7-1-1987

Reproductive and life history characteristics of a North American prairie orchid Spiranthescernua cernua (Linnaeus) L.C. Richard.

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REPRODUCTIVE AND LIFE HISTORY CHARACTERISTICS OF A NORTH AMERICAN PRAIRIE ORCHID SPIRANTHES CERNUA (Linnaeus) L. C. Richard

A Thesis
Presented to the
Department of Biology
and the
Faculty of the Graduate College
University of Nebraska

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts
University of Nebraska at Omaha

by
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July, 1987

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ACKNOWLEDGMENTS

I would like to thank the University Committee on Research and the Department of Biology for their financial support of this project. Mercy Hospital of Council Bluffs also deserves thanks for paying part of my tuition.

Special thanks goes to Dr. Ann Antlfinger for her tolerance and forbearance as my academic advisor and mentor. I would also like to thank her for allowing me to use 1987 data supported by the University Committee on Research.

I am grateful to my committee members: Dr. David Sutherland, Dr. Thomas Weber and Dr. James Van Etten for their helpful comments. I wish to thank my mother and father for their help with the genetic and behavioral engineering. Finally, I would like to thank my friends and fellow students for their moral support during the rough going.

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INTRODUCTION

Reproductive systems have a profound effect on the genetic structure of plant populations. How genes are passed from one generation to the next influences the amount and structure of genetic variation in succeeding generations (Clegg, 1980; Bell, 1982). Sexual reproduction increases the genetic variation within a population through recombination while asexual reproduction duplicates the maternal genome (Williams, 1975). A variety of systems have evolved in plants which combine both sexual and asexual reproduction (Lloyd, 1984).

Asexual reproduction can occur through vegetative reproduction and/or agamospermy (Gustafsson, 1946). Except in rare cases, both means of asexual reproduction result in progeny that are genetically identical to the parent (Stebbins, 1950). More recent discussions consider vegetative reproduction to be a form of growth (Harper, 1977). Agamospermy is the formation of seeds without fertilization (Grant, 1981). In agamospermy, seeds are derived from structures of the ovule. There are two types of agamospermy, gametophytic apomixis and adventitious embryony (Stebbins, 1950; Grant, 1981). In gametophytic apomixis, seed embryos develop from an unreduced cell within an embryo sac. In adventitious embryony, embryos develop from the nucellus or integument and the embryo sac is "bypassed" (Grant, 1981). Agamospermy may be obligate, if there is a complete loss of

sexual function, or facultative, if some sexual reproduction occurs. Most if not all agamospermous plants are facultative (Clausen, 1954; Asker, 1979).

The adaptive significance of asexual reproduction is currently under intense investigation (Maynard Smith, 1978; Marshall and Brown, 1981). Agamospermy may be advantageous in the following situations: (1) partially or completely sterile populations (Darlington, 1939, 1958; Stebbins, 1950; Grant, 1981); (2) zones of interspecific hybridization (Darlington, 1932; Williams, 1975; Grant, 1981); and (3) peripheral populations where plants are isolated or under a loss or reduction in pollinators (Manning, 1981). Since agamospermy eliminates the need for male gametes, resources can be redirected into the production of additional female progeny, with a potential two fold increase in fecundity (Williams, 1975; Maynard Smith, 1971, 1978).

This study was designed to quantify the expression of a multiple reproductive strategy under different pollination regimes. It was predicted that pollen limitation would favor agamospermy and that pollination would induce a sexual response. The experimental organism was the terrestrial orchid, Spiranthes cernua (L.) L. C. Richard.

Spiranthes cernua exhibits both sexual and asexual reproduction. Asexually, it is agamospermous by means of adventitious embryony (Leavitt, 1900; Ames, 1921; Swamy, 1948; Catling, 1980; 1982). Sexual reproduction occurs by

geitonogamy and outcrossing. This multiple mating strategy allows comparison of sexually and asexually produced seeds. Previous studies of sexual versus asexual reproduction have compared genets and ramets (Harper, 1977). S. cernua is interesting because seeds of different reproductive origin can be compared. In the past, attempts to quantify agamospermy have involved microscopic examination of embryo sacs during megasporogenesis, restricting sample size (Marshall and Brown, 1974). As will be described below, differences in seed morphology were used to determine the presence of agamospermy in a large number of individuals of S. cernua.

Reproduction in Orchids

Orchidaceae is one of the largest families of angiosperms. Only Compositae rivals it in number of genera and species (Dressler, 1981; Benzing, 1986). It is the largest family of monocotyledons and is considered the most specialized in its interactions with pollinators (Darwin, 1877; van der Pijl and Dodson, 1966; Cronquist, 1981; Stebbins, 1984). Seventy-five percent of the species are epiphytes, native to the forests of the tropics and subtropics (Dressler, 1981; Benzing, 1986).

In general, orchids are outcrossing species. Autogamy is usually prevented by the rostellum which separates the anther from the stigma (Cronquist, 1981; Stort and Galdino, 1984). Many orchids are long-lived perennials with high chromosome

numbers, a situation favoring outcrossing (Dressler, 1981). Orchids attract pollinators using myriad forms of visual and olfactory deception. Pollen is bound in a coherent mass called the pollinium. Pollinia, together with the caudicles and the viscidium, form the pollinarium (van der Pijl and Dodson, 1966). The floral parts of orchids are arranged such that the pollinarium attaches to the vector, usually an insect, as it leaves the flower (Darwin, 1877; van der Pijl and Dodson, 1966). Flowers are pollinated upon entry. Though they are often self-compatible, fertilization requires the reentry of a flower by a pollinator. Therefore, self-fertilization is rare in nature (Darwin, 1877; Schemske, 1980). Pollination and fertilization can be separated by several days to several months (Swamy, 1949).

Nygren (1954) thought agamospermy was likely to occur in orchids, "because no endosperm is formed in this family, which makes double fertilization unnecessary." Asker (1979) described the condition as "common" in orchids. At that time, adventitious embryony had been documented in four of the estimated 20,000 species of orchids: Spiranthes cernua (Leavitt, 1901; Swamy, 1948;), Nigritella nigra (Afzelius, 1928, 1932), Zueuxine sulcata (Sesshagiriah, 1941; Swamy, 1946) and Zygopetalum intermedium Lodd. ex Lindl. (Suessenguth, 1923; Gustafsson, 1946). Since then it has been confirmed in Spiranthes sinensis (Pers.) Ames (Sharma and Vij, 1981), S. casei (Catling, 1982), S. magnicamporum (Catling, 1982), S.

ochrolueca (Catling, 1982) and Epidendrum nocturnum Jacq (Stort and Pavanelli, 1985). Of these, only Spiranthes is native to temperate North America, although some form of agamospermy is suspected in Aplectrum hyemale (Muhl. ex Willd.) Nutt. (Hogan, 1983).

Reproduction in Spiranthes cernua

Spiranthes cernua is pollinated by bees of the genus Bombus (Ames, 1921; Catling, 1983b; Sheviak, 1982). They are attracted to the secretion of callosities at the base of the column (Ames, 1921; Correll, 1950). S. cernua is protandrous. The lower flowers on the spike open first and receive pollen. Younger flowers, further up the spike, act as pollen donors before the stigma is exposed. The bees work up the inflorescence depositing their pollen in the lower female flowers and collecting pollen from the upper, less mature, male flowers (Cating, 1983b). The sticky viscidium of the pollinarium attaches to the bee's galea as it searches for nectar within the flower. It is uncertain whether S. cernua is self-compatible. The anther remains intact until the flower withers. Thus, self-fertilization does not involve alteration of the pollen structure (Hagerup, 1952; Mosquin, 1970; Catling, 1983a; Mehrhoff, 1983). Considering the pollinator behavior, geitonogamy and/or autogamy seem possible (Pyke, 1978). Some orchids, like Zygopetalum intermedium (Gustafsson, 1946) and

possibly Epidendrum nocturnum (Stort and Pavanelli, 1985), require pollination to effect adventitious embryony, i.e. pseudogamy. Pollination does not appear to be required in S. cernua (Leavitt, 1901).

Asexual embryos in Spiranthes cernua develop from the inner and outer integument of the ovule (Swamy, 1948; Catling, 1982). Some studies suggest that adventitious embryony begins before anthesis (Swamy, 1948; Catling, 1982). One result of adventitious embryony is that many of seeds are polyembryonic, with up to six embryos per seed (Swamy, 1948; Sheviak, 1976; 1982; Catling, 1980; 1982). Polyembryony has been used to distinguish S. cernua from related species (Leavitt, 1900; Ames, 1921; Correll, 1950; Sheviak, 1976; Catling, 1980; 1982).

Systematics of Spiranthes

Spiranthes is the type genus for the subtribe Spiranthinae. It contains approximately 42 species. Most are native to the temperate regions of North America and Eurasia (Garay, 1980). All are terrestrial. The classification of Spiranthes cernua is complicated by a high degree of polymorphism (Correll, 1950; Sheviak, 1976; Catling, 1980). What was once thought to be a single species has been divided into S. cernua, S. odorata (Nuttall) Lindley, S. orchroleuca (Rydberg) Rydberg, S. magnicamporum Sheviak (Sheviak, 1973) and S. casei Catling & Cruise (Catling and Cruise, 1974). Of

these, only S. cernua and S. magnicamporum are native to prairie. Sheviak (1982) maintains that only the polyploid (2n = 60, 61, or 45) S. cernua is agamospermous. S. cernua is considered an agamic complex and has been further subdivided into morphologically distinct variants (Sheviak, 1982) S. vernalis Engelmann & Gray, a closely related, sympatric species, is entirely sexual and is useful for comparison with S. cernua.

Life History of Spiranthes cernua

The population dynamics of Spiranthes cernua were investigated to understand the demographic consequences of agamospermy. Asexual species have been characterized as superior colonizers (Stebbins, 1950; Levin, 1975) and inferior competitors (Williams, 1975; Cuellar, 1977; Ellstrand and Antonovics, 1985). Further, it has been suggested that reproductive strategy is related to environmental disturbance (Michaels and Bazzaz, 1986). Species should exhibit a greater degree of asexual reproduction in disturbed sites, where an entire population could be descended from a single agamospermic individual (Baker, 1974). S. cernua is found in undisturbed climax prairies ranging west through much of Nebraska, Kansas and Oklahoma (Magrath, 1973; Luer, 1975; Sheviak, 1976). In this stable environment S. cernua should show increased sexual reproduction. Many asexual species have greater fecundity

(Williams, 1975) but lower survivorship (Gadgil and Bossert, 1970) than sexual species. Thus, the survivorship, growth and fecundity of an individual may be a function of its reproductive origin (Sarukhan et al, 1984).

Although members of the orchid family are considered endangered (Ayensu, 1975; Ayensu and DeFilipps 1978; Gade, 1987), Spiranthes cernua is locally abundant. S. cernua produces thousands of very small seeds. In October and November, the capsules dehisce and the seeds are dispersed by the wind. The embryos lack endosperm, cotyledons or root initials and rely on fungal infection for germination (Arditti, 1979). Dead testa cells form a net around the embryo (Arditti, 1979). Seeds must locate a "safe site" with suitable moisture, light, soil and fungal associates (Cook, 1979). Germination rates and juvenile establishment may be largely determined by the microenvironment (Harper, 1977; Schmitt and Antonovics, 1986). Unlike other species of orchids, the seeds of S. cernua can germinate immediately, becoming photosynthetic within three weeks (Stoutamire, 1974). Curtis (1937) reported that refrigerated seeds remain viable for three years. It is not known how long seeds remain viable in nature or if there is a dormant period. Once a seedling is established, the fungus may no longer be necessary for growth (Ames, 1921; Stoutamire, 1974). This is disputed by Sheviak (1976) who "found prominent mycorrhiza nearly throughout roots studied during the period of root growth". Since vegetative reproduction in S. cernua from

the prairies is extremely rare (Sheviak, 1976), each plant likely represents a genet.

The phenology of Spiranthes cernua begins in early spring with a rosette of one to three leaves. This occurs before the growth of surrounding vegetation. The leaves grow and persist until an inflorescence appears in the fall. Each spiralling inflorescence may have as many as 60 flowers (Williams and Williams, 1983). The period between germination and sexual maturity has been estimated at two (Ames, 1921) to three years (Stoutamire, 1974). Seedlings are most often observed near flowering plants (Ames, 1921). It is likely that the seedling population size is greater than the adult population (Cook, 1979). There is no method for determining the exact age of individuals of S. cernua, except by studying cohorts. Ames (1921) believed the number of roots was related to a plant's age, a single root representing a yearling.

Very little quantitative information is available on the survivorship and fecundity of Spiranthes cernua. Environmental factors, such as soil moisture, temperature and shading may account for some of the within-population or between-year variation in demographic parameters (Ames, 1921; Sheviak, 1974; 1976; Catling, 1980). The age structure of populations of S. cernua has not been studied. Other terrestrial orchid species, including the related Spiranthes spiralis (L.) Chevall, have life spans of 20 to 30 years (Wells, 1967; Tamm, 1972). Fire stimulates flower production in many terrestrial orchids

(Stoutamire, 1974; Bernhardt and Balogh, 1986) and this may be true of S. cernua (Sheviak, 1982). The effects of predation and competition on fecundity and survivorship are also unknown. Many questions about the life history of S. cernua remain to be addressed. Such demographic data are essential to preserving a species such as S. cernua, which exists in small numbers (Wells, 1981).

MATERIALS AND METHODS

Description of the Study Site

The study site was located at Nine Mile Prairie approximately 14.5 km. northeast of Lincoln, NE, Lancaster County (lat. 40° 52' long. 96° 46'). This unplowed tallgrass prairie has been studied by many investigators, including Clements (1924; 1929) and Weaver (1954). It supports a diverse flora of approximately 350 species dominated by Andropogon gerardii Vitm. and A. scoparius Michx. (Steiger, 1930; Harrison and Bolick, 1979). A population of Spiranthes cernua was found in an upland area (Appendix A). Flowering individuals were flagged and numbered with all-weather plastic labels.

The topsoil forms a deep, even layer of finely textured silty and loamy soils (Barnes, 1984). Drainage varies from moderately well to poor. Average precipitation is 68.38 cm per year. The mean annual temperature is 10.28° C. Temperature and precipitation were measured at Lincoln, NE, (WSO AP) within 2 km of the study site (NOAA, 1985). The average elevation is 360 m.

Pollination Experiments

Pollination treatments. Seven pollination experiments were designed to test specific hypotheses regarding reproductive strategy (Table 1). In treatment 1, plants were bagged before anthesis. Under these conditions, seeds could develop by agamospermy and/or autogamy. Treatment 2 prevented self-fertilization. Since all flowers on a spike were emasculated, sexual reproduction was possible by outcrossing alone. Agamospermy could also occur. In treatment 3, plants were bagged and emasculated before anthesis. Seeds could develop only by agamospermy. In treatment 4, plants were bagged before anthesis. When the flowers opened, they were self-pollinated. Seed production was the result of selfing and/or agamospermy. Treatment 5 was similar to treatment 4 except flowers were pollinated with pollen from different flowers on the same plant. In this case, reproduction resulted from geitonogamy and/or agamospermy. In treatment 6, plants were bagged before anthesis and emasculated. Later, the flowers were outcrossed. Seeds could be produced by outcrossing and/or agamospermy. The plants in treatment 7 were not manipulated, representing the natural condition. Reproduction could occur by autogamy, geitonogamy, xenogamy and/or agamospermy.

Pollinators were excluded with nylon mesh bags (1mm), supported on a wire frame (Whigham and McWethy, 1980; Hogan,

Table 1. Description of pollination manipulations conducted on *Spiranthes cernua* in 1985.

Treatment	Number of Plants Scored[1]
1 Autogamy (bagged)	15
2 Outcrossing (emasculated)	19
3 Agamospermy (bagged, emasculated)	8
4 Self-compatibility (bagged, self-pollinated)	16
5 Geitonogamy (bagged, cross pollinated with a different flower on the same plant)	16
6 Xenogamy (bagged, cross pollinated with a different plant)	14
7 Unmanipulated	19

[1] Five plants from each treatment were scored. Ten capsules from each plant were counted.

1983; Cole and Firmage, 1984). The spike was firmly tied to the wire support before anthesis. The bags were large enough for the flowers to grow and mature. A 2.5X binocular magnifying lens (Optivisor, Donnegan Optical Co. Lenexa, KS) was used for pollination manipulations. Flowers were emasculated by depressing the labellum with forceps, exposing the rostellum. Intact pollinaria were removed in an upward motion away from the rostellum. This prevented contact with the stigmatic surface. The technique was also used on unopened flowers. In crosses, an entire pollinarium was deposited on the sticky surface of the stigma and held there by repositioning the labellum under the rostellum. The manipulations were completed over a nine day period. At least 20 flowers were manipulated on each inflorescence. If a treatment was unsuccessful, the flower was removed. Some plants were too mature to manipulate, especially the bagged, emasculated flowers which were the last to be treated. If this occurred, new individuals were located and randomly assigned to the treatment.

Inflorescences were collected before dehiscence in early October. At least 10 capsules were separated from the inflorescence and stored at 4° C. Extra capsules were refrigerated for seed germination experiments.

Seed morphology. Initial observations of seeds resulted in six seed types (Table 2). Polyembryonic, extruded and unknown seed categories represent seeds of asexual origin (Swamy, 1948;

Table 2. Categories of seeds of Spiranthes cernua.

Seed Type	Description
Monoembryonic	Seeds with a single, well defined oval or circular embryo.
Polyembryonic	Seeds with two or more distinct embryos.
Unknown	Seeds with embryos of indefinite shape and or number.
Extruded	Seeds with one or more embryos protruding from the seed coat.
Free	Free embryos without a seed coat.
Empty	Seed coats with no apparent embryo.

Sheviak, 1982; Catling, 1982). Monoembryonic seeds can develop either sexually or asexually. The origin of free and empty seeds could not be determined.

Slide preparation. Seeds were removed from the capsule and stained in concentrated phyloxine B (1 ml) for 48 hours (See Appendix B for reagent concentrations). The stain was removed with a pipet. The seeds were washed twice in 50% ETOH (2 ml). Most of the alcohol was decanted. Since the number of seeds per capsule varied, two to eight drops of the solution were placed on a slide and allowed to air dry. Melted glycerol jelly (4 drops) was placed on the dry seeds. The seeds were stirred into the mounting media until an even distribution was achieved. The slides could be read after cooling. Seed embryos stained red. The slides were read using Hoffman optics (100X) in a Z pattern beginning in the upper left corner of the slide.

Sampling. Five plants, from each of the seven pollination treatments, were selected at random, for a total of 35 plants. Ten capsules from each plant were assigned a random number and prepared in numerical order. The treatment was unknown to the observer. An average of 150 seeds were classified per slide. Only one capsule lacked seeds. A total of 349 slides were read. The date the slide was scored was recorded. The size, color and condition were noted for 155 capsules.

Seeds of Spiranthes cernua were collected in 1985 and 1986. The 1985 S. cernua sample (i.e. collected in 1985, but

scored in 1986) contained 5 capsules from 7 unmanipulated plants. These data were added to the 50 slides in the unmanipulated treatment scored in 1985. The 1986 S. cernua sample (i.e. collected and scored in 1986) included 5 capsules from each of 10 unmanipulated plants. These plants were used to test for the possible effect of capsule size and position on seed type. The sexual species, S. vernalis, was sampled in 1986. Five capsules were collected from 10 unmanipulated individuals. Slides were prepared as described above.

Data analysis. A Chi-square contingency analysis was used to detect differences among the seven treatments. Two seed types were considered: (1) monoembryonic, and (2) agamospermic, which included polyembryonic, extruded and unknown seed categories. Since the empty and free embryo categories were of unknown origin, they were not included in the analysis. The data were subdivided for hypothesis testing using contingency analysis. Hypotheses were tested by comparing the obligate agamospermic condition (treatment 3) with the other pollination treatments. Further, differences between the two selfing treatments (autogamy and geitonogamy) and the two outcrossing treatments (artificial and natural) were tested.

A Chi-square contingency analysis was used to test: (1) yearly differences in seed morphology of unmanipulated plants; (2) differences in slides of unmanipulated plants prepared and read in different years; and (3) differences in seed type between Spiranthes cernua and S. vernalis. All 2X2 contingency

Chi-square statistics were corrected for continuity (Zar, 1984).

The frequencies of each seed type were converted to percentages. The percentages were transformed with an arcsine transformation to improve normality. Treatment differences were examined with an ANOVA. A nested factor, plant within pollination treatment, was included to test for heterogeneity among capsules within plants. The date a slide was scored was a significant source of variation. This was not surprising since there were 56 reading dates. The condition of the capsule, recorded for 155 slides, was used as a covariate. In the analysis of covariance, the reading date was not a significant source of variation. Apparently capsule condition varied among reading dates and accounted for differences among reading dates. The reading date was not included as a source of variation in the final model. A VAX version of SAS (1985) was used for the data analysis.

Pollen Viability

Successful pollination treatments require viable pollen. Therefore, pollinaria, collected from September 16-18, 1985, were tested for viability. The pollen was refrigerated at 4^{o} C in microfuge tubes. Pollen stainability was determined with cotton blue in lacto-phenol (Radford et al, 1974). A pollinium was squashed under a coverslip in an even layer and 3 drops of

cotton blue in lacto-phenol (Appendix B) were added to the slide. Pollen tetrads that stained dark blue were considered positively stained. As an additional test of viability, pollen was germinated in a 0.5% sucrose solution. Preliminary experiments showed this concentration to be optimal (Appendix D). A pollinium was macerated in a depression slide, covered with solution and sealed with vespar. Slides were read after 2 and 24 hours of growth. Thirty pollinaria, two from each of 15 different plants, were followed. From each pollinarium, one pollinium was stained and the other was used in the germination experiment.

Seed Germination

Several methods of seed germination have been described for orchids (Arditti et al, 1981; Arditti, 1982, Oliva and Arditti, 1984). The following procedure represents a modification of these methods (Appendix E). Green capsules were removed from the inflorescence. The capsules were sterilized in freshly prepared 1/4 strength calcium hypochlorite for ten minutes in a laminar flow hood. They were then rinsed twice with distilled water. Capsules were dissected and the immature seeds were dropped into 250 ml Ehrlenmeyer flasks containing nutrient agar. Each flask contained the seeds of a single capsule. Four nutrient media were tested: (1) Orchid Agar (Bacto Orchid Agar #0242-02, Difco Labs. Detroit, MI); (2) Curtis solution 5

(Curtis, 1936; Arditti, 1982); (3) Knudson solution C (Knudson, 1946; Stoutamire, 1974) and (4) Modified Curtis Full strength (Arditti et al, 1981). Seeds were taken from three individuals of Spiranthes cernua and one Spiranthes vernalis. Two replicates of each combination of seed source and nutrient media were placed in an incubator. The temperature was maintained at a constant 23.5° C. The light intensity was 16 uEin/m^2/s (PAR) for 12 hours a day. Light is required for germination (Henrich et al, 1981)

Population Dynamics

Population flux. The population of Spiranthes cernua was censused in 1985, 1986 and 1987. The 1987 data were provided by Antlfinger (unpublished data). In August, 1985, 121 flowering plants were permanently marked. The survival of these individuals was followed for two years. Leaf (N=117, including newly marked plants) and flower herbivory (N=51) were documented beginning in 1986. The reproductive fate of each individual was recorded in 1986, including inflorescence, flower and capsule production. Linear regression was used to determine the relationship between flower number and total capsule weight (N=10).

Plant growth. Vegetative and reproductive growth of each plant was recorded during the 1986 growing season. Morphological characteristics, including leaf number, plant

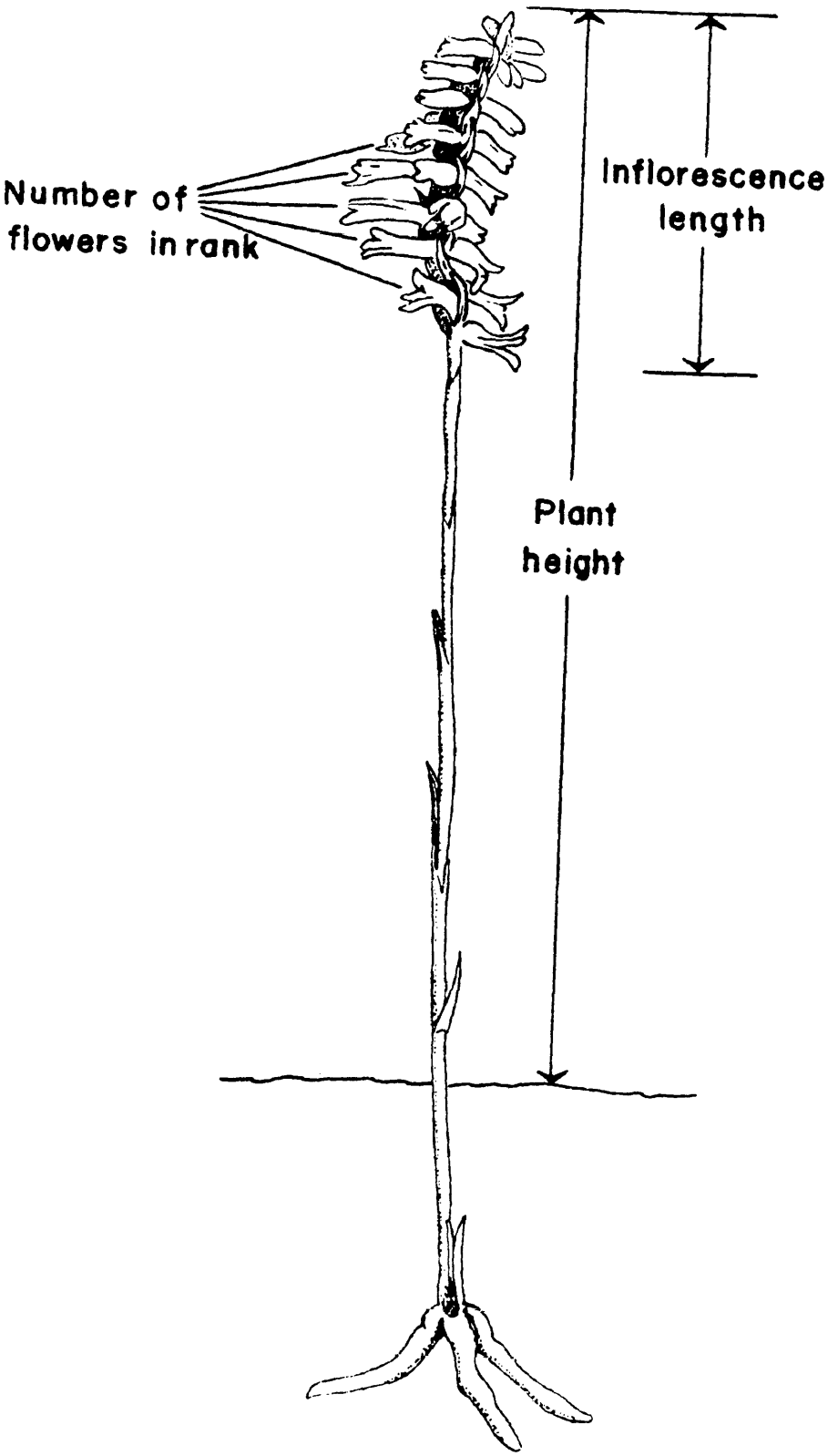
height, inflorescence length, number of flowers per rank and the direction of floral rotation (Figure 1) were measured at two to three week intervals through October. Similar traits were included in a study of Spiranthes spiralis (Wells, 1967). Leaves were numbered on a subset of the population (N=65), as they expanded. The length and width of these leaves were measured. Leaf production was recorded for the entire population. Leaves, from plants not in the census, were sampled to determine leaf area and leaf dry weight (N=65). Leaf area was estimated by tracing a fresh leaf on graph paper (Maddox and Antonovics, 1983). Leaves were dried to constant weight at 60° C. Linear regression was used to estimate leaf area and leaf dry weight from the product of leaf length times leaf width. The relationships are given below:

(1) leaf area (cm) = 0.6225(length X width) + 0.0541
$r^2 = 0.98$ $P < .0001$

(2) leaf dry weight (g) = 0.0030(length X width) - 0.0069 $r^2 = 0.95$ $P < .0001$

Leaf number and estimated leaf area for three similar dates in 1986 and 1987 were compared using paired t-tests. The 1987 data were provided by Antlfinger (unpublished data). Linear regression was also used to determine the relationship between measures of fecundity and plant size.

Figure 1. Non-destructive measurements of morphological characters of Spiranthes cernua.


Number of
flowers in rank
Inflorescence
length
Plant
height

Resource allocation. Whole plants were collected from Nine Mile Prairie to determine biomass (N=19). Capsules were numbered and weighed, with number 1 being the lowest capsule on the inflorescence. Roots and inflorescences were separated and dried to a constant weight. Shoot weights could not be determined because of the fugacious habit of the plant.

RESULTS

Seed morphology. Significant differences were found between pollination treatment and seed type (Table 3). The proportion of monoembryonic seeds ranged from 10.72% in treatment 4 (a test of self-compatibility) to 13.70% in treatment 6 (outcrossing). The detection of such small differences among treatments was due to the large sample sizes. These results were confirmed in the ANOVA of transformed data (Table 4). Further, there were significant differences among plants within treatments. Seed type was not correlated with capsule weight ($r = 0.017$, $P = 0.91$) or position of the flower on the inflorescence ($r = .108$, $P = 0.45$).

The data were partitioned to test specific hypotheses concerning reproduction (Table 5). Treatment 3, obligate agamospermy, was compared to the other pollination treatments. The proportion of mono and polyembryonic seeds in treatments 1, 2 and 5 (tests for autogamy, outcrossing in nature and geitonogamy, respectively) were statistically identical to obligate agamospermy, i.e. the pollination treatment had no effect. The unmanipulated plants (treatment 7) were also not significantly different from treatment 3, suggesting a high incidence of agamospermic reproduction within the natural population. Plants that were selfed had significantly fewer monoembryonic seeds than plants in treatment 3. There may be a

Table 3. Contingency table analysis of pollination treatment differences.

Treatment	Seed Categories	
	Monoembryonic	Agamospermic (Polyembryonic + Extruded + Unknown)
1 Autogamy	886 (12.58%)	6159 (87.42%)
2 Outcrossing	825 (11.95%)	6076 (88.05%)
3 Agamospermy	800 (11.97%)	5881 (88.03%)
4 Self-compatibility	692 (10.72%)	5765 (89.28%)
5 Geitonogamy	807 (12.91%)	5444 (87.09%)
6 Xenogamy	747 (13.70%)	4705 (86.30%)
7 Control	732 (11.92%)	5411 (88.08%)

H_o: Seed type is independent of treatment. (7 X 2)

$X^2_{calc,\ 6} = 29.72^{***}$

* P < 0.05, ** P < 0.01, *** P < 0.001.

Table 4. Analysis of variance of the frequency of monoembryonic seeds. The data were transformed prior to analysis.

Source	df	Sum of Square	F	P
Model	34	0.690	3.86	0.0001
Treatment	6	0.069	2.20	0.0432
Plant(Treatment)	28	0.620	4.22	0.0001
Error	314	1.650		
Total	348	2.340		

Table 5. Contingency analysis of association between seed morphology and pollination treatments.

1. H_o: Seed type is independent of emasculation.
(bagged, emasculated vs. bagged)

$$X^2_{calc,\ 1} = 1.098\ NS$$

2. H_o: Seed type is independent of pollinator exclusion.
(bagged, emasculated vs. unbagged, emasculated)

$$X^2_{calc,\ 1} = >0.001\ NS$$

3. H_o: Seed type is independent of selfing.
(bagged, emasculated vs. bagged, selfed)

$$X^2_{calc,\ 1} = 4.989\ ^{*}$$

4. H_o: Seed type is independent of geitonogamy
(bagged, emasculated vs. bagged, geitonogamy)

$$X^2_{calc,\ 1} = 2.513\ NS$$

5. H_o: Seed type is independent of outcrossing.
(bagged, emasculated vs. outcrossed)

$$X^2_{calc,\ 1} = 7.896\ ^{**}$$

6. H_o: Seed type is independent of manipulation.
(bagged, emasculated vs. unmanipulated)

$$X^2_{calc,\ 1} = .006\ NS$$

7. H_o: Seed type is independent of selfing mechanism.
(bagged, selfed vs. bagged, geitonogamy)

$$X^2_{calc,\ 1} = 14.291\ ^{***}$$

8. H_o: Seed type is independent of outcrossing mechanism.
(emasculated vs. outcrossed)

$$X^2_{calc,\ 1} = 8.209\ ^{**}$$

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

self-incompatibility mechanism which reduces sexual reproduction by self-fertilization. Outcrossing should increase the amount of sexual reproduction. As would be expected, outcrossed plants produced the largest proportion of monoembryonic seeds.

Sexual reproduction in Spiranthes cernua was examined in hypotheses 7 and 8. The type of self-fertilization (autogamy or geitonogamy) had a significant effect on seed type. Autogamy produced a smaller proportion of monoembryonic seeds than geitonogamy. As previously stated, the geitonogamy treatment was essentially agamospermic. Plants which outcrossed naturally produced fewer monoembryonic seeds than plants crossed by hand. This result might occur if pollinators were limited, or if the artificial crosses were more effective in pollination.

There were significant between year differences in unmanipulated plants ($X^2 = 419.534$, $P < .001$). The mean percent monoembryony varied from 13.2% in 1985 (N=85) to 26.1% in 1986 (N=50). Significant differences were also found in seeds collected in 1985 and scored in 1985 and 1986 ($X^2 = 21.825$, $P < .001$). Of the seeds scored in 1985, 11.9% were monoembryonic compared to 15.1% scored in 1986. These results may reflect differences among plants within the unmanipulated condition and/or observer differences between years.

Seed morphology readily distinguished seeds from Spiranthes cernua and Spiranthes vernalis, a sexually reproducing species

($X = 4000.247$, $P < .001$). Most of the seeds of S. vernalis were monoembryonic (87.4%). Of 4905 seeds counted only three were polyembryonic. The remaining seeds had single embryos with irregular shapes or positions within the testa. Many empty seeds were observed in the slides of S. vernalis. As above, empty seeds were not included in the analysis.

Pollen viability. Pollen from all fifteen plants tested stained positive (Table 6). These data suggest that the pollen used in the crosses was viable. However, only half of the pollinia produced pollen tubes (Table 6). Pollen stainability and germination often gave different results for the same pollinarium. It is possible that pollen tube growth was reduced because the pollinia had been refrigerated for several months.

Seed germination. Successful germination was observed in 16 of 36 cultures (44%). While germination frequency could not be determined, it appeared that germination was high. The unsuccessful cultures had bacterial and/or fungal contamination. This was likely due to partially dehisced capsules becoming contaminated before external sterilization. All of the Spiranthes vernalis cultures were contaminated. The greatest and most rapid growth was observed on Curtis' modified media. The seeds began to germinate at eight weeks. Seedlings were transferred as the media became dehydrated. The largest plants were 1 cm tall at seven months. Some seedlings were lost because of exposure to high light intensity during the

Table 6. Pollen stainability and germination of Spiranthes cernua.

Plant number	Pollinium	Viability (Stainability)	Pollen tubes per slide[2]
6	1	+	18
	2	+	16
13	1	+	8
	2	+	0
20	1	+	97
	2	+	77
27	1	+	33
	2	+[1]	52
34	1	+	0
	2	+	0
41	1	+	0
	2	+	0
48	1	+	0
	2	+	0
55	1	+	0
	2	+	0
62	1	+	66
	2	+	52
76	1	+	0
	2	+	0
83	1	+	65
	2	+	42
90	1	+	34
	2	+	11
97	1	+	0
	2	+	0
111	1	+	17
	2	+	32
118	1	+	0
	2	+	0

[1] Four tetrads from this slide did not stain.

[2] Pollen was germinated in a 0.5% sucrose solution. Slides were read 24 hours later. The pollen was four months old.

first month of growth.

Population flux. The survivorship of Spiranthes cernua was over 95% for the three years of the study (Table 7). A significant number of plants were "lost" in the first year when their tags could not be located. "Absent" plants did not produce leaves. Absence does not necessarily denote mortality since terrestrial orchids are known to reappear after surviving several years below ground (Tamm, 1972; Wells, 1967; Bowles, 1983). One source of mortality may be leaf herbivory. Leaf damage ranged from zero to complete defoliation (Figure 2). Most plants showed little damage (Mean = 0.23).

Inflorescence production decreased in 1986 (Table 7). However, more than half of the plants that flowered in 1985 produced flowers in 1986. Of these, 55% were predated before anthesis. Other flowers were lost to unknown causes. Surviving flowers produced capsules at a very high rate (92%). The mean values of reproductive structures, plant height, inflorescence length and flower number, were similar in 1985 and 1986 (Table 8).

There were significant positive correlations between years for inflorescence length and number of flowers (Figure 3). The correlation for plant height was also positive but not significant. Plants which produced the most flowers had the highest total capsule weight (i.e. there was a significant relationship between flower number and capsule weight per plant, N=10, r = 0.92). Flowers at the bottom of the

Table 7. Population dynamics of Spiranthes cernua, 1985-1987. The 1987 data were courtesy of Antlfinger (unpublished data).

	1985	1986	1987
Total number of marked plants	121	97	89
Number of lost plants		24	28
Number of plants with inflorescences	121	51	
Number of vegetative plants		45	
Number of absent plants		1	4

Figure 2. Frequency distribution of leaf predation of Spiranthes cernua in 1986. Percentage leaf predation was the number of leaves predated per plant/number of leaves per plant. Plants in the 5% class had no damage.

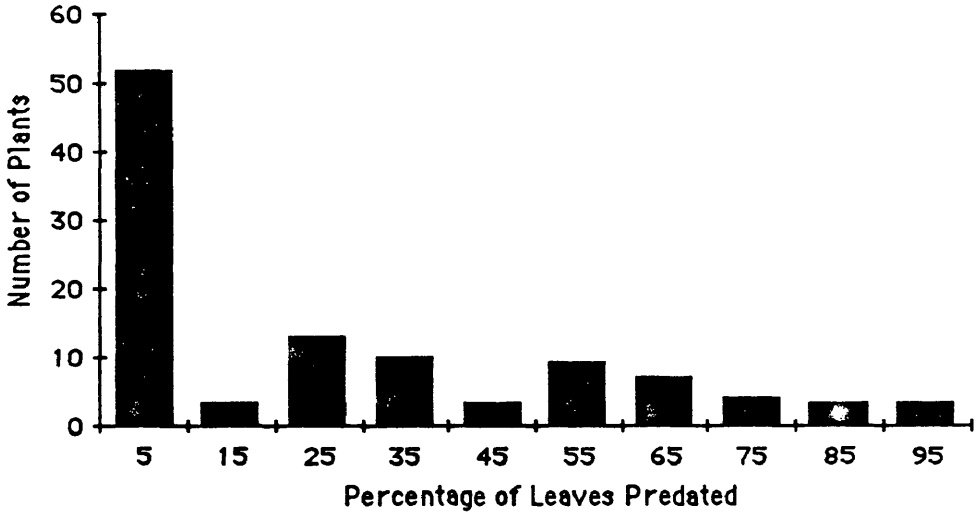

60
50
40
30
20
10
0
Number of Plants
5
15
25
35
45
55
65
75
85
95
Percentage of Leaves Predated

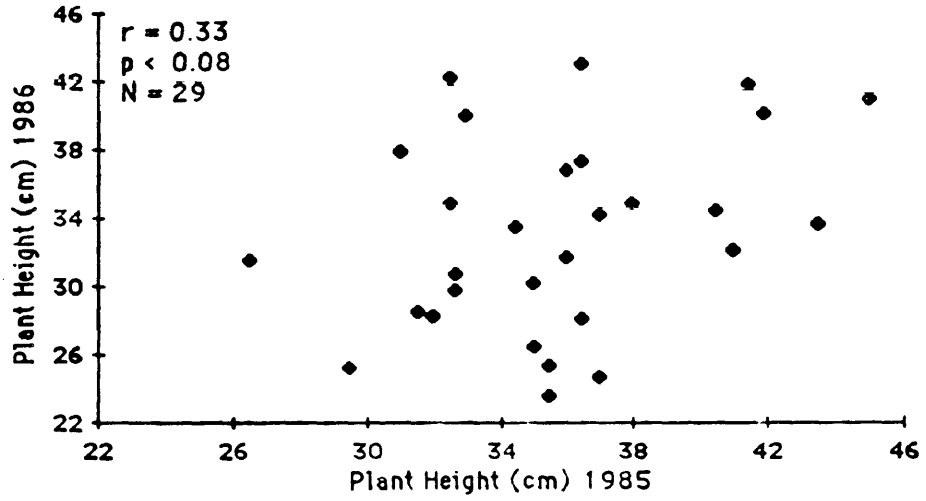
Table 8. Yearly variation in reproductive morphological characters in Spiranthes cernua. Values represent the mean ± SE.

Character	1985 (N=92)	1986 (N=32)
Plant Height	34.25 ± 0.52	32.50 ± 0.97
Inflorescence Length	8.94 ± 0.31	7.92 ± 0.43
Flower Number	31.99 ± 1.02	32.44 ± 1.66

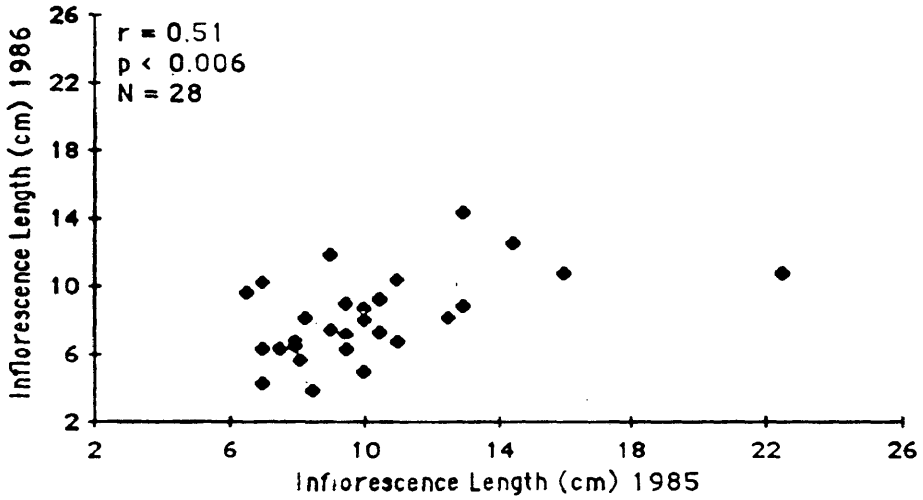
Difference between means was not statistically significant at the .0001 level using t-tests.

Figure 3. Correlation between years for three reproductive morphological characters, (A) plant height, (B) inflorescence length and (C) flower number.

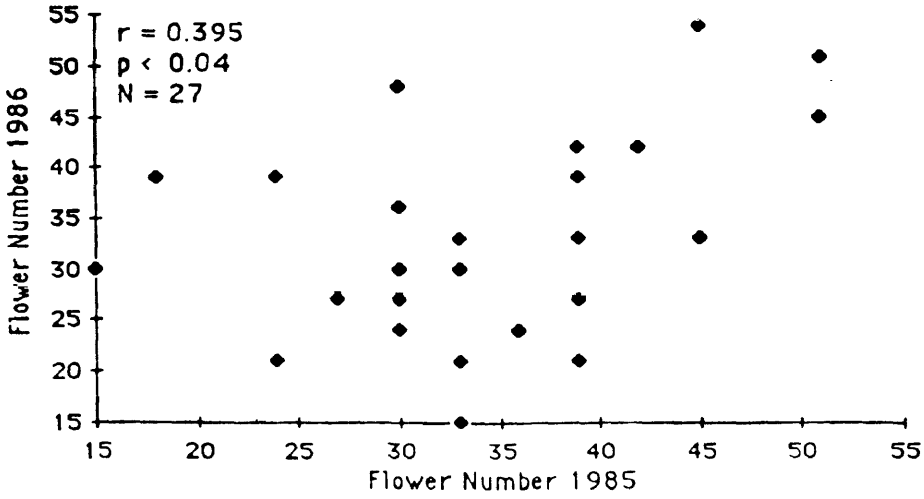
A


r = 0.33
p < 0.08
N = 29
Plant Height (cm) 1986
Plant Height (cm) 1985
46
42
38
34
30
26
22
22
26
30
34
38
42
46

B


r = 0.51
p < 0.006
N = 28
Inflorescence Length (cm) 1986
Inflorescence Length (cm) 1985
26
22
18
14
10
6
2
2
6
10
14
18
22
26

C


r = 0.395
p < 0.04
N = 27
Flower Number 1986
Flower Number 1985
55
50
45
40
35
30
25
20
15
15
20
25
30
35
40
45
50
55

inflorescence produced the largest capsules by weight (Table 9). Flower position accounted for approximately 50 to 95% of the variation in capsule weight. The slopes for each plant were significantly different from zero.

Phenology. Spiranthes cernua overwinters as a rosette of 1-3 leaves. Vegetative growth begins in early spring. Plant leaf area increased as new leaves were produced and reached a maximum in early June (Figure 4). Few or no leaves were present when the inflorescence appeared in late August. Thus, vegetative and reproductive growth were separated in time. The loss of leaves before anthesis is typical of S. magnicamporum but is unusual in populations of S. cernua (Sheviak, 1976).

Plant size. Leaf production (N=117) varied among plants throughout the growing season reaching a maximum of six leaves (Figure 5). The winter leaves senesced as primary leaves developed. Most plants averaged 3 to 4 leaves at peak growth. The distributions became significantly skewed as leaves were lost in mid to late summer. The frequency distributions change to normal when leaf production resumed in the fall. Plants produced significantly fewer leaves and less leaf area ($P < 0.05$) in 1987 than 1986 at comparable times in the growing season. The 1987 data were provided by Antlfinger (unpublished data).

Size distributions based on leaf area are shown in Figure 6. Few plants attained a leaf area greater than 65 cm^2. The distribution of leaf area became significantly skewed as the

Table 9. Regression of capsule weight versus flower position in Spiranthes cernua.

Plant Number	# of capsules	slope	P	r^2
9	20	-0.0011	0.0001	.90
11	28	-0.0005	0.0001	.50
22	25	-0.0010	0.0001	.78
25	22	-0.0009	0.0001	.89
37	34	-0.0008	0.0001	.80
64	54	-0.0006	0.0001	.75
71	31	-0.0009	0.0001	.92
73	37	-0.0007	0.0001	.78
74	28	-0.0005	0.0001	.46
78	17	-0.0016	0.0001	.81
89	30	-0.0005	0.0001	.72
91	43	-0.0007	0.0001	.85
99	31	-0.0012	0.0001	.95
111	30	-0.0005	0.0001	.93
144	30	-0.0006	0.0001	.76
150	18	-0.0016	0.0001	.85
151	30	-0.0014	0.0001	.93
152	20	-0.0008	0.0001	.67
163	21	-0.0005	0.0009	.46

Figure 4. Growth of Spiranthes cernua in 1986 and 1987. Leaf area and inflorescence height were measured biweekly during the growing season. The 1987 data were courtesy of Antlfinger (unpublished data).

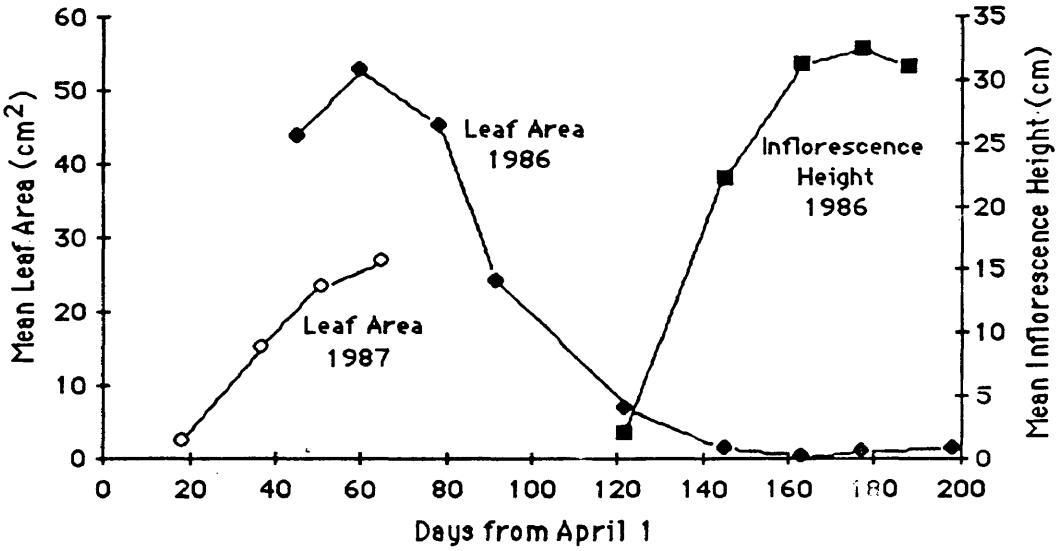
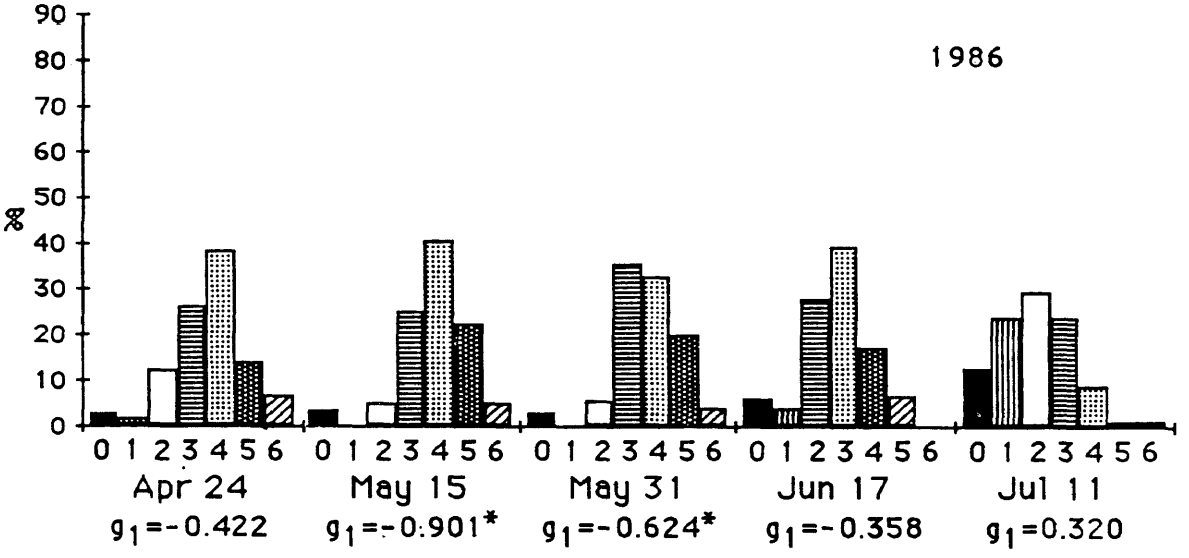
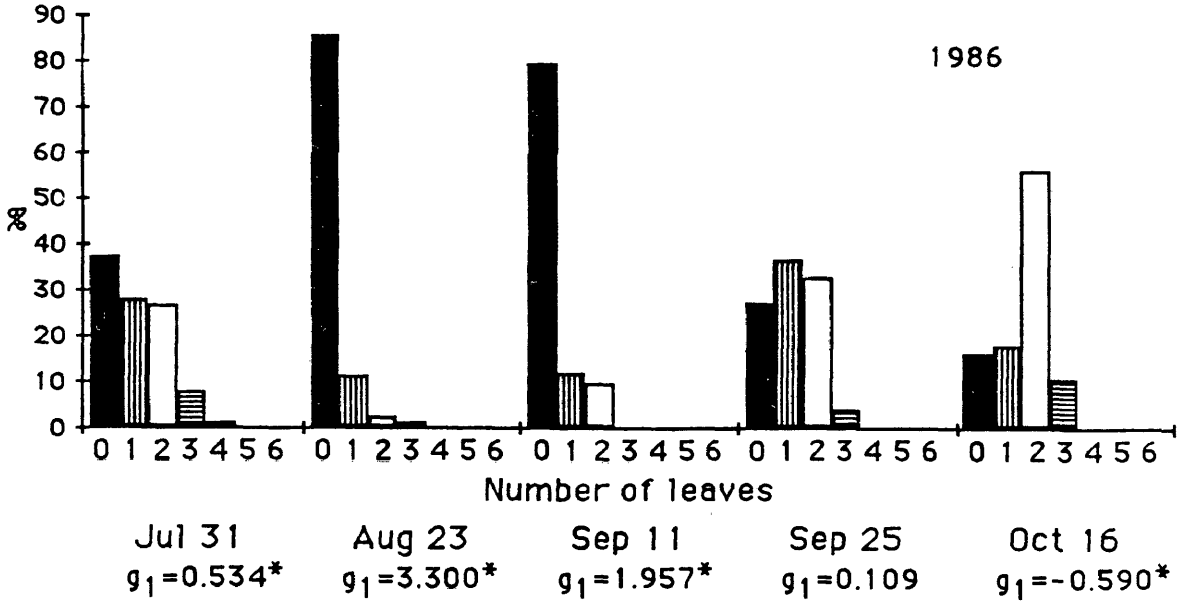
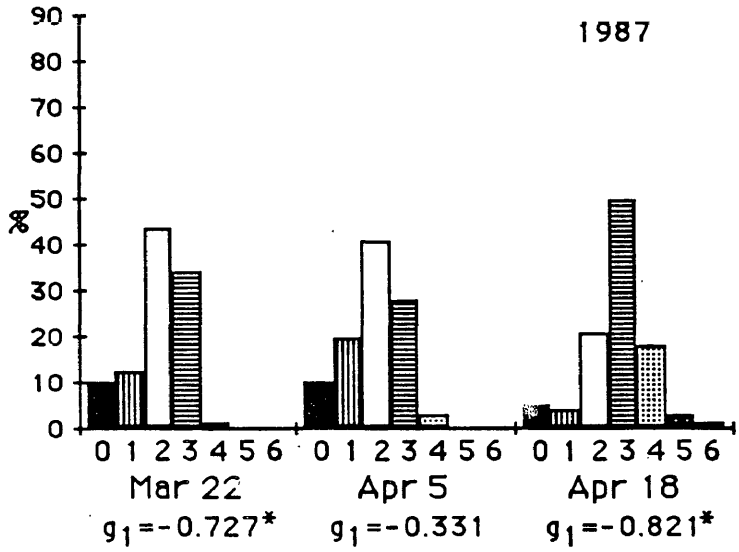

Mean Leaf Area (cm²)
Mean Inflorescence Height (cm)
Days from April 1
Leaf Area 1986
Leaf Area 1987
Inflorescence Height 1986
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
140
160
180
200

Figure 5. Frequency distributions of leaf number in Spiranthes cernua for 1986 and 1987. The 1987 data were courtesy of Antlfinger (unpublished data).


1986
%
0 10 20 30 40 50 60 70 80 90
0 1 2 3 4 5 6
Apr 24
g_1=-0.422
May 15
g_1=-0.901*
May 31
g_1=-0.624*
Jun 17
g_1=-0.358
Jul 11
g_1=0.320


1986
%
0 10 20 30 40 50 60 70 80 90
0 1 2 3 4 5 6
Number of leaves
Jul 31
g_1=0.534*
Aug 23
g_1=3.300*
Sep 11
g_1=1.957*
Sep 25
g_1=0.109
Oct 16
g_1=-0.590*


1987
90
80
70
60
50
40
30
20
10
0
%
0 1 2 3 4 5 6 0 1 2 3 4 5 6 0 1 2 3 4 5 6
Mar 22
Apr 5
Apr 18
g1=-0.727*
g1=-0.331
g1=-0.821*

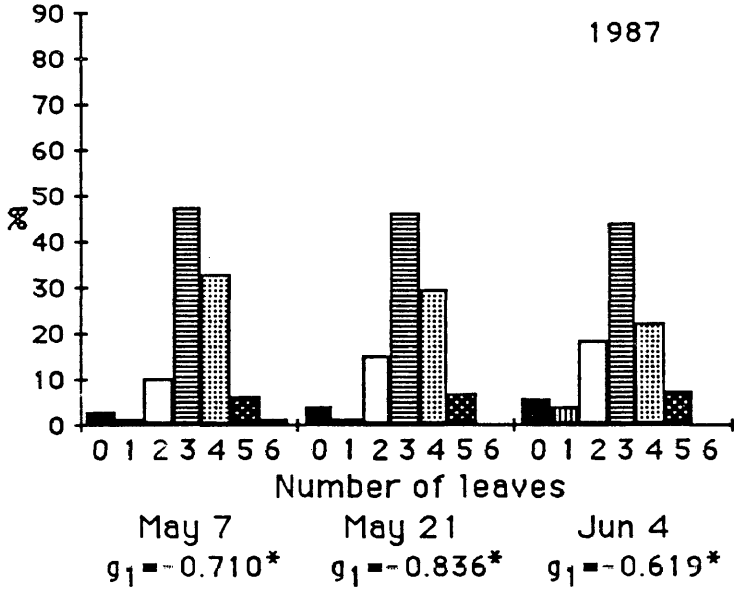
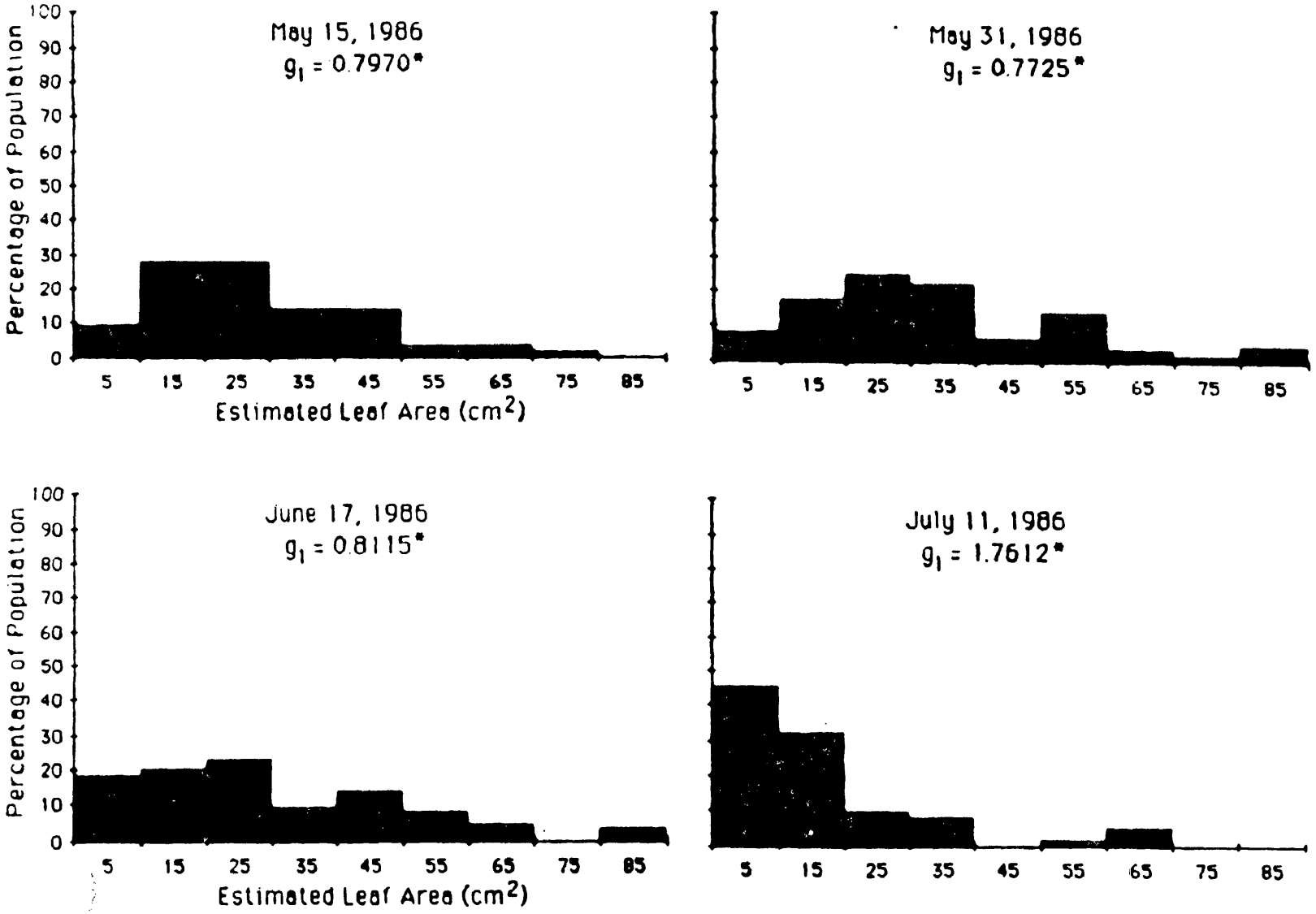
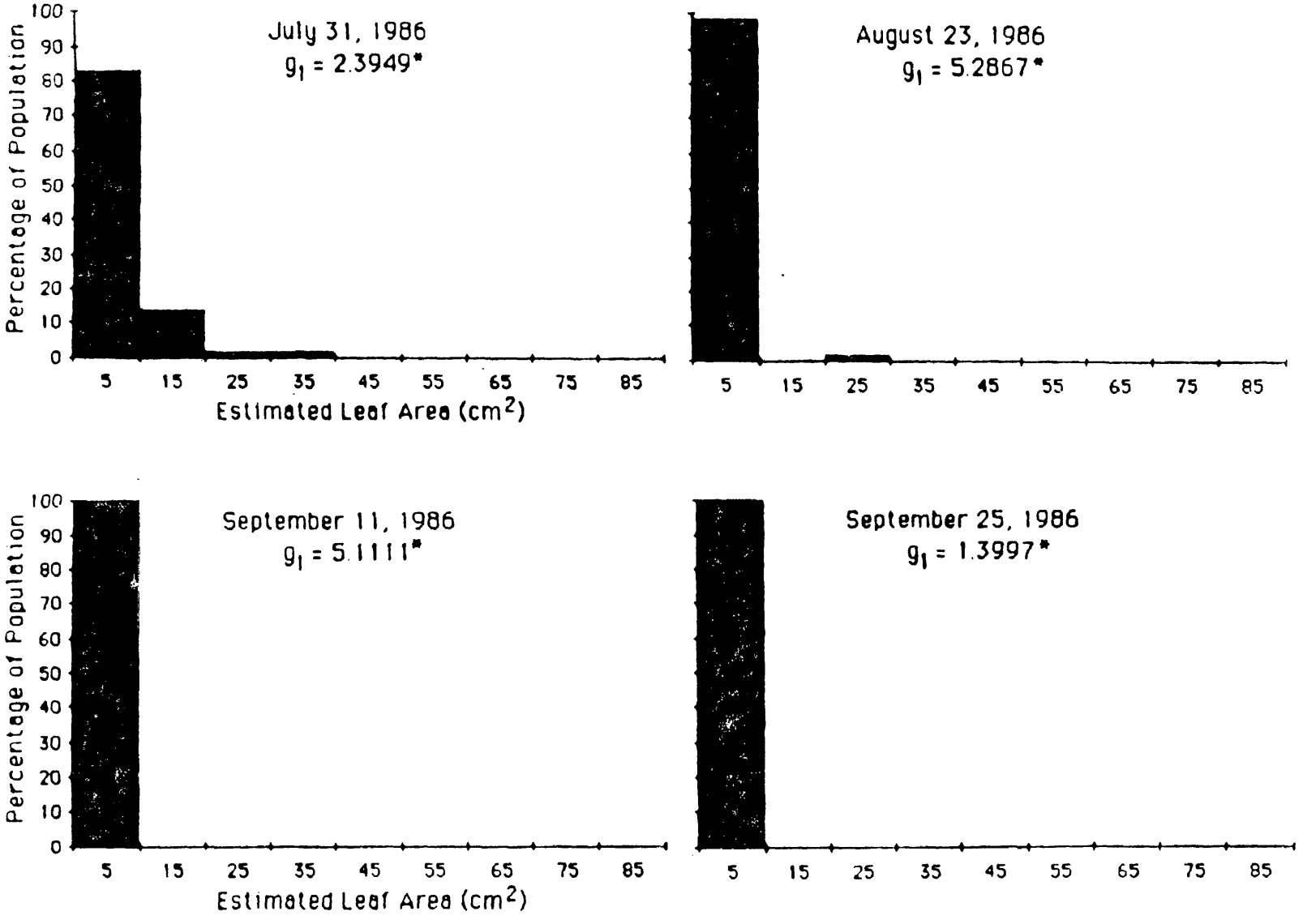
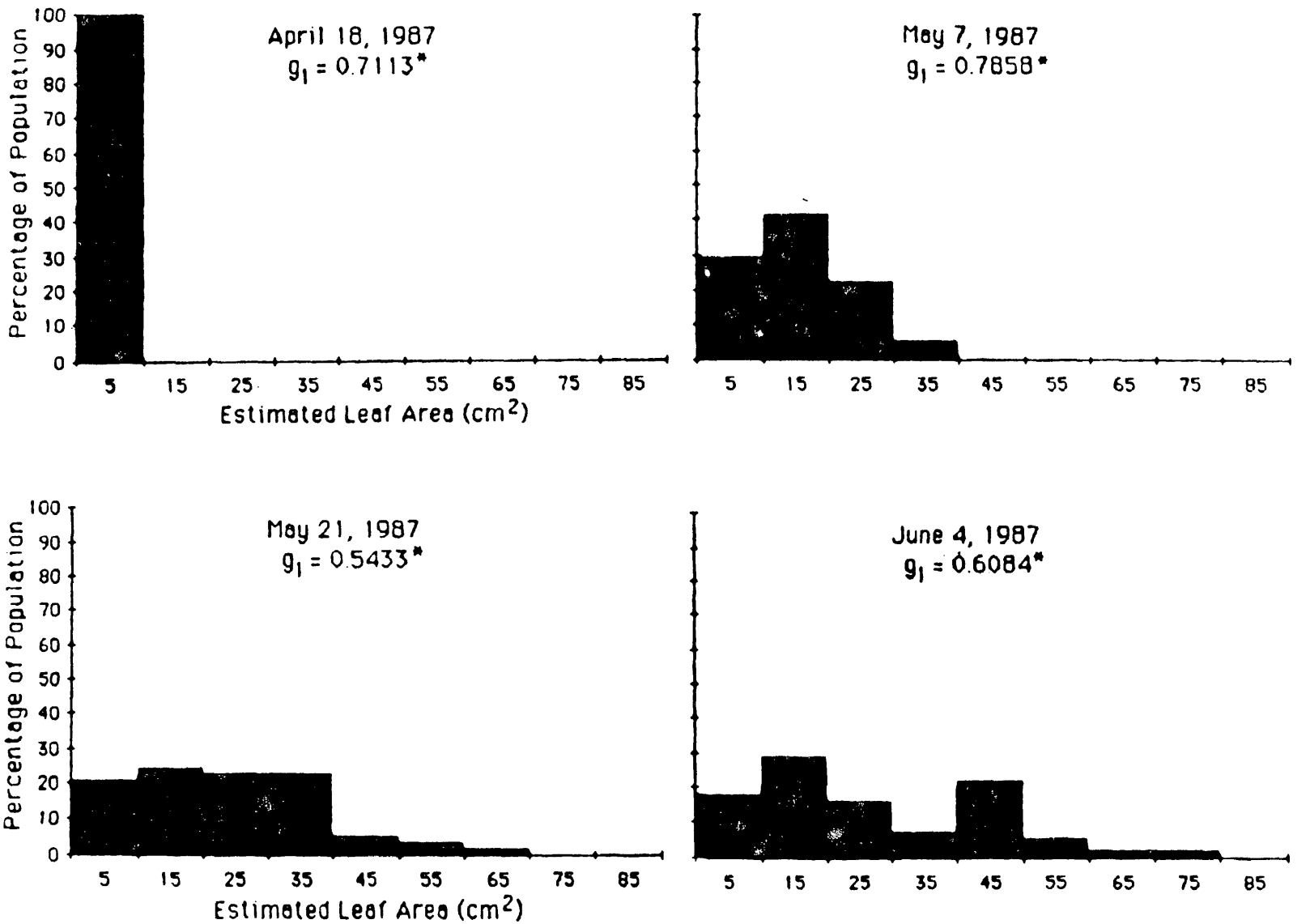

1987
90
80
70
60
50
40
30
20
10
0
%
0 1 2 3 4 5 6 0 1 2 3 4 5 6 0 1 2 3 4 5 6
Number of leaves
May 7
May 21
Jun 4
g1=-0.710*
g1=-0.836*
g1=-0.619*

Figure 6. Frequency distributions of leaf area in Spiranthes cernua, May, 1986 - June, 1987. The 1987 data were courtesy of Antlfinger (unpublished data).


May 15, 1986
g_1 = 0.7970*
May 31, 1986
g_1 = 0.7725*
June 17, 1986
g_1 = 0.8115*
July 11, 1986
g_1 = 1.7612*
Percentage of Population
0
10
20
30
40
50
60
70
80
90
100
5
15
25
35
45
55
65
75
85
Estimated Leaf Area (cm^2)


July 31, 1986
g_1 = 2.3949*
August 23, 1986
g_1 = 5.2867*
September 11, 1986
g_1 = 5.1111*
September 25, 1986
g_1 = 1.3997*
Percentage of Population
0
10
20
30
40
50
60
70
80
90
100
5
15
25
35
45
55
65
75
85
Estimated Leaf Area (cm^2)


April 18, 1987
g_1 = 0.7113*
May 7, 1987
g_1 = 0.7858*
May 21, 1987
g_1 = 0.5433*
June 4, 1987
g_1 = 0.6084*
Percentage of Population
Estimated Leaf Area (cm^2)
0
10
20
30
40
50
60
70
80
90
100
5
15
25
35
45
55
65
75
85

plants lost their leaves in July. The leaves that developed in the fall remained small until spring. They never grew as large as primary leaves. The period of leaf development was similar in 1986 and 1987 but total leaf area was less in 1987. Changes in leaf area corresponded to changes in leaf production.

Estimates of fecundity, number of flowers and inflorescence weight (gdw), were related to plant size (Figures 7 and 8). Maximum leaf area accounted for 56% of the variation in flower number. Small plants (< 20 cm) did not produce flowers. This may represent a size threshold for reproduction. Large, non-flowering plants could have been victims of predation which went unobserved. If plants which failed to flower were removed from the regression, the r increased to 0.65. Reproductive output was also related to dry root weight. Root weight was a better predictor of flower number than leaf area ($r = .70$). Root weight accounted for 79% of the variation in inflorescence weight. To determine the distribution of biomass in Spiranthes cernua, 19 individuals were excavated at the time of flowering. At anthesis, 46% of the biomass was allocated to the inflorescence (Table 10). The root/inflorescence ratio was not significantly different than one. This suggests that much of a plant's resources are allocated to reproduction.

Figure 7. Flower production as a function of leaf area for Spiranthes cernua (N=41). The maximum leaf area during the growing season was used.

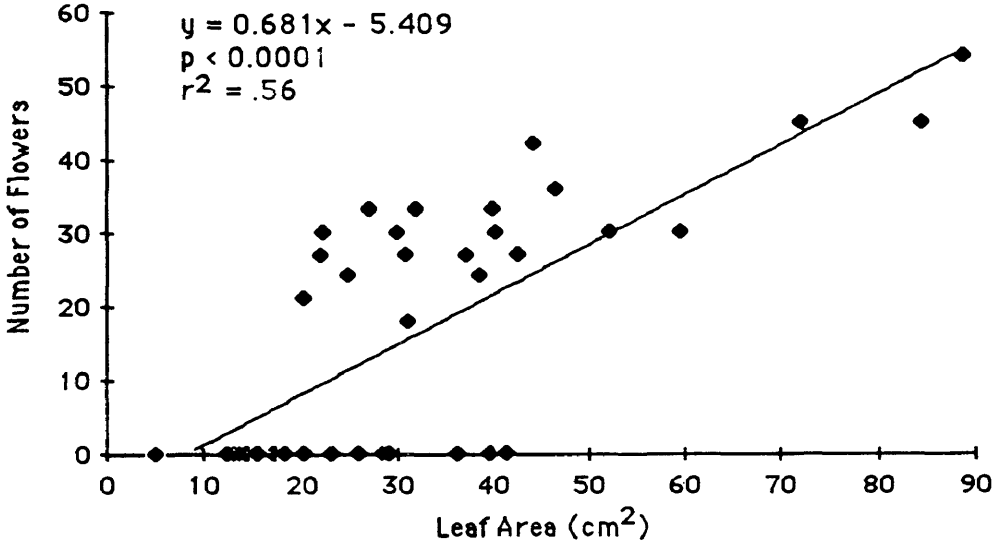
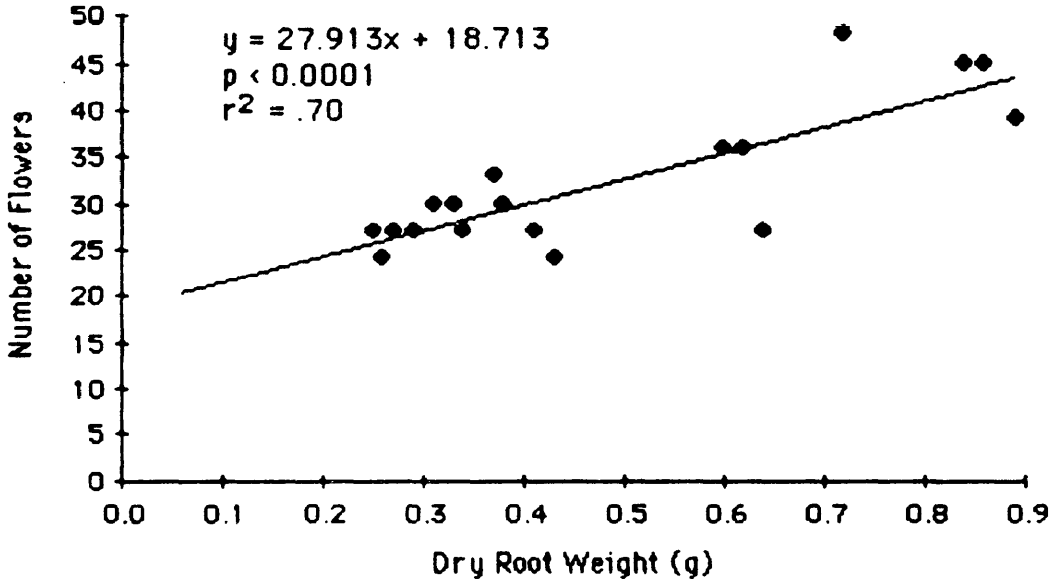

y = 0.681x - 5.409
p < 0.0001
r2 = .56
Number of Flowers
Leaf Area (cm2)
0
10
20
30
40
50
60
70
80
90

Figure 8. Flower production and inflorescence weight as a function of root weight for Spiranthes cernua (N=19).


y = 27.913x + 18.713
p < 0.0001
r2 = .70
Number of Flowers
Dry Root Weight (g)
0
5
10
15
20
25
30
35
40
45
50
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

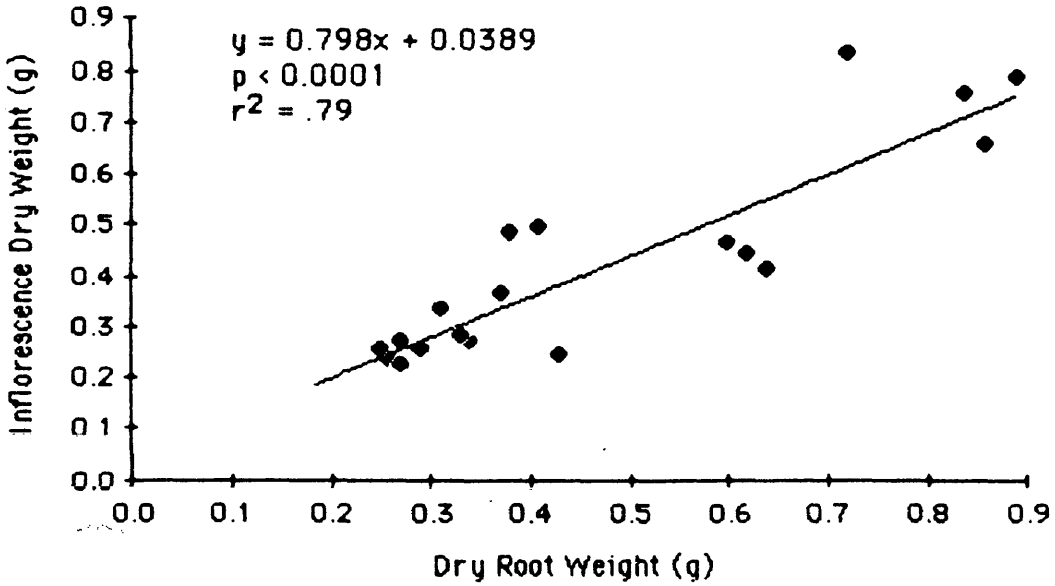

y = 0.798x + 0.0389
p < 0.0001
r2 = .79
Inflorescence Dry Weight (g)
Dry Root Weight (g)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

Table 10. Allocation of biomass in Spiranthes cernua during flowering. Values represent the mean ± 1 SE. N = 19.

Proportion of total biomass (gdw)	
Roots	0.54 ± 0.012
Inflorescence	0.46 ± 0.021
Root/Inflorescence (gdw)	1.22 ± 0.632

DISCUSSION

The North American tall grass prairie supports a diverse flora. Compared to the dominant grasses, little is known about the more numerous perennial forbs (Kerster, 1968). Spiranthes cernua is one of the few orchids adapted to the prairie environment. An important objective of this study was to investigate its breeding system. Pollination treatments are typically performed to determine the breeding system of orchids (Thien and Marcks, 1972; Smith and Snow, 1976; Nilsson, 1978; Ackerman and Mesler, 1979; Whigham and McWethy, 1980; Catling, 1982; Keddy et al, 1983; Mehrhoff, 1983; Cole and Firmage, 1984). In previous investigations pollen viability was not tested. Attempts were made in this study to determine viability. Positive staining indicated the presence of cytoplasm in the pollen grains. Half the pollinia produced pollen tubes. The correlation between staining and germination might have been better if fresh pollen had been used. Viable pollen was essential in the selfing and outcrossing treatments. If the pollen was viable in at least half of these crosses, there would be an adequate sample size. It is probable that more than half of the crosses were successful.

Reproduction in Spiranthes cernua was largely asexual. There are at least two explanations for the high level of agamospermy. First, pollinators may have been limited in 1985. There was evidence that pollinators were not visiting S.

cernua. Flowers were carefully examined at the time of manipulation and none appeared to be pollinated. Only one pollinator was observed with pollen during the two years of study. Low rates of pollination have also been reported in other terrestrial orchid species (Mosquin, 1970; Thien and Marcks, 1972; Ackerman, 1975, 1981; Boyden, 1982; Mehrhoff, 1983; Cole and Firmage, 1984). Polination of S. cernua has been reported as "occasional" or "rare" by other investigators (Sheviak, 1982; Catling, 1983). Further evidence of low natural pollination was shown by the fact that the frequency of monoembryonic and polyembryonic seeds of emasculated unbagged plants was identical to bagged plants. Pollinators may be limited generally. Dissection of tall grass prairie into small remnants may have reduced the number of pollinators. Also, other species may compete with S. cernua for pollinators. The second explanation for significant levels of agamospermy is that adventitious embryos may develop before anthesis. Capsule development before anthesis has been observed in some individuals of S. cernua (Sheviak, 1982). Examination of ovules at different stages of flower development would help clarify this issue.

The level of agamospermy in a population represents a balance between genetic and environmental factors (Clausen, 1954; Marshall and Brown, 1981). "Timing" may play a key role in regulating the amount of sexual versus asexual reproduction in Spiranthes cernua (Catling, 1982). Perhaps the longer

asexual reproduction is postponed in the flowering period, the greater the probability of sexual reproduction. The mechanism of timing is unknown. While the degree of polyembryony varies among populations (Swamy, 1948; Magrath, 1973; Catling, 1982), within a population, it may be relatively fixed (this study). The significant between-year differences in polyembryony observed here suggest environmental factors may be important. For example, changes in photoperiod with latitude are known to effect rates of agamospermy in grasses (Knox, 1967).

Spiranthes cernua is a facultative apomict (Catling, 1982; Sheviak, 1982). This type of reproduction has been described as a "versatile" breeding system (Clausen, 1954; Saran and de Wit, 1970; Catling, 1982). Sexual reproduction is infrequent but can sustain ample variability (Marshall and Weir, 1979). This system leads to taxonomic schemes variously referred to as agamic complexes (Stebbins, 1950; Grant, 1982), compilospecies (de Wit and Harlan, 1966; Sheviak, 1982) or agamospecies (Richards, 1973; Ford, 1981). S. cernua exhibits many of the characteristics of such complexes, including a broad range, extensive morphological variation and geographically isolated variants. Considerable morphological variation and floral variants were observed in the Nine Mile population but since only a single population was examined, the generality of these results is unknown.

Sexual reproduction, though infrequent, may occur primarily by outcrossing and/or geitonogamy. Autogamy is avoided in

Spiranthes cernua. There are several mechanisms which, discourage self-fertilization. (1) A physical barrier separates the sexual parts of the flower. (2) A pollinator must reenter the same flower to effect autogamy, an unlikely event (Smith and Snow, 1976; Pyke, 1978). (3) The flowers are protandrous, with pollen maturing before the stigma is receptive (Catling, 1983b). (4) Finally, the results of this study suggested that individual flowers may be self-incompatible. Thus, geitonogamy and outcrossing are favored in S. cernua. There was a detectable increase in monoembryony in plants that were artificially outcrossed. Since geitonogamous crosses were not significantly different than agamospermy, sexual reproduction was probably the result of cross-fertilization.

The results of this study can be used to evaluate the use of seed morphology to quantify the breeding system of Spiranthes cernua. The major problem was that the origin of monoembryonic seeds could not be determined unambiguously (Catling, 1982; Sheviak, 1982). Agamospermic monoembryonic seeds were identical to sexual monoembryonic seeds. In contrast, polyembryonic seeds were the result of asexual reproduction. Further, distinctions among seed types were not always clear. The technique did detect differences among plants, between years and between species. One advantage was that the proportion of monoembryonic and polyembryonic seeds was not related to the position of the capsule on the

inflorescence or its development.

Germination of terrestrial orchid seed has not been as broadly studied as the commercially important epiphytes. Spiranthes cernua was first cultured asymbiotically in 1933 (Curtis, 1936, 1937). Since then many North American species have been germinated (Stoutamire, 1964, 1974; Linden, 1980; Arditti, 1967; Arditti et al, 1981; Oliva and Arditti, 1984). Asymbiotic culture methods are relatively similar, with slight differences in media, sterilization and light exposure (Arditti, 1982) Few plants have been grown to maturity in the laboratory because of the long time between germination and flowering. The time to germination of Spiranthes cernua in this study was eight weeks. Stoutamire (1964) reports germination in two weeks. Compared to other orchids, S. cernua develops rapidly. Thus it is a good candidate for study. The method of culture employed here was similar to that used to germinate S. gracilis and S. romanzoffinia (Oliva and Arditti, 1984) and originally described by Arditti et al (1981). A less concentrated sterilant gave adequate germination with less risk to the seeds. Light intensities greater than 40 uEin/m /s (PAR) were fatal to developing S. cernua seedlings. Sensitivity to light has been described in other terrestrial orchid seedlings (Linden, 1980).

Survivorship in Spiranthes cernua is best described by a Deevey Type III survivorship curve (Deevey, 1947). Most seeds probably fail to reach a suitable environment for germination

(Cook, 1980). Though species which produce numerous airborne seeds have a greater chance of locating "safe" sites. The juvenile stage is also a period of high mortality (White and Harper, 1970; Cook, 1979; Schmitt and Antonovics, 1986). Once established, survivorship in many terrestrial orchids is high (Curtis, 1954; Tamm, 1972; Harper, 1977; Wells, 1981; Bowles, 1983). Orchids like Spiranthes cernua typically renew above and belowground structures on an annual basis (Harper, 1977; Wells, 1981). However, "absent" plants have been reported. Vegetative dormancy was not found in this population of S. cernua, though it has been observed in similar orchids (Wells, 1967; Tamm, 1972; Bowles, 1983). The adult population probably experiences mortality from herbivory, interspecific competition and loss of its fungal associate.

Plant size within the population varied considerably at each census date. Size frequency distributions of leaf area were skewed, i.e. the population consisted of many small and a few large plants. Hierarchical distributions are a common feature of plant populations. Variation in plant size has been attributed to differences in age (Ford, 1975; White, 1980), seed size, time of germination (Harper and White, 1974) and relative growth rate (Burdon and Harper, 1980; Antlfinger et al, 1985). Predation, intra and interspecific competition can also affect plant size (Harper, 1977; Weiner, 1985). Since Spiranthes cernua can not be aged, cohort studies will be needed to understand the effect of age on plant size. The

seeds of S. cernua are microscopic. Seed length and width could be measured but seed weight only estimated. Germination has not been observed in the field. Individual differences in germination could not be determined in this study, because laboratory results were qualitative rather than quantitative. Relative growth rates can be calculated for leaf area and are currently being analyzed. A study is underway to investigate the spatial distribution of S. cernua. Hopefully, this study will indicate the importance of competition in this population.

Fecundity is often size dependent rather than age dependent in many plants (Harper and White, 1974; Harper, 1977; Cook, 1980; Solbrig, 1981). In Spiranthes cernua plant size accounted for most of the variation observed in reproduction. Large plants produced the most flowers and the largest capsules year after year. The disproportionate contribution of these individuals to the gene pool may alter the genetic structure of the population through inbreeding and drift. The effective population size may be smaller than the actual census indicated. Combined with the ability of S. cernua to reproduce asexually, the skewed distribution of size might decrease variation in the population. Since plant size decreased in 1987, reduced reproductive output is predicted.

The proportion of a population flowering varies from year to year in several terrestrial orchids (Curtis, 1954; Wells, 1967; Tamm, 1972; Bowles, 1983; Cole and Firmage, 1984). This was also true in Spiranthes cernua. The average number of

flowers produced per plant was the same in 1985 and 1986 for Spiranthes cernua. Similarly, flower production per plant did not change in Spiranthes spiralis over a three year period (Wells, 1967). The number of flowers per plant was correlated in different years (i.e. individuals produced a similar number of flowers in consecutive years). Flower number may have a significant genetic component based on the positive between-year correlation for this character. Spiranthes cernua allocated 39% of its biomass to reproduction. This was considerably more than Tipularia discolor, a fugacious terrestrial orchid whose inflorescence biomass never exceeds 20% (Whigham, 1984). The reproductive structures of S. cernua are probably energetically expensive to produce. This may account for some of the variation in flowering between years. A minimum or threshold energy level, necessary to produce an inflorescence, may not be available every year.

The phenology of Spiranthes cernua is well adapted to prairies dominated by warm-season grasses. It avoids competition for light by beginning vegetative growth early. The winter rosette of leaves carry over until new leaves are produced. The high temperature and drought conditions of midsummer are avoided by this pattern of growth. The erect inflorescence with its leafy bracts is probably photosynthetic. Since the leaves have senesced before flowering, the inflorescence may provide an important source of carbon fixation at this time (Reekie and Bazzaz, 1987).

Flowering was staggered with some flowers open from late August to early October. This lengthened the time of exposure to pollinators and increased the likelihood of sexual reproduction.

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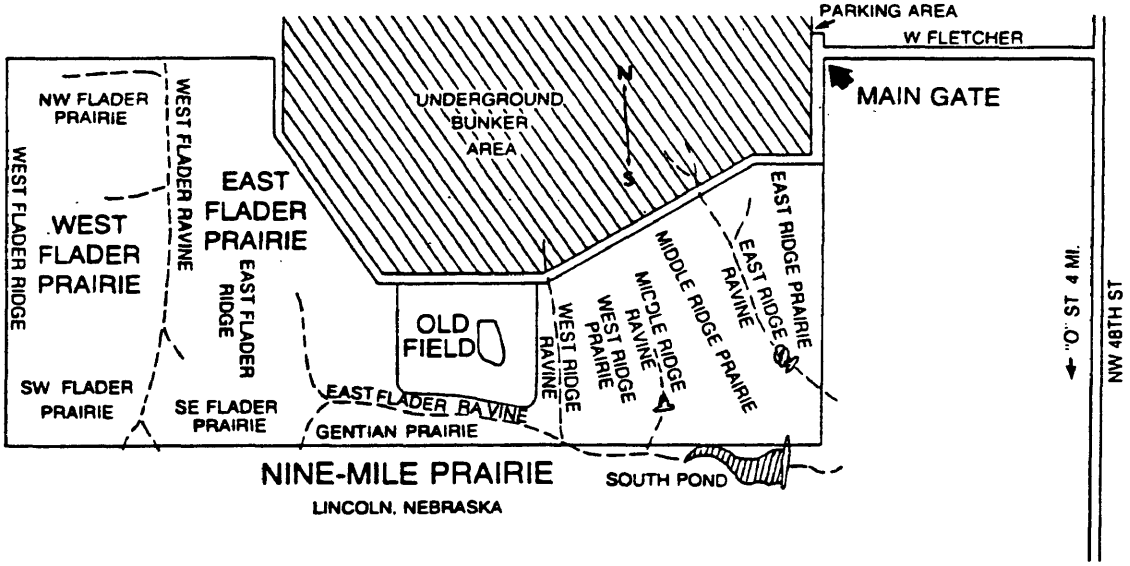
Appendix A

Nine Mile Prairie

The study site was located in East Flader Prairie (T10N; R5E; NW quarter of section 1 and NE quarter of section 2).



Appendix B

Solutions and Media

Wetting agent

Aerosol-OT (detergent)	1 g.
formalin	5 ml.
denatured ethanol	95 ml.

Concentrated phyloxine B (Owczarzak, 1952)

phyloxine B	2 g.
50% ethanol	100 ml.

Glycerol jelly (Owczarzak, 1952)

Knox unflavored gelatine	10 g.
phenol	0.25 g.
glycerol	15 ml.

Vespar

petroleum jelly	1/2 volume
parafin	1/2 volume

The mixture is heated and applied with a brush.

Cotton Blue in Lactophenol (Radford et al., 1974)

lactic acid	40 ml.
glycerin	40 ml.

phenol (melted)	20 ml.
1 % aqueous Cotton Blue	1-5 ml.

Hoagland's solution (Noggle and Fritz, 1976)

1. Molar stock solutions of the following were prepared.

	in 1 liter
1 M KH_2PO_4, potassium dihydrogen phosphate (13.609 g./100 ml. H_2O)	1 ml.
1 M KNO_3, potassium nitrate (10.11 g./100 ml. H_2O)	5 ml.
1 M $Ca(NO_3)_2$, calcium nitrate (23.615 g./100 ml. H_2O)	5 ml.
1M $MgSO_4$, magnesium sulfate (24.648 g./100 ml. H_2O)	2 ml.

2. A stock solution of the following was prepared. 1 ml. was added to the Hoagland's solution.

	in 500 ml.
H_3BO_3, boric acid	1.43 g.
$MnCl_2 \cdot 4H_2O$, magnesium chloride	0.905 g.
$ZnSO_4 \cdot 7H_2O$, zinc sulfate	0.110 g.
$CuSO_4 \cdot 5H_2O$, copper sulfate	0.040 g.
$H_2MoO_4 \cdot H_2O$,molybdic acid	0.010 g.

3. A stock solution of ferric salts of EDTA was prepared and van Winden, 1970).

1 ml provides 5ppm of FeEDTA to one liter of nutrient solution.

A. Dissolve 2.62 g of EDTA in 50ml of hot water (about 70 C) containing 28.16 ml of 1 N NaOH.

B. Dissolve 2.49 g of $FeSO_4 \cdot 7H_2O$ in 30 ml of hot water (about 70 C), containing 4 ml of 1 N H_2SO_4.

C. Mix A and B and distilled add water to about 95 ml; aerate vigorously for 12 hr; increase to 100 ml with distilled water.

Orchid Agar (Bacto Orchid Agar #0242-02, Difco Labs. Detroit, MI)

18.5 g agar

500 ml hot H_2O

Stir until dissolved. Adjust pH 5.0 with H_2SO_4.

Knudson's C media (Knudson, 1946)

KH_2PO_4, monopotassium acid phosphate	0.25 g.
$Ca(NO_3)_2$, calcium nitrate	1.00 g.
$(NH_4)_2SO_4$), ammonium sulfate	0.50 g.
$FeSO_4$, ferrous sulfate	0.025 g.
$MnSO_4 \cdot 4H_2O$, manganese sulfate	0.0075 g.
Sucrose	20.00 g.
Agar	17.50 g.
Distilled water	to 1000 ml.

Curtis solution 5 media (Curtis, 1936; Arditti, 1982)

1. Stock solutions.

	in 1 liter
KH_2PO_4, potassium phosphate (1.2 g./100 ml. H_2O)	10 ml.
$MgSO_4 \cdot 7H_20$, magnesium sulfate (2.6 g./100 ml. H_2O)	10 ml.
NH_4NO_3, ammonium nitrate (2.2 g./100 ml. H_2O)	10 ml.
$Ca(NO_3)_2$, calcium nitrate (3.5 g./100 ml. H_2O)	10 ml.
$FeSO_4 \cdot 7H_2O$, ferrous sulfate (0.553 g./100 ml. H_2O)	1 ml.

2. 800 ml. of nutrient solution was adjusted to pH 4.7. 10 g. of glucose was added and the volume brought up to 1000 ml. with distilled water.

3. The solution was heated to a gentle boil with stirring. 14 g. of agar was added. The media was autoclaved for 30 min at 270°.

Curtis Full strength media (Arditti et al, 1981)

1. Stock solutions.

	in 1 liter
KH_2PO_4, potassium phosphate (1.2 g./100 ml. H_2O)	10 ml.
$MgSO_4 \cdot 7H_20$, magnesium sulfate (2.6 g./100 ml. H_2O)	10 ml.
NH_4NO_3, ammonium nitrate (2.2 g./100 ml. H_2O)	10 ml.
$Ca(NO_3)_2$, calcium nitrate (3.5 g./100 ml. H_2O)	10 ml.
$FeSO_4 \cdot 7H_2O$, ferrous sulfate (0.553 g./100 ml. H_2O)	1 ml.

2. Stock solution of microelements. 1 ml used in nutrient solution.

$AlCl_3$, aluminum chloride	0.03 g.
$CoCl_2 \cdot$, cobalt chloride	0.025 g.
$CuSO_4 \cdot 5H_2O$, copper sulfate	0.055 g.
$FeCl_3 \cdot 6H_2O$, ferric chloride	1.00 g.
H_3BO_3, boric acid	7.20 g.
KI, potassium iodide	0.84 g.
$MnSO_4 \cdot 6H_2O$, manganese sulfate	22.31 g.
$Na_2MoO_4 \cdot 2H_2O$, molybdic acid	0.25 g.
$NiCl_2 \cdot 2H_2O$, nickel chloride	0.03 g.
$ZnCl_2$, zinc chloride	3.93 g.
$ZnSO_4 \cdot 7H_2O$, zinc sulfate	1.00 g.
Distilled water	to 1000 ml

3. Stock solution of hormones. 1 ml used in nutrient solution.

Benzyladenine	100 mg.
6-dimethyl-aminopurine	25 mg.
Kinetin	100 mg.
Zeatin	25 mg.
Napthaleneacetic acid	10 mg.
Distilled water	to 100 ml.

4. Stock solution of vitamins. 1 ml used in nutrient solution.

Biotin	100 mg.
Ca·pantothenate	100 mg.
Folic acid	100 mg.
Niacin	100 mg.
Pyridoxine·HCl	100 mg.
Thyamine·HCl	100 mg.
Distilled water	to 100 ml.

5. The following ingredients were then added to complete the media.

Coconut water from unripe nuts	50 ml.
Banana homogenate unripe banana distilled water (1:1 weight by volume)	75 g.

6. 800 ml. of nutrient solution was adjusted to pH to 5.0. 10 g. of glucose was added and the volume was brought up to 1000 ml. with distilled water.

7. The solution was heated to a gentle boil and 14 g.

of agar was added with stirring. The media was autoclaved.

Sterilizing solutions

Concentrated calcium hypochorite: 7 g. $Ca(OCl)_2$ in 100 ml. distilled H_2O. Heat, stir and filter using Whatman #1 quality paper. Add 2-3 drops of household detergent. Prepare within 12 hours of use (Harrison, 1968; Arditti, 1982).

$\frac{1}{2}$ strength calcium hypochlorite: 3.5 g. $C(OCl)_2$.

$\frac{1}{4}$ strength calcium hypochlorite: 1.75 g. $C(OCl)_2$.

Bleach: 5 ml. household bleach in 95 ml H_2O.

Appendix C

Development of Slide Methodology

Experiment #1: This experiment was designed to test observer reliability in distinguishing morphological differences among seeds. The seeds represented capsules from different plants. Since orchid seeds float, an organic wetting agent was used. The wetting solution (see Appendix B) was added to test tubes containing seeds. The tube was vortexed until the seeds sank to the bottom. A Pasteur pipet was used to agitate the seeds into solution. Drops (7-10) of the mixture were placed onto a slide and covered with a coverslip. The coverslip was ringed with nail polish. Five slides were prepared on each of five consecutive days and were read within 24 hours of preparation.

Seeds were classified into three categories. Monoembryonic seeds were seeds with a single embryo encased in a seed coat. Polyembryonic seeds had two or more embryos. Agamospermous-monoembryonic seeds were seeds with a single embryo protruding from the seed coat. Other types of seeds were observed but not counted. Approximately 100 seeds were counted per slide. One observer scanned the slides under 100X magnification moving in a Z pattern. The other observer read four quadrats of the slide under 40X magnification. Special Hoffman optics were used by both observers. Chi-square analysis was used to partition experimental variation among

slides, among days and between observers. The results are given in Table 1C.

Experiment #2: In this experiment, the observers used the same magnification (100X). Eight slides were prepared on each of five consecutive days and were read within 24 hours of preparation. Four slides were prepared from the mixture of seeds of experiment #1. The other four were made using seeds from an individual capsule from a single plant. Slide preparation was as in Experiment #1. Vespar (see Appendix B) was used to preserve the slides. On average, 80 seeds were counted per slide. The slides were scanned in a Z pattern, avoiding the edges. The seed classes were as in the first experiment. The data were analyzed with Chi-square tests for the following differences: among slides, among days, between observers and among seed sources. The results are given in Table 2C.

Experiment #3: In this experiment, the seed categories were redefined. Further, the seeds were stained to increase the contrast. Seeds were classified into three categories: monoembryonic, agamospermic and unknown. In this experiment, agamospermic seed included both polyembryonic and agamospermous-monoembryonic seeds. In addition, unknown seeds were counted. Four slides were prepared on each of four consecutive days and were read within 24 hours of preparation.

Table 1C. Contingency analysis of experiment 1.

---

1. H_o: Seed type is independent of the day slides are read. (5 days X 3 seed types)

$$X^2_{calc,\ 8} = 49.48\ ^{***}$$

2. H_o: Seed type is independent of the observer. (2 X 3)

$$X^2_{calc,\ 2} = 61.70\ ^{***}$$

3. H_o: Seed type is independent of the day slides are read for observer 1.

$$X^2_{calc,\ 8} = 51.59\ ^{***}$$

H_o: Seed type is independent of the day slides are read for observer 2.

$$X^2_{calc,\ 8} = 17.73\ ^{*}$$

---

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2C. Contingency analysis of experiment 2.

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1. H_o: Seed type is independent of the day slides are read. (5 days X 3 seed types)

$$X^2_{calc,\ 8} = 21.99\ ^{**}$$

2. H_o: Seed type is independent of the observer. (2 X 3)

$$X^2_{calc,\ 2} = 42.36\ ^{***}$$

3. H_o: Seed type is independent of the day slides are read for observer 1.

$$X^2_{calc,\ 8} = 21.33\ ^{**}$$

H_o: Seed type is independent of the day slides are read for observer 2.

$$X^2_{calc,\ 8} = 8.83$$

---

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Seeds from four capsules of a single plant were placed in test tubes. The seeds were stained over night in concentrated phyloxine B stain (1 ml) (see Appendix B). The stain was removed with a pipet and the seeds were rinsed twice with 50% ETOH. This procedure is a modification of a differential stain technique (Owczarzak, 1952). Four to five drops of the seed-ETOH mixture were placed on a slide. The slides were again sealed with vespar. Eighty seeds per slide were counted. Data analysis was the same as in experiment #1. The results are given in Table 3C.

Experiment 4: In this experiment, seeds were dropped onto the slide and the ethanol allowed to evaporate. The dry seeds were mixed with four drops of hot glycerol jelly (see Appendix B) using a dissecting needle to evenly disperse the seeds. The coverslip was added before the jelly solidified (Owczarzak, 1952; Catling, 1982). This semipermanent slide could be read several months after preparation. Slides were read on three consecutive days. Each seed was classified into two categories: monoembryonic and agamospermous. Analysis was the same as in previous experiments. The results are given in Table 4C.

Table 3C. Contingency analysis of experiment 3.

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1. H_o: Seed type is independent of the day slides are read. (4 days X 3 seed types)

$$X^2_{calc,\ 6} = 24.92^{***}$$

2. H_o: Seed type is independent of the observer. (2 X 3)

$$X^2_{calc,\ 2} = 16.98^{***}$$

3. H_o: Seed type is independent of the day slides are read for observer 1.

$$X^2_{calc,\ 6} = 28.40^{***}$$

H_o: Seed type is independent of the day slides are read for observer 2.

$$X^2_{calc,\ 6} = 30.22^{***}$$

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* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 4C. Contingency analysis of experiment 4.

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1. H_o: Seed type is independent of the day slides are read. (3 days X 2 seed types)

$$X^2_{calc,\ 2} = 5.58$$

2. H_o: Seed type is independent of the observer. (2 X 2)

$$X^2_{calc,\ 1} = 0.31$$

3. H_o: Seed type is independent of the day slides are read for observer 1.

$$X^2_{calc,\ 2} = 4.01$$

H_o: Seed type is independent of the day slides are read for observer 2.

$$X^2_{calc,\ 2} = 2.05$$

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* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Appendix D

Pollen Germination

Experiment #1: Four pollinia, from each of two plants, were macerated on a slide. Four different concentrations of sucrose (0.25%, 0.50%, 0.75%, 1.00%) were tested. The slides were covered and sealed with vespar. The slides were checked for pollen tube germination at 1, 2, 24 and 48 hours.

Experiment #2: In this experiment, higher sucrose concentrations were tested (1.00%, 1.50%, 2.00% and 2.50%). The slides were examined for pollen tube germination at 2 and 24 hours.

A summarry of the results of these experiments is given in Table 1D.

Table 1D. Pollen germination of Spiranthes cernua

Plant number	Sucrose Concentration	Pollen tubes per slide 1 hr.	2 hr.	24 hr.	48 hr.
90	0.25%	0	0	0	0
	0.50%	0	0	CONT[1]	CONT
	0.75%	0	0	4	1
	1.00%	0	0	0	0
111	0.25%	0	0	0	0
	0.50%	0	0	12	19
	0.75%	0	0	5	12
	1.00%	0	0	4	2
6	1.00%		0	3	
	1.50%		0	0	
	2.00%		0	0	
	2.50%		0	7	
41	1.00%		0	0	
	1.50%		0	0	
	2.00%		0	0	
	2.50%		0	1	

[1] CONT = Contaminated

Appendix E

Seed Germination

Experiment 1. The first experiment was to germinate seeds (1) with distilled water in sterile petri plates (Stoutamire, 1974), (2) with Hoagland's solution (see Appendix B) and (3) in soil from the study site. The experiment was performed in the greenhouse. Within a week all plates were contaminated with bacteria and fungi. The seeds planted in soil had not germinated after 10 weeks.

Experiment 2. In this experiment, the seeds were sterilized with a 5% solution of bleach (see Appendix B). The germination conditions were the same as experiment #1 except that a sterile, commercial orchid media was used instead of soil (see Appendix B). The seeds were germinated in the greenhouse. The seeds in Hoagland's solution were contaminated after two weeks. In general, the distilled H_2O and orchid agar treatments did not become contaminated. However, after two months, none of the seeds were photosynthetic.

Experiment 3. In this experiment, attempts were made to reduce contamination through asymbiotic culture. Seeds were sterilized with concentrated calcium hypochlorite solution (see Appendix B). Three asymbiotic culture media were tested (1) the commercial orchid agar, (2) Curtis number 5 media and (3)

Knudson's C media (see Appendix B). Plates were poured and the seeds were sown under a laminar flow hood. The seed cultures were grown in an incubator at 25° C. with a 12hr light/dark cycle. All plates showed evidence of dehydration at one month. After two months, none of the seeds were photosynthetic. Five of 21 plates were contaminated.

Experiment 4. The seed sterilant appeared to suppress germination. An experiment was therefore conducted to compare methods of seed sterilization on Knudson's C media. The treatments were (1) strength $Ca(OCl)_2$ - 4 replicates, (2) strength $Ca(OCl)_2$ - 4 replicates, (3) 5% bleach - 4 replicates and (4) controls - 1 replicate (see Appendix B). Ehrlenmeyer flasks were used instead of petri plates in this experiment to reduce water loss. After two months, none of the seeds were photosynthetic. None of the flasks showed contamination.