship between the number of pollen grains deposited on stigmas and seed set. Realized seeds set to number of pollen grains deposited may be much lower than a 1:1 relationship, and several studies have documented such instances of reduced seed set due to low pollination intensity in the field (Snow 1982, McDade 1983). While this study did not explicitly explore the link between pollination intensity and seed set, the data from pollen grain number on the two flower groups does point to pollination intensity limiting seed set. For the self and cross treatments, the entire stigmatic surface was covered with pollen, most likely by hundreds of pollen grains. Data from the two flower groups show an average of less than one pollen grain per stigma for the ''corolla on'' group and an average of 27 pollen grains per stigma for the ''corolla off'' group. It appears that enough pollen is deposited on the stigma by the abscission of the corolla to effect fruit set with seed, but seed set following this delayed, autogamous self-pollination is not equivalent to seed set following the self and cross hand pollination treatments. These findings support the idea that low pollination intensity from delayed self-pollination results in reduced seed set for this species.

CONCLUSION The *Ruellia succulenta* populations studied are fully self-compatible and

show no signs of inbreeding depression in the earliest life history stages. Plants exhibited a high capacity for autofertility, and the mechanism appears to be the direct contact of the adnate anthers with the persistent stigma as the corolla abscises. This reproductive assurance mechanism may be especially important as the pine rockland habitat in Everglades National Park is known to be depauperate of insect pollinators at certain times e.g., when pinelands become overgrown due to fire exclusion (Koptur 2006), and after hurricanes (Pascarella 1998, Koptur et al. 2002). Further research could be conducted to determine the relationship between pollination intensity and seed set. Additionally, field assessments are needed to establish whether primarily selfing, primarily outcrossing, or a mixed mating system occurs for these populations in nature.

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