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Root morphology, distribution of mycorrhizae, and nutrient status of the terrestrial orchid Spiranthes cernua.

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ROOT MORPHOLOGY, DISTRIBUTION OF MYCORRHIZAE, AND NUTRIENT STATUS OF THE TERRESTRIAL ORCHID SPIRANTHES CERNUA

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by

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THESIS ACCEPTANCE

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Committee

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ABSTRACT

The rhizosphere is a complex of biotic and abiotic factors and their interactions. It includes the soil, the micro- and megafauna, and a variety of autotrophic species. The goal of this study is to understand the belowground dynamics of the terrestrial orchid, Spiranthes cernua, through the characterization of soil nutrients, root system morphology, and mycorrhizal infection. Interrelationships among the soil, the mycorrhizae and S. cernua may explain the role of mycorrhizae in adult chlorophyllous orchids as well as the influence of soil nutrients on mycorrhizae. Understanding mycorrhizal relationships, will also contribute to the conservation and reestablishment of threatened and endangered terrestrial orchids.

Spiranthes cernua was studied at Nine-Mile Prairie, near Lincoln, NE, in 1997. The soil is a silt loam to silty clay loam with a pH range of 5.4 - 5.95. The soil contained 4.54 ± 0.61 μg/gds ammonium-N, 1.23 ± 0.11 μg/gds nitrate-N, 5.62 ± 0.13 μg/gds phosphorus (Bray), and 3.38 ± 0.07 g/gds total carbon. Inorganic nitrogen decreased through the growing season while carbon and phosphorus remained stationary.

The size and structure of the root system of S. cernua changed during the period April - July. During this vegetative phase the root system was composed of young and old mature roots and bud roots. Bud roots and old roots were not observed after July.

Mycorrhizae infected approximately 12.1% of root cortical cells in S. cernua, however, less than 5% was active infection. Mature roots were significantly less infected than bud roots (10.4 ± 0.01% versus 31.1 ± 0.04%). It is possible that S. cernua, like its congener S. sinensis, has two types of roots, one primarily for mycorrhizal infection and one for storage. In S. cernua bud roots may represent the "mycorrhizal" roots.

During the vegetative phase the plant must manufacture/acquire resources for the growth and maintenance of the current shoot as well as store resources for future growth and reproduction. In S. cernua mycorrhizal activity is greatest when the plant is

vegetative and soil inorganic nitrogen levels are highest. This temporal pattern may allow the plant to acquire nutrients at the lowest possible cost.

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INTRODUCTION

Mycorrhizae play a role at many levels of biological organization from increasing the growth of individual plants (Carlile and Watkinson, 1994) to influencing community structure (van der Heijden et al., 1998). Mycorrhizal associations are common in the family Orchidaceae, especially in terrestrial orchids. The terrestrial orchid, Spiranthes cernua (L.) Rich., has been extensively studied (Schmidt, 1987; Schmidt and Antlfinger, 1992; Wendel, 1994; Wendel and Antlfinger, 1996; Antlfinger and Wendel, 1997) in Nebraska and is the experimental organism in this research.

Antlfinger and Wendel (1997) found that there was a critical aboveground size necessary for flowering in S. cernua. While current photosynthesis met about 50% of the expense of flowering, stored reserves would be needed for the remainder, in addition to supporting vegetative growth the next year. Therefore, to understand resource allocation in S. cernua, belowground dynamics must be considered. In addition, Alexander (1987) and Rasmussen (1995) have called for investigations which relate mycorrhizal infection to plant and soil nutrient levels in adult orchids. Based on these reports, the goals of this study were to: (1) describe the development of the root system and its contribution to resource allocation; (2) quantify the mycorrhizal infection of roots of adult plants in space and time; and (3) analyze the nutrient concentrations of soil and plant tissues.

Knowledge of the relationship between mycorrhizal infection, root system morphology and nutrients will contribute to our understanding of growth and reproduction in S. cernua and other mycorrhizal species. Further, these data are essential for understanding the environmental requirements for terrestrial orchid conservation and reestablishment.

Research involving root systems is difficult because it relies on excavation which disrupts normal growth and is labor intensive. The following sections review the information available on the goals stated above and introduce the specific objectives and methodology of this study.

Mycorrhizae--Underground there is a world of organisms rarely seen or considered but vital to the growth of individual plants and to the establishment of plant communities. Fungi are one component of this underground world. Fungi are important decomposers, pathogens, and symbionts of plants. Symbiotic associations between fungi and the underground parts of plants are known as mycorrhizae. Approximately 90% of vascular plants are known to be mycorrhizal (Selosse and Le Tacon, 1998). The significance of mycorrhizal associations is recognized by both scientists and practitioners (St. John and Coleman, 1983; Cook, Jastrow, and Miller, 1988; Gange, Brown, and Farmer, 1990). In most mycorrhizae, the fungal partner provides the plant with increased absorption of nutrients, especially phosphorus, and water from the soil in exchange for

carbon from the plant. Greenhouse studies have shown that the host plant may exhibit increased growth and/or increased levels of nutrients in the tissues (i.e., phosphorus: Hetrick, Wilson, and Schwab, 1994). Plants growing in nutrient-poor soils tend to benefit more from their mycorrhizae than plants of the same species in nutrient-rich soils.

Most of what is known about orchid mycorrhizae comes from seed germination and seedling studies. Unlike most mycorrhizae, the orchid mycorrhiza transfers carbon from the fungus to the seedling (Smith, 1966, 1967; Alexander and Hadley, 1985; Beyrle and Smith, 1993). It is not known whether adult plants also receive carbon via the fungus, though the symbiotic relationship is maintained for the entire life span of the plant (Poole and Sheenan, 1982). The fungus is able to utilize a wide range of carbon compounds from simple sugars (i.e., glucose) to cellulose (Harley, 1959; Hadley, 1969; Smerciu and Currah, 1989). It is thought that cellulose is the major carbon source available to the fungus in nature (Hadley, 1969). A higher incidence of fungal parasitism relative to symbiosis is observed on nutrient-rich media (Hadley, 1969; Beryl, Penningsfeld, and Hock, 1991).

Orchid mycorrhizae are typically basidiomycete fungi in the form genus Rhizoctinia though a few ascomycetes are known (Rasmussen, 1995). Basidiomycete fungi are septate and have two to many nuclei per cell. The orchid mycorrhizae are

mostly asexual. A few telomorph genera have been identified including Ceratobasidium, Tulasnella, Sebacina, and Thanatephorus (Filipello Marchisio et al., 1985; Currah, Sigler, and Hambleton, 1987; Smreciu and Currah, 1989; Currah, Smreciu, and Hambleton, 1990). The classification of these mycorrhizal fungi is based on morphological features of the hyphae and septal pore, but is not well understood (Rasmussen, 1995). Several Rhizoctinia species are known plant pathogens of non-orchid species (Carlile and Watkinson, 1994).

Fungi used in symbiotic germination of seeds are isolated from the roots of adult orchids. It is possible to isolate several fungal species from the roots of one orchid species, or even a single individual (Curtis, 1939). Seed germination experiments have demonstrated a range of fungal specificity (Curtis, 1939; Warcup, 1981; Smerciu and Currah, 1989; Zelmer, Cuthbertson, and Currah, 1996). Williamson and Hadley (1970) found that the fungi respond to crucifer seedlings differently than to orchid seedlings. This indicates recognition between the fungi and orchid that directs the association toward symbiosis and not parasitism. It has been suggested that bacteria associated with orchid mycorrhizae assist in recognition (personal communication in Wilkinson, Dixon, and Sivasithamparam, 1989). The symbiotic association is thought to be controlled by the orchid (Harley, 1959; Hadley, Johnson, and John, 1971). Infection is restricted to the

basal portion of the protocorm, away from the meristem, in symbiotic associations. Masuhara and Katsuya (1992) observed restricted infection in roots of Spiranthes sinensis suggesting that adult orchids are also able to exert some control over their fungal partner.

Orchid mycorrhizae infect orchid tissues through epidermal hairs (many orchids lack true root hairs) or exodermal passage cells (Esnault, Masuhara, and McGee, 1994). Active infection is greatest in young roots and may also occur in the rhizome (Liparis: Rasmussen, 1995). The infection is tolypophagous, that is, the hyphae invade host cells and form a mass of coiled hyphae (called a peloton) before they are digested. During residence in the orchid cells the fungus breaks down carbon storage products (i.e., starch) and may use some of this carbon (Breddy, 1991). The orchid receives nutrients from the fungus upon fungal digestion (i.e., necrotrophically); biotrophic transfer of nutrients has not been clearly demonstrated (Rasmussen, 1995). Two types of pelotons are visible in orchid root cells: living, hyphal pelotons and amorphous pelotons which have been digested (Curtis, 1939; Currah, 1991; Stoutamire, 1991; Masuhara and Katsuya, 1992). Hyphae are thought to remain viable as pelotons for a short period of time (Stoutamire, 1991). It is not known how long amorphous pelotons persist.

Terrestrial orchids have higher levels of mycorrhizal infection than epiphytes. While infection in epiphytes is generally limited to those portions of the root in contact

with the substrate, infection in terrestrial orchids is found throughout the entire root (Hadley and Williamson, 1972; Alexander, 1987; Goh, Sim, and Lim, 1992).

Very few studies have quantified the infection of adult orchids through time (Alexander, 1987; Rasmussen, 1995). Masuhara and Katsuya (1992) examined roots of S. sinensis across 13 months. They found that in heavily-infected roots, peloton numbers peaked at the time of flowering and then living pelotons decreased markedly. One objective of this study was to characterize the mycorrhizal infection in roots of adult plants through the growing season. Because S. cernua is a chlorophyllous orchid, I hypothesized that the mycorrhizal infection functions like a "typical" mycorrhizae, in that the orchid obtains mineral nutrients (e.g., nitrogen and phosphorus) and water in exchange for carbon. Plants need additional nutrients and water during rapid growth, therefore increases in mycorrhizal infection would be expected early in the season when new leaves develop. Further, infection can be tolerated during the vegetative period when photosynthesis is able to cover the cost of carbon used by the fungus. Peloton number should decrease with leaf senescence, because the orchid should limit carbon loss, especially if the plant is going to flower.

Orchid root systems--Vascular plant roots are another component of the rhizosphere. Roots change the physical and chemical aspects of the soil and conduct

essential nutrients into the plant. Terrestrial orchids typically have simple root systems with unbranched roots (one explanation for the necessity of mycorrhizae; Rasmussen, 1995; but see Curtis, 1939). Though roots are simple, a variety of underground structures are observed. These structures are typically adaptations for storage and, in terrestrial orchids, include cormous rhizomes (Tipularia: Stoutamire, 1991; Zimmerman and Whigham, 1992), root-stem tubers (in orchidoid species: Rasmussen, 1995) and tuberoid roots, which function in mycotrophy as well as storage (Spiranthes: Ames, 1921; Stern et al., 1993). Masuhara and Katsuya (1992) observed two types of roots in S. sinensis which differed in function and time of development but were relatively indistinguishable morphologically. Pseudobulbs are typical "underground" storage organs found in epiphytic orchids.

Underground storage organs are common in herbaceous perennials and are thought to provide necessary resources for periods of rapid growth (i.e., breaking of seasonal dormancy or reproduction). In orchids, research on epiphytes has demonstrated the importance of storage organs. Zimmerman (1991) found that storage of non-structural carbon, nitrogen and phosphorus in the pseudobulbs was important for vegetative growth and flowering in Catasetum viridiflavum. In the hybrid epiphyte Oncidium 'Goldiana', Hew and Ng (1996) also reported that pseudobulbs function in

storage of carbon, nitrogen, phosphorus, and potassium, and that these nutrients are shunted from pseudobulbs to developing tissues. In the terrestrial orchid Tipularia discolor, Whigham (1984) found that the cormous rhizome was mainly involved in carbohydrate storage. It is possible that the higher levels of mycorrhizal infection in terrestrial orchids have reduced the need for mineral storage as compared to epiphytes.

Prairie populations of S. cernua are usually fugacious, that is, without leaves at the time of flowering (Sheviak, 1991). Therefore, resources stored in the root system could be mobilized twice within a growing season: in early spring for vegetative growth and for inflorescence development during the fall. I examined the morphology of the root system to try to detect this reallocation and to monitor root system size. Also, root and leaf nutrient levels (carbon, nitrogen and phosphorus) were determined during the vegetative and reproductive stages to understand patterns of nutrient allocation related to reproduction.

Prairie soils and soil interactions--Relative to other ecosystems, prairies have limited and variable nutrient and water supplies, and there is intense competition for resources. In grasslands nitrogen has been shown to be important in affecting structure and function of biogeochemical cycles (Risser and Parton, 1982). Nitrogen levels in temperate grasslands are highest in spring and decrease through the summer (Turner et

al., 1997; DeLuca and Keeney, 1994). Soil moisture and temperature are the primary abiotic factors influencing nitrogen mineralization and cycling within the plant-soil system (Risser and Parton, 1982). Nitrogen is often a limiting resource for plants in tallgrass prairie (Bentivenga and Hetrick, 1992).

Soil is a complex medium formed by both abiotic and biotic factors and their interactions (Coleman and Crossley, 1996). Soil nutrient interactions are often complex and cyclic. For example, plants with high nitrogen use efficiency tend to have high tissue C:N ratios, which produces poor quality litter and depresses nitrogen mineralization rates, and consequently less nitrogen becomes available in the soil (Tateno and Chapin, 1997). The plant "environment" is both above and below ground. Thus, plants contribute to and are affected by various soil interactions. Beaver (1994) and Beaver, Westover, and Antonovics (1997) suggest that even interactions between plant species may be mediated by the soil community.

Mycorrhizae also contribute to belowground interactions. Prairies are rich in mycorrhizal species, dominated by vessicular-arbuscular mycorrhizae (VAM). VAM supply phosphorus to their plant hosts and thereby increase the host's competitive ability (Bentivenga and Hetrick, 1992). Phosphorus mobility in the soil matrix is more limited than that of other minerals (White, 1987). VAM confer a benefit to their host because

fungi are able to search large volumes of soil in addition to having increased absorptive abilities compared to vascular plants (Carlile and Watkinson, 1994).

Though many studies have examined nutrient relations between VAM fungi and the host plant (Bentivenga and Hetrick, 1992; Hetrick, Wilson, and Schwab, 1994; Wilson and Hartnett, 1997), few studies of orchid mycorrhizae have attempted to relate infection with the nutrient levels of adult plants (Rasmussen, 1995). In this study the tallgrass prairie site was unammended. It was necessary to characterize the soil nutrient levels at the site to establish a base-line for interpreting plant nutrient levels. The goal was to relate the degree of mycorrhizal infection to nutrient levels in plant tissues and soil through the growing season. Higher root infection was expected in soil with lower nutrient levels or in plants with lower tissue nutrient levels. Plants that were going to flower were expected to have increased infection during the vegetative phase and a marked decline at or around the time of flowering regardless of soil nutrient levels. This study is unique in that multiple components affecting the mycorrhizal relationship were investigated simultaneously in order to understand the complex relationships between mycorrhizal fungi, the host plant, and the immediate environment.

MATERIALS AND METHODS

The species--Spiranthes cernua (L.) Rich. (Lady's-tresses) is a terrestrial orchid found in the eastern half of the United States and southeastern Canada in wet to dry prairies and occasionally open woodlands (Kaul, 1986). Plants are perennial and emerge in mid to late April. Plants have a basal rosette of 2 – 5 lanceolate leaves. Prairie populations are fugacious, losing their foliage in late July (Sheviak, 1991). The root system is composed of one to several fleshy fasiculate roots that are known to be mycorrhizal (Stern et al., 1993). Plants flower in mid-September through October. The inflorescence is composed of a spike with several tight twisting ranks of white flowers. Seeds can be produced both sexually and asexually (Ames, 1921; Sheviak, 1991; Schmidt and Antlfinger, 1992). Like many orchids, S. cernua has dust seeds and thousands of seeds are produced in a single capsule (Rasmussen, 1995). Sheviak (1991) also notes that vegetative reproduction occurs via roots acting as a stolon.

Description of study site--Nine-Mile Prairie is a 97 ha virgin tall-grass prairie owned by the University of Nebraska Foundation and located in Lancaster County, Nebraska. The dominant grass species are Andropogon gerardii, A. scoparius, Sorghastrum nutans, and Koeleria pyramidata (Kaul and Rolfsmeier, 1987). The S. cernua study population is located on a well-drained, north-facing upland in the East

Flader section of Nine-Mile Prairie (see map in Schmidt, 1987; Wendel, 1994). The soil is a silt loam to silty clay loam with a pH range of 5.4 – 5.95 (Antlfinger, unpublished data). Most of the data collection for this study occurred 29 April through 31 October, 1997. Mean daily maximum temperature during this period ranged from -5.6° C to 32.2° C and averaged 17.2° C during the vegetative phase (April through July) and 18.3° C during the reproductive phase (August through October). Total precipitation during the 1997 growing season was 1 127.78 mm.

The age of the population is not known. Individual S. cernua plants were first flagged and numbered in 1985 and have been regularly censused since that time (Schmidt, 1987; Schmidt and Antlfinger, 1992 ; Antlfinger and Wendel, 1997). Most plants in the population are located in seven 10 m × 10 m plots (see map in Appendix A). In 1997 there were 81 plants in the population with at least one leaf and only 4 plants flowered. The study site was last burned in the spring of 1995.

Morphological examination--Herbarium study-Herbarium specimens were obtained from the University of Nebraska-Lincoln (NEB), University of Nebraska at Omaha (OMA), South Dakota State University (SDC), University of Kansas (KANU), and Kansas State University (KSC). Only plants from prairie habitats were used. Due to morphological similarities between S. cernua and other congeners, only annotated plants

were included in the survey of herbarium specimens to ensure proper identification. Traits measured on herbarium plants included: total plant length, inflorescence length, number of flowers, number of roots, and length and width of roots. All measurements were in millimeters. Length measurements were only taken on roots that did not appear broken. The presence of a small shoot was noted when present. The herbarium survey was completed before the beginning of the 1997 field season.

Harvested plants-A trial excavation was performed on 29 April 1997 to determine a method of excavation and develop staining techniques for mycorrhizal examination. Two plants were harvested, photographed, weighed, and measured. Using these plants, different root clearing and staining procedures (Appendix B) were evaluated to establish a protocol for light microscopy of mycorrhizae.

Twelve plants were harvested on four dates during the growing season of 1997 (three plants per date). There were two collection dates during the vegetative phase of the life cycle (19 May and 30 June), and two collection dates during the reproductive phase of the life cycle (17 August and 16 September). Plants for this project were selected based on their status in 1996. In order to survey the range of variability in morphology, and mycorrhizal and nutrient status at each date, a sample included a plant that had flowered in 1996, a large vegetative plant, and a small vegetative plant. "Large

plants" had a leaf area greater than 20 cm^2 and "small plants" had a leaf area less than 20 cm^2. Orchids were excavated by removing a cylinder approximately 15 cm in diameter and 20 cm deep of surrounding soil and vegetation. The entire cylinder was placed in a plastic bag and transported to the laboratory.

In the laboratory, plants were removed from the soil, rinsed with distilled water, blotted dry, and photographed. Length and width measurements of roots, leaves, and reproductive structures were made with digital calipers. Total plant fresh weight, and fresh and dry mass of individual structures were measured to the nearest 0.0001 g. Usually one root was prepared for light microscopy of mycorrhizae and one root for scanning electron microscopy. Plants that were harvested during the vegetative phase had small, apparently new, roots. When possible, a new or "bud" root was taken for mycorrhizal examination in addition to the mature root. Any remaining roots, leaves, and reproductive structures were dried at 70° C to a constant weight for nutrient analysis. Bud and mature roots were not separated for nutrient analysis.

Replanted plants-Three plants were excavated and replanted in their original location on each of four dates. As with the harvested plants, there were two dates during the vegetative phase (5 June and 15 July), and two dates during the reproductive phase (5 September and 30 September). Plants were excavated a second time to determine

growth. Plants excavated during the vegetative phase were reexamined on 17 August, and plants excavated during the reproductive phase were reexamined on 31 October. This schedule allowed the second excavation to occur after 1 and 2 months of growth. Plants were removed from the soil, rinsed, photographed, measured, and weighed in the field as above. Care was taken to keep roots moist and return them to the soil quickly.

Plants were censused in 1998 to determine the survival and growth of transplanted individuals. Aboveground structures were measured in May and June. Plants that had not emerged by 16 June were excavated, photographed, weighed, and measured. None of the replanted plants flowered in 1998.

Mycorrhizal examination--Light microscopy-Roots that were examined for mycorrhizae were separated from the rest of the plant with a small portion of the hypocotyl. Previous studies indicated that infection was limited to the area at the base of the stem (Ames, 1921). Roots were placed in a 10% KOH solution for 24 hours to clear the root (Stoutamire, 1991). The root was divided into 0.5 cm blocks and hand sectioned; each block yielding 10 – 16 sections. Sections were stained with warm 0.05% trypan blue in lactophenol and rinsed once with plain lactophenol (Alexander and Hadley, 1984). Sections were mounted in distilled water and coverslips were ringed with several layers of clear nail polish. It was necessary to reapply nail polish to some slides as gaps

formed and caused some drying. In general, the sections from one 0.5 cm block were mounted onto two slides. Sections were not kept in serial order. There were 292 slides made from 22 plants.

One randomly-chosen slide from each block was used to determine fungal infection (N = 158). Each slide was assigned a random number and read in numerical order to reduce bias. Slides were read at 100X with a 10×10 mm^2 ocular grid (0.5 mm divisions) to determine quadrant location and to assist in cell counting. Mycorrhizal infection was estimated by counting all cortical cells in one systematically assigned quadrant of each root section. Cells were classified as uninfected, amorphous, or hyphal infection. The presence of infection in any area of the section was also noted.

Scanning electron microscopy (SEM)-Cleaned roots were immersed in phosphate-buffered 5% glutaraldehyde (pH = 6.8) after separation from the root system. Roots were cut into sections taking care to keep the tissue immersed and then fixed for a minimum of three hours. Tissue was then post-fixed in phosphate-buffered 1% osmium tetraoxide (pH = 6.8) for 1.5 hours. Fixation was followed with an ethanol dilution series (five steps from 30-100%) and then immersed in acetone before critical point drying with CO_2 as the transitional fluid. Some sections were cut after critical point drying. Specimens were

mounted and gold sputter-coated for 2 – 4 minutes with a Technics Hummer IV (Anatech, Ltd., Alexandria, VA).

Sections were viewed using a Phillips 515 scanning electron microscope (Eind Hoven, The Netherlands) at magnifications from 2.64 X 10^1 to 3.86 X 10^3. Pelotons were typically viewed at 2.0 X 10^2 to 4.0 X 10^2. Micrographs were made using Polaroid Type 55 positive/negative sheet film.

Nutrient analysis--Soil-During all plant excavations, soil was collected from immediately around the roots of the orchid. On the same day, a soil core was collected at the base of Helianthus rigidus (Asteraceae) as a non-mycorrhizal control. Core samples were likely not from the rhizosphere and therefore were not entirely equivelent to orchid soil samples. Helianthus rigidus is abundant at the S. cernua site and has a non-fibrous root system. Soil cores were placed in Whirl-pak bags, transported to the lab, and stored at 4° C until processed (not more than five days). Soil was sifted through multiple sieves, the smallest with a 1 mm pore size. Soil was dried at 60° C for 24 to 48 hours to a constant weight. Dry soil was analyzed for available phosphorus (Bray test) and total carbon. A 2M KCl soil extract was prepared for nitrate-N and ammonium-N determination. 4 g fresh weight of soil was added to 20 ml of 2M KCl. The soil solution was mixed on a rotary shaker for one hour. The suspension was allowed to settle for ten

minutes. The solution was transferred to 15 ml tubes and centrifuged at 4 000 RPM for five minutes at room temperature. The supernatant was poured into 20 ml scintillation vials and frozen until analyzed. All soil analyses were performed by the University of Nebraska-Lincoln, Department of Agronomy, Soil Testing Service (Lincoln, NE). Nitrate-N and ammonium-N values were expressed as μg analyte per gram dry soil using the following conversion: "in solution" value (mg/L) * 1 000 μg/mg * 0.02 L/ g soil dry weight.

Plant tissue-Plant tissue was dried at 70° C to a constant weight. The tissue was ground to a fine powder using a ball mill grinder (Model 5100 Spex CertiPrep, Metuchen, NJ). Due to small amounts of tissue, samples from some individual plants were combined to meet the mass required for the analyses. Total C, total N, and the C:N ratio were determined by micro-Dumas combustion elemental analysis. Total phosphorus was determined by continuous flow anion colorimetry of an ash/double acid extraction. Ground tissue was sent to the University of Georgia, Institute of Ecology, Stable Isotope Laboratory (Athens, GA) where extractions and analyses were performed.

Data analysis--All statistical analyses were performed using the Statistical Analysis System (SAS, 1990) general linear model, t-test, correlation, means and univariate procedures. Student-Newman-Keuls (SNK) multiple comparison tests were

performed on significant main effects. Analyses were tested at $\alpha = 0.05$. Significant values are indicated by an asterisk in tables and figures. Details of each analysis are presented in the Results section. Means and standard errors are presented throughout. Figures were prepared with CricketGraph (version 1.3).

Plant status in 1997 was based on root volume and flowering history. Leaf area could not be used in 1997, as it was in 1996, because the data were incomplete. Root volume was estimated using the formula $2/3\pi r^2h$. The volumes of individual roots were summed for total root system volume. Three classes were defined: 1) flowered in 1997, 2) vegetative in 1997 with a root system volume greater than 1.5 cm^3, and 3) vegetative in 1997 with a root system volume less than 1.5 cm^3.

Few plants in the population flowered during the 1997 season. To increase the sample size of plants that were reproductive in 1997 for light microscopy and nutrient analysis, a single root from two plants in the replanted group that flowered and a new plant found in flower were harvested.

Hyphal and amorphous peloton counts were expressed as proportions to remove the effect of root size, i.e., counts were divided by the total number of cortical cells. The proportion of infected cells included the number of cells with hyphal and amorphous pelotons per total number of cortical cells. Only hyphal pelotons are thought to be

biologically active (Curtis, 1939; Currah, 1991; Stoutamire, 1991). To estimate the amount of active infection, the number of hyphal pelotons was divided by the total number of pelotons. Proportions were arcsine transformed to improve normality. All infection proportions were analyzed separately.

Results

Herbarium specimens--Antlfinger and Wendel (1997) found that S. cernua must reach a critical aboveground size before plants will flower. Herbarium specimens represent a unique sample because usually the plants have flowered and are therefore robust. All selected herbarium specimens were from prairie habitats and were not in leaf, but a developing shoot was visible in addition to the flowering stalk. For this sample, the mean total inflorescence height was 263.14 ± 3.81 mm (N = 257), mean flower number was 9.18 ± 0.23 (N = 257) per rank and mean root number was 3.07 ± 0.08 (N = 257). With the exception of four herbarium specimens collected from the Nine-Mile Prairie population in 1985, few plants had unbroken roots. Average root system volume of these four plants was 1.48 ± 0.068 cm^3.

Shoot morphology--Plant excavations allowed observations of both root and shoot development. In 1997, leaves of the basal rosette first appeared aboveground in April

and all leaves had senesced by the end of July. Of the three to five leaves of the basal rosette, one was noticeably smaller and may represent an overwintering leaf. After the senescence of the basal rosette, the developing overwintering shoot became visible. One to two small leaves then developed. The shoot was not heavily pigmented and showed an increase in pigmentation at the distal end as it lengthened and approached the soil surface. When two leaves were present on the shoot, only one was pigmented and expanded. By September, some overwintering leaves had reached the surface. The overwintering shoot of flowering plants tended to be smaller than that of vegetative plants until flowering was terminated.

The developing inflorescence became visible aboveground in early August. In a plant that later flowered, no belowground structures were noted that indicated flowering would occur. Flowering in the Nine-Mile Prairie population of S. cernua has been recorded as early as August, but in 1997, open flowers were not observed until September. Of the four plants that flowered in 1997, three of were included in this study.

Root morphology--The roots of S. cernua were often entangled with the fibrous root systems of neighboring grass species. Some roots were oddly shaped and appeared to have grown in response to these space limitations. Several times the excavation of a "single" orchid aboveground disclosed multiple plants belowground. These roots were in

close proximity and appeared to have divided belowground. Vegetative propagation in Spiranthes by root separation may occur when plants are dormant (Brickell and Zuk, 1996).

The proportion of total biomass in the root system varied through the growing season: 0.816 ± 0.05 for plants in leaf (N = 7), 0.979 ± 0.01 for plants with a developing shoot (N = 7) and 0.556 for plants with an inflorescence (N = 1). The biomass of roots used for mycorrhizal examination was estimated with the regression equation: dryweight = 0.110333 * freshweight + 0.0019 (r^2 = 0.8504, P = 0.0001). The biomass of leaves was obtained from the regression equation: dryweight = 0.090409 * freshweight - 0.000171 (r^2 = 0.8299, P = 0.0006). As with many herbaceous perennials, a large proportion of the biomass of S. cernua is belowground.

On average the root system of S. cernua was composed of 3.45 ± 0.29 (N = 33) roots. There were young and old mature roots, bud roots, and shriveled roots, possibly from the previous year. Young and old mature roots were the same size, but young roots were lighter in color and had no evidence of herbivory. Emerging bud roots were observed in plants excavated on 29 April. By May, bud roots ranged in length from 2.51 to 19.16 mm. They continued to increase through July, but no bud roots were found after

July. The "disappearance" of bud roots suggests that not all buds develop into mature roots.

Temporal changes in the root system--An obvious change in the root system was the change in root number. There was a significant decrease in mean root number between the vegetative and reproductive phases (Fig. 1). This change is likely due to the senescence of bud roots. Plants excavated during the vegetative phase had young and old mature roots, as well as bud roots. Plants that were reexamined in August had only young mature roots present.

The roots of S. cernua are storage organs for both water and the products of photosynthesis. Therefore, the size of the root system was expected to reflect the changing resource demands of the plant during the growing season. To detect these changes, plants were excavated May through September. Root system volume was used to estimate root system size. Temporal changes in root system volume were analyzed with ANOCOV using leaf area in 1996 as the covariate. There was no significant effect of time on root system size (Table 1) whether based on month or growing season phase. This is particularly interesting since there was a significant decrease in the number of roots between the vegetative and reproductive phases of the growing season. Apparently

Figure 1. Change in mean root number during the vegetative and reproductive phases of the growing season. Both bud and mature roots are included. Bars indicate one SE of the mean. Sample sizes were N = 18 in the vegetative phase and N = 15 in the reproductive phase.

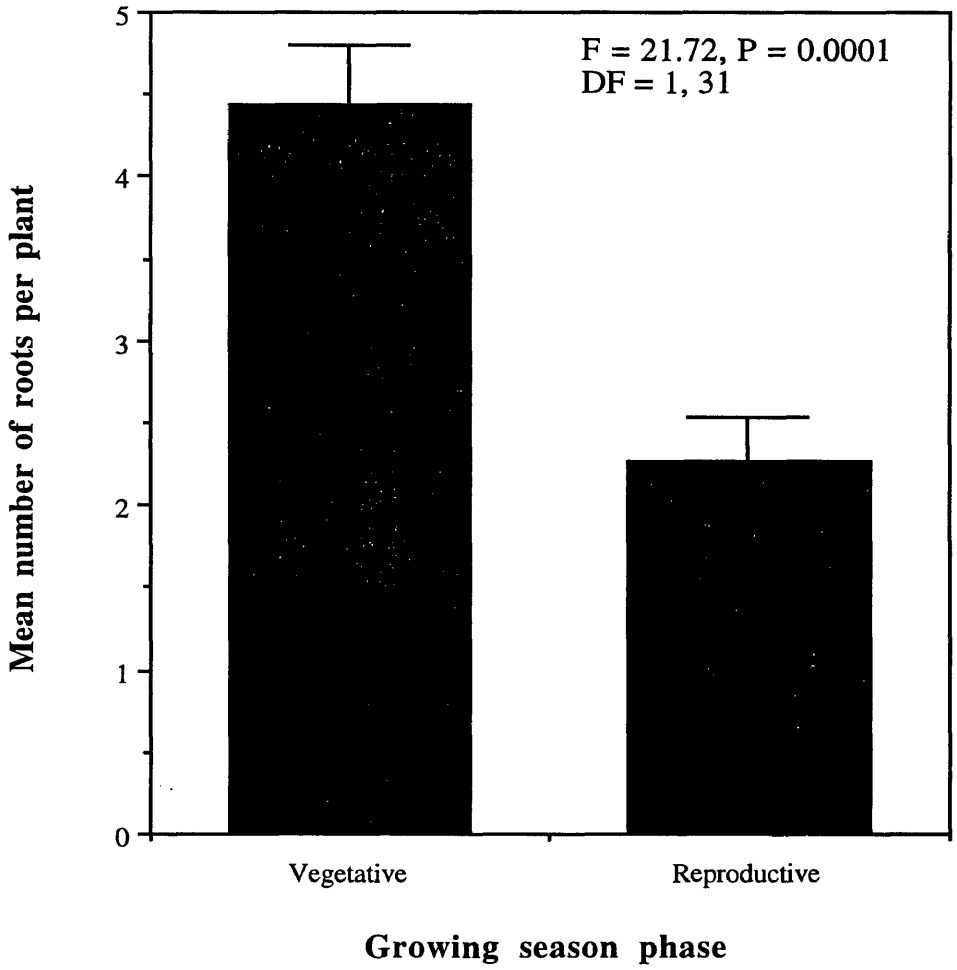

F = 21.72, P = 0.0001
DF = 1, 31
Mean number of roots per plant
5
4
3
2
1
0
Vegetative
Reproductive
Growing season phase

Table 1. ANOCOV of the effect of time (month or phase) on root system volume. Leaf area in 1996 is the covariate.

Source	DF	F	P
Month	4, 1	0.79	0.3583
Covariate	1, 16	0.08	0.7862
Phase	1, 1	1.13	0.3001
Covariate	1, 19	0.89	0.3582

the bud roots make a small contribution to the total root system volume. It is also possible that the small sample sizes prevented detection of differences in root system volume. Further, because the plants selected at each date represented different life history and size categories, variation at each date was maximized.

Significant differences in root system volume were found among plants with different reproductive histories and sizes (one-factor ANOVA, $F = 5.87$, $P = 0.0094$, $DF = 2, 21$). Plants that were vegetative and large in 1996 had a significantly larger root system volume than plants that were vegetative and small (Fig. 2). The root system of plants that reproduced in 1996 was intermediate in size. This may reflect a cost of flowering.

In order to study changes in the root system of the same individual, a group of plants were excavated and reexamined at later dates. The change in root number and root system volume between the first and second excavations was analyzed with a two-factor ANOVA to assess the effect of excavation regime (one or two months between the first and second excavation), growing season phase (vegetative or reproductive) and their interaction. There was a significant decrease in root number between the vegetative and reproductive phases, but excavation regime had no effect (Table 2). Root number decreased by an average of 2.83 ± 0.98 during the vegetative phase, but there was no

Figure 2. The effect of status in 1996 on mean root system volume in 1997. Status in 1996 is based on leaf area (plant size) and reproduction in 1996. Bars represent one SE of the mean. Means with the same letter are not significantly different. Sample sizes were N = 5 for flowering, N = 10 for large vegetative and N = 7 for small vegetative.

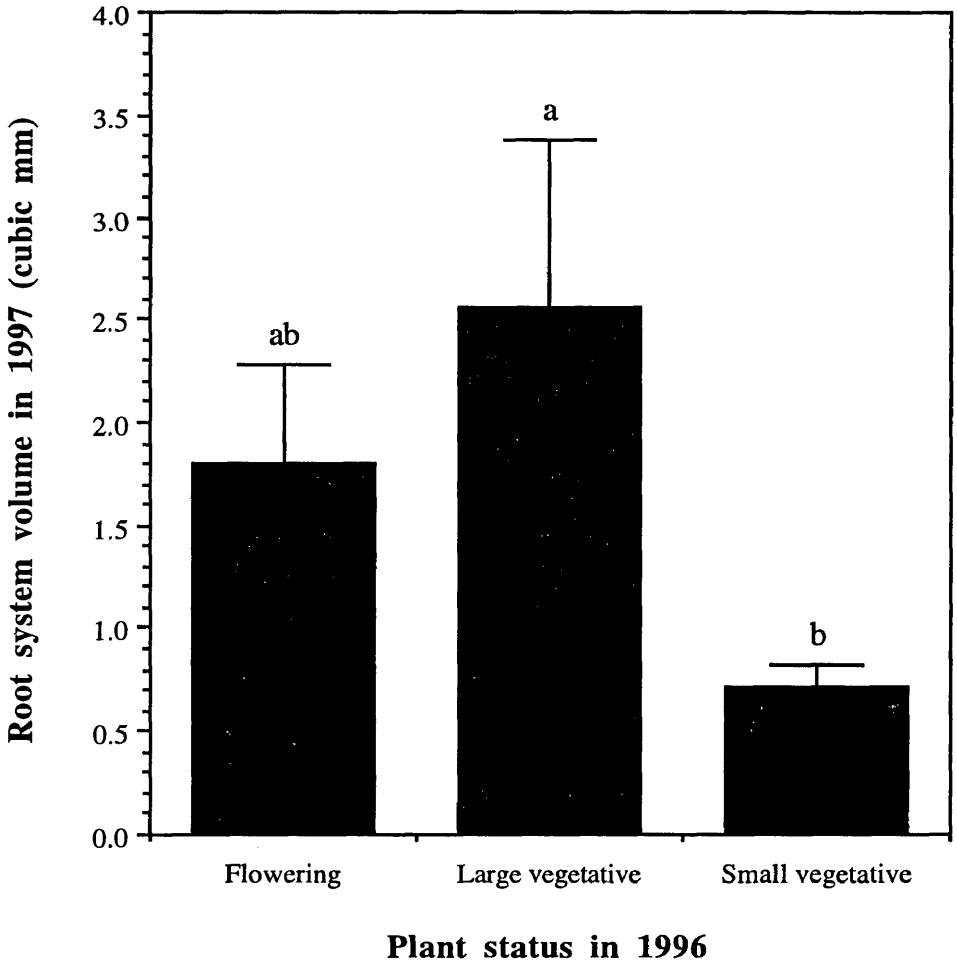

Root system volume in 1997 (cubic mm)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
Flowering
Large vegetative
Small vegetative
Plant status in 1996

Table 2. Two-factor ANOVA of the effect of growing season phase and excavation regime (one or two months between excavations) on the change in number of roots between the first and second excavations.

Source	DF	F	P
Phase	1, 8	12.57	0.0076*
Excavation regime	1, 8	3.52	0.0974
Interaction	1, 8	3.52	0.0974

Table 3. Two-factor ANOVA of the effect of growing season phase and excavation regime (one or two months between excavations) on the change in root system volume between the first and second excavations.

Source	DF	F	P
Phase	1, 8	0.00	0.9604
Excavation regime	1, 8	0.91	0.3684
Interaction	1, 8	0.52	0.4911

change in root number between excavations during the reproductive phase. Changes in root number during the vegetative phase were similar to those observed in plants that were destructively sampled. Change in root system volume was not affected by either excavation regime or by growing season phase (Table 3).

All plants excavated and replanted during the vegetative phase emerged in 1998 and grew normally. However, the plants excavated during the reproductive phase did not. To determine if these plants were dead or dormant, they were excavated on 16 June 1998. Five of the six plants were alive. Shoots appeared etiolated and were found growing horizontally, as well as vertically, in the soil. Average shoot length was 84.68 ± 0.92 mm. It is possible that by exposing shoots late in the season a developmental cue was altered, or that soil upheaval caused some mechanical interference with normal shoot expansion. The sixth plant was not found and a gopher mound was observed adjacent to its flag.

Root internal morphology--Several layers of cells were visible in S. cernua root cross-sections. Cells of the epidermis had scalariform thickenings on the inner cell wall as described by Stern et al. (1993). Projecting from the epidermis were epidermal hairs. Epidermal hairs appeared to be elongated epidermal cells and also had scalariform thickenings. The hairs tended to tear in a pattern consistent with these thickenings. Stern

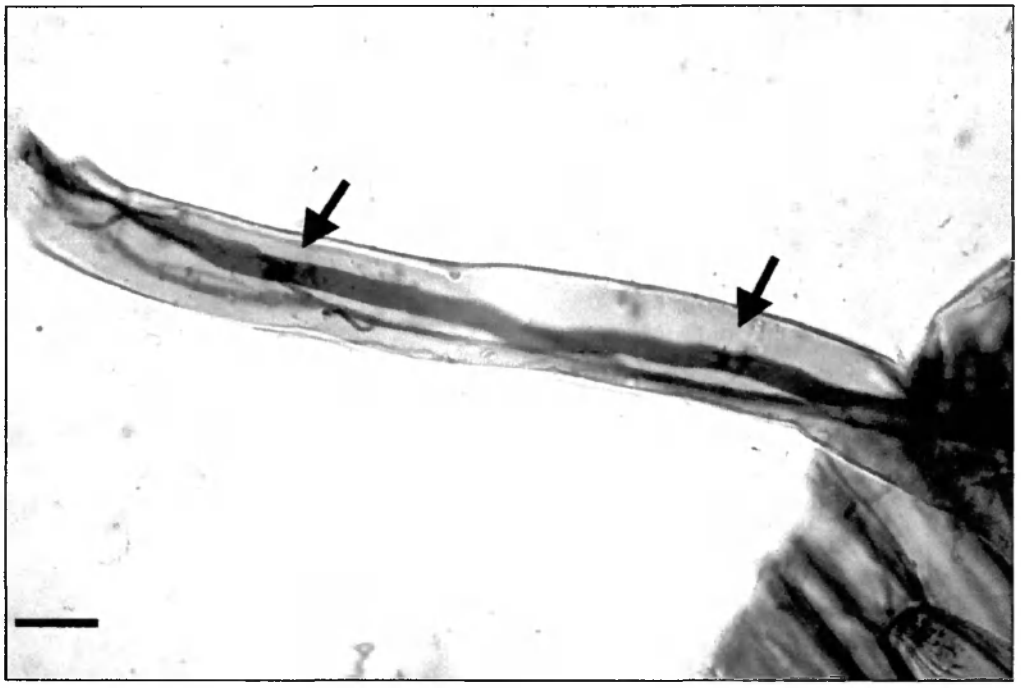
et al. (1993) suggested the thickenings add support and are needed because the roots have thin epidermal cell walls. Cortical cells were polygonal and homogeneous. The cortex radius was generally 9 – 17 cells wide. The endodermis and stele were well defined.

Other structures viewed within the root included raphid bundles, starch granules, and starch granule aggregations known as spiranthosomes (Stern et al., 1993). Starch granules and spiranthosomes were visible with SEM. Because stained roots were cleared with 10% KOH, no starch was observed in sections observed with light microscopy.

Mycorrhizae--The mycorrhizae found in S. cernua were similar to mycorrhizae described from other orchid species. Fungal septa were observed with light microscopy (Fig. 3) indicating the fungus was not a zygomycete, such as VAM, which are commonly found in prairies. Two forms of orchid mycorrhizae were observed with both light microscopy and SEM. "Hyphal" pelotons had distinct filaments in a coiled bundle within root cortical cells (Figs. 4 - 5) and "amorphous" pelotons lacked distinct hyphal filaments but did have filaments "anchoring" the pelotons within the cortical cells (Figs. 6 - 7). We assumed hyphal pelotons were biologically active and amorphous pelotons were nonfunctional (Curtis, 1939; Stoutamire, 1991). Amorphous pelotons, though non-functional, persist within the cortical cells providing a history of infection for the life of

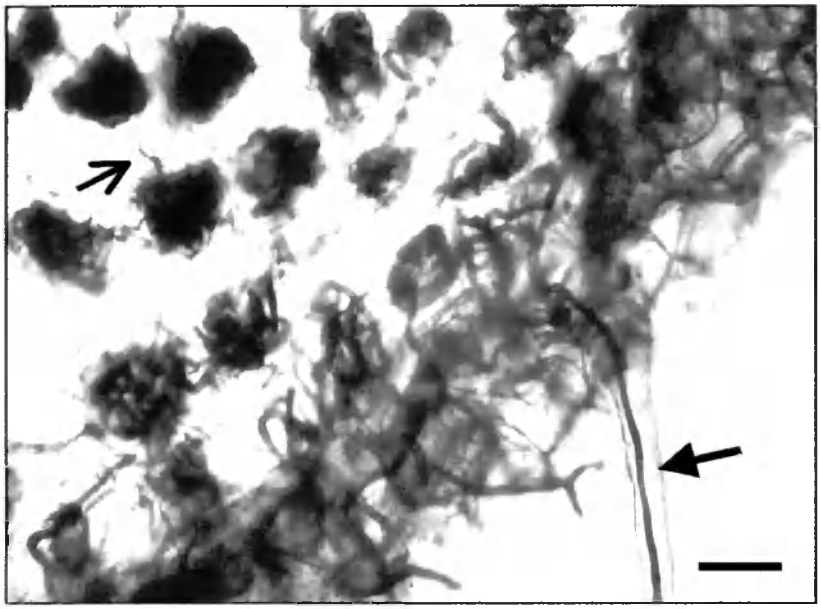
Figure 3. Infected root epidermal hair showing fungal septa (arrows). Bar = 15 μm.

3

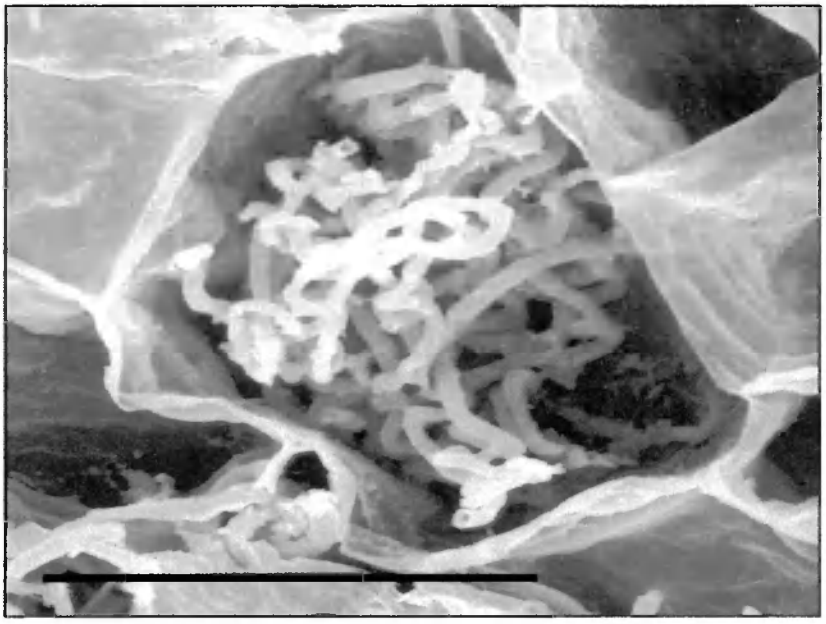


Figures 4 – 5. Hyphal pelotons. Individual hyphae are distinct within the cortical cells. (4) Infection viewed with light microscopy. Note that the infected epidermal hair (closed arrow) is adjacent to an infection site. Cell to cell infection is visible (open arrow). Bar = 30 μm. (5) Peloton viewed with SEM. Bar = 100 μm.

4

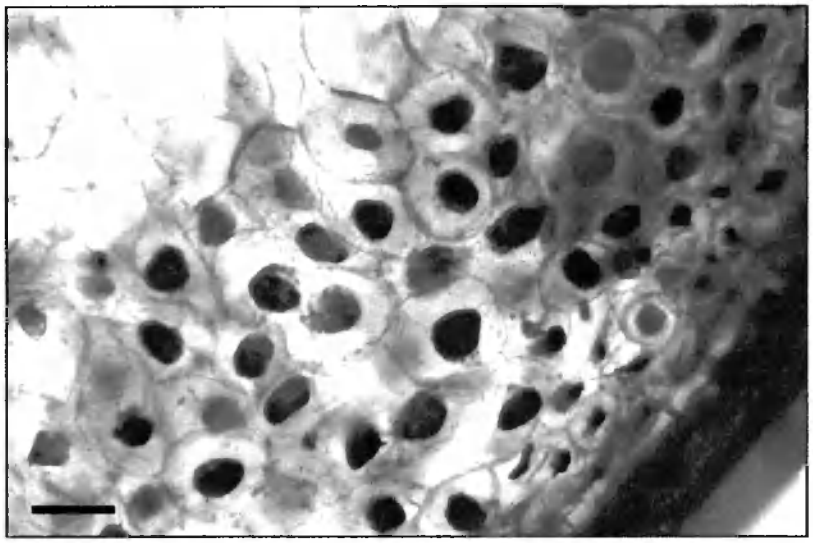


5

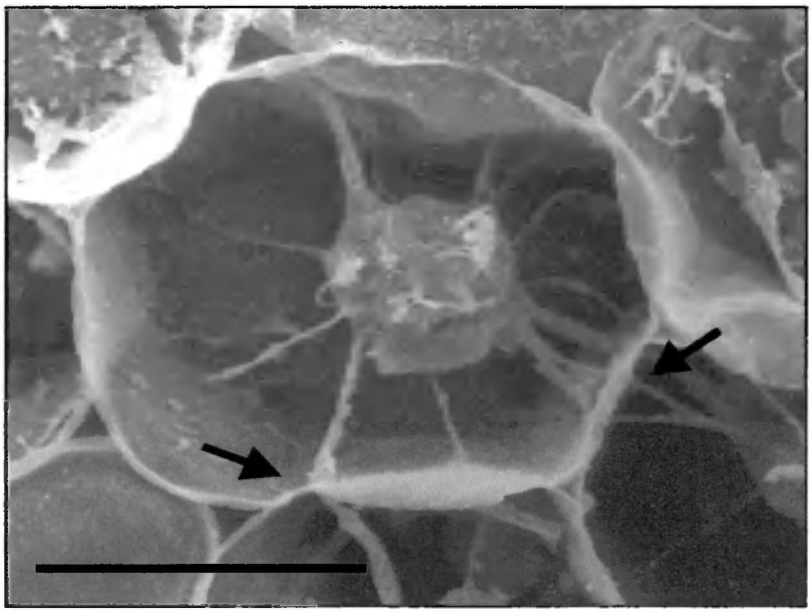


Figures 6 – 7. Amorphous pelotons. Fungal hyphae are digested and no longer distinct. Filaments anchoring the peloton within the cortical cell are clearly visible. (6) Infection site viewed with light microscopy. Pelotons are synchronous in the infection site. Bar = 65 μm. (7) Peloton viewed with SEM. Note the cell to cell infection (arrows). Bar = 100 μm.

6



7



the root. Cortical cells that were infected more than once were noted in S. cernua and have been observed in other orchids (Harley, 1959; Rasmussen, 1995).

Roots are thought to be infected through epidermal hairs or at the base of hairs (Peterson and Currah, 1990; Szendrak, 1997). Infected epidermal hairs were observed (Fig. 8). Infection within the root was restricted to the cortex. Figures 4 and 7 show cell-to-cell hyphal connections, demonstrating how infection is spread within the cortex. There was always a layer of cells directly under the epidermis where pelotons were not found. Pelotons were also never observed in cells with raphid bundles.

Mycorrhizal infection was observed along the entire length of the roots. The mean proportion of cortical cells of all roots infected with mycorrhizae was 12.1 ± 0.01% (7.4 ± 0.01% with amorphous pelotons and 4.7± 0.01% with hyphal pelotons). When buds and mature roots were analyzed separately, an average of 31.1 ± 0.04% of bud root cortical cells were infected while 10.4 ± 0.01% of mature root cortical cells were infected. Root buds had significantly higher proportions of all infection types than did mature roots (Table 4).

In the Nine-Mile population, biologically-active hyphal infection was found in all mature roots but at very low levels (2.13 ± 0.005%). Figures 9 and 10 show profiles of cortical cell infection for a "typical" mature root and bud root, respectively, from the

Figure 8. Infected epidermal hairs. Section of orchid root showing a group of infected epidermal hairs (arrows). Bar = 65 μm

8

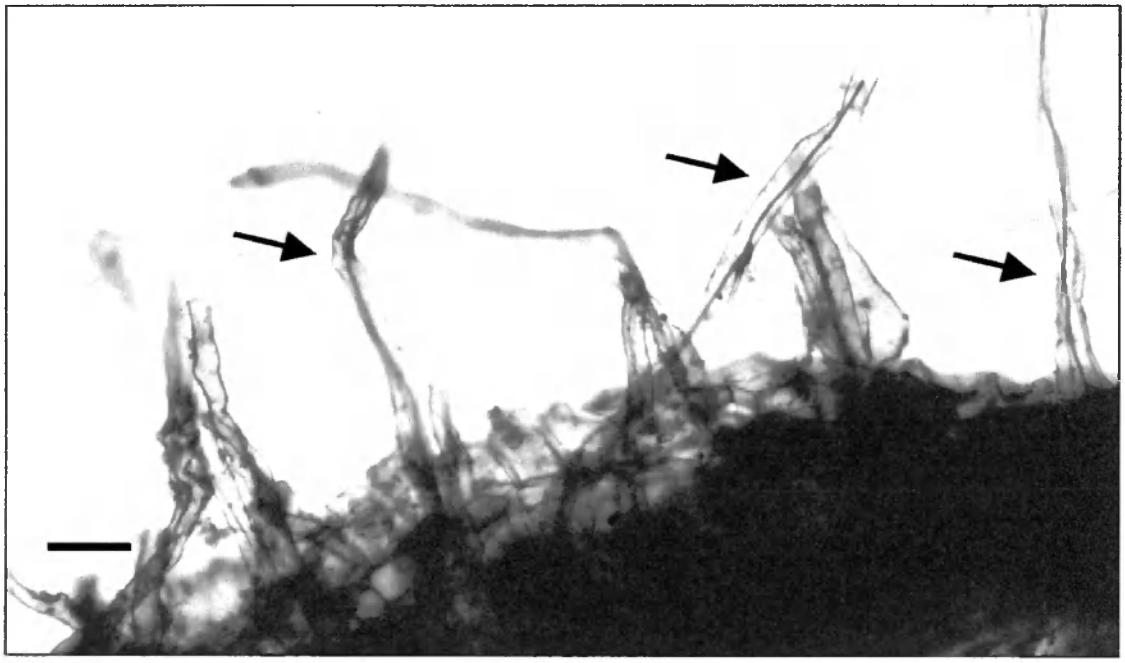
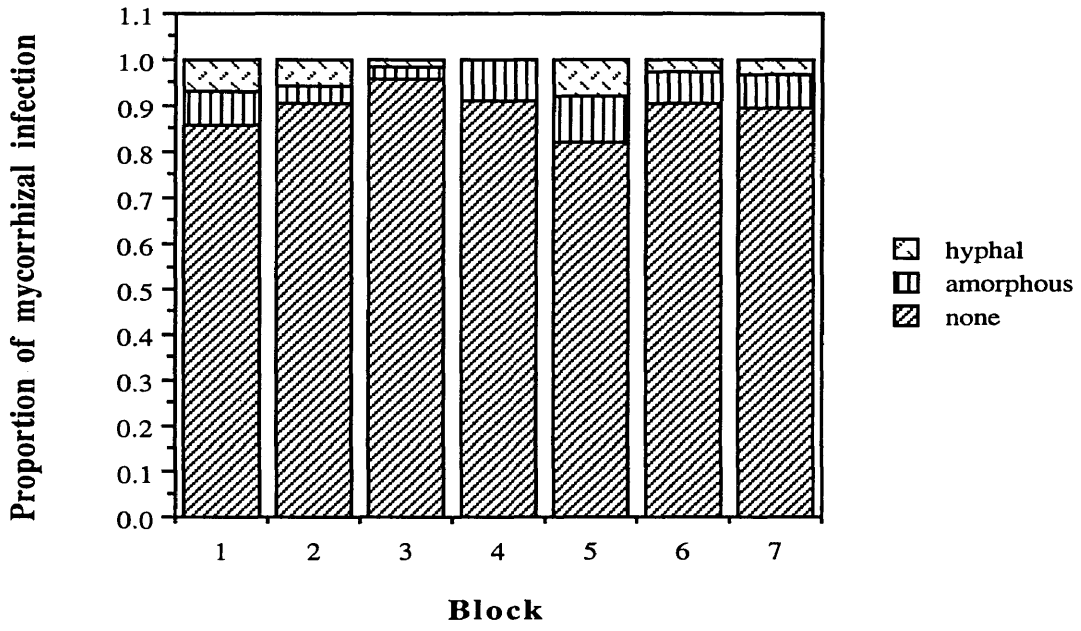


Table 4. One-factor ANOVA of the effect of root type (bud or mature) on cortical cell infection proportions.

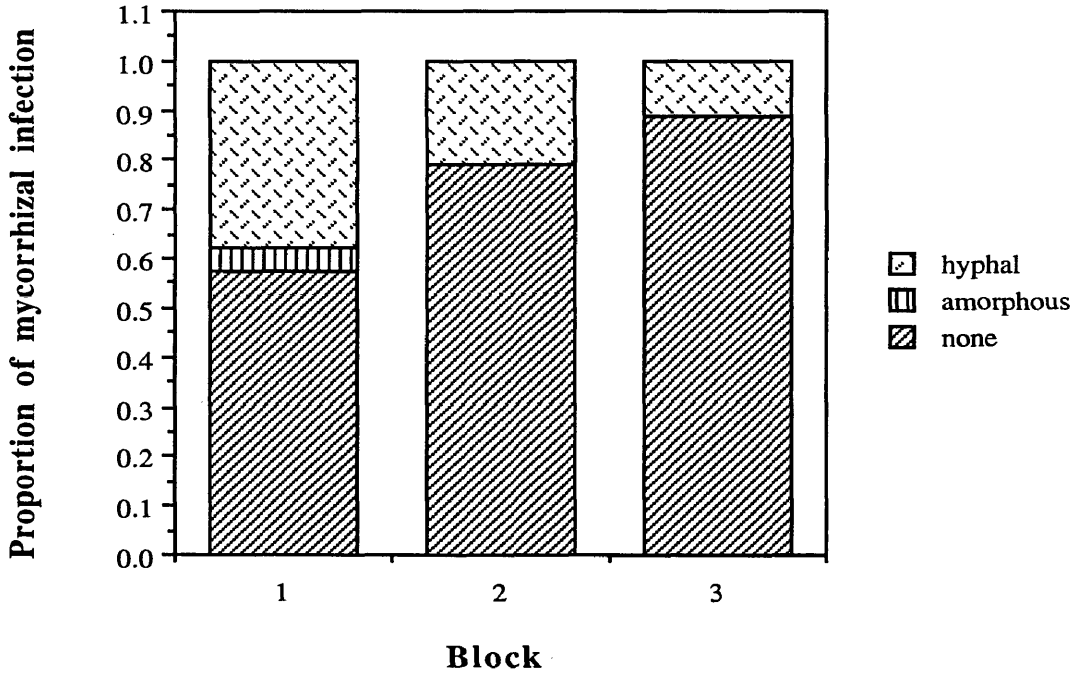
Infection type	DF	F	P
Hyphal	1, 649	139.26	0.0001*
Amorphous	1, 649	4.43	0.0357*
Total	1, 649	47.41	0.0001*
Hyphal/Total	1, 344	84.54	0.0001*

Figures 9 – 10. Variation in infection type along the length of the root for a bud and mature root from the same plant (# 139). A block equals 0.5 cm. Block one is at the proximal end of the root. (9) Mature root. The majority of the cortical cells in the mature root are uninfected. (10) Bud root. Bud roots have a high proportion of hyphal (living) infection.

9


Proportion of mycorrhizal infection
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
7
Block
hyphal
amorphous
none

10


Proportion of mycorrhizal infection
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
Block
hyphal
amorphous
none

same plant. The hyphal infection in blocks 1 – 3 and blocks 5 – 7 probably represents two separate infection events in the mature root (Fig. 9). The mature root had a mean hyphal infection of 3.74 ± 0.01% versus 32.41 ± 0.06% for the root bud. The mechanism controlling infection in the two types of roots requires further investigation.

Pattern of mycorrhizal infection along the length of the root--Richardson et al. (1993) examined the mycorrhizae of tropical epiphytic orchids and found that the percent infection varied along the length of the root. Differences in infection proportions among 0.5 cm blocks were examined to try to find a pattern of exogenous infection. Infection proportions were analyzed with a one-factor ANOVA separately for each root. Twelve of nineteen roots had significant differences among blocks for the cortical cell infection proportions (Table 1, Appendix C). A greater number of roots (eight of nineteen) had significant differences among blocks in amorphous infection proportions than in hyphal infection proportions (seven of nineteen). Though pelotons were found throughout the root, no distinct pattern of infection along the length of the root was detected.

Seasonal variation in mycorrhizal infection--Mycorrhizae are known to be important in the acquisition of nutrients and water by roots of the host plants. Because the dependence of S. cernua on root storage may vary within the growing season, it is possible that the level of mycorrhizal infection would also change seasonally.

Mycorrhizal infection might show a seasonal pattern due to the influence of the host plant, the fungus, or some interaction of the two (or more) species. To detect changes in infection proportions over time, a nested ANOVA was performed with harvest date as the main effect. Nested factors included plant within harvest date and block within plant. Harvest date was not a significant effect for any of the infection proportions (Table 5). Except for the hyphal proportion, differences among plants were not significant. Block was significant for all of the infection proportions. A nested ANOVA was also used to examine differences between the vegetative and reproductive phases of the growing season (Table 2, Appendix C). As above, plant nested within phase and block nested within plant were tested. Plants were replicates for all infection types and differences among blocks were significant for all infection proportions. There was no significant effect of season on any of the infection proportions.

Effect of life history and season on amorphous infection--Masuhara and Katsuya (1992) observed a decrease in active infection at the time of flowering in S. sinensis. If infection in S. cernua responds similarly, plants which flower would have a lower number of pelotons than vegetative plants. To address this question, plants that flowered in 1996 were analyzed. Too few plants flowered in 1997. Since flowering occurred the previous year, amorphous pelotons would reflect this association better than hyphal

Table 5. Nested ANOVA of the effect of harvest date on cortical cell infection.

Infection type	Effect	DF	F	P
Hyphal[a]	Harvest date	3, 18	1.09	0.3796
	Plant	18, 99	2.79	0.0006*
	Block	99, 530	.94	0.0001*
Amorphous[b]	Harvest date	3, 18	1.64	0.2145
	Plant	18, 99	1.41	0.1455
	Block	99, 530	3.64	0.0001*
Total[c]	Harvest date	3, 16	0.27	0.8461
	Plant	16, 20	0.89	0.5896
	Block	20, 609	6.30	0.0001*
Hyphal/Total	Harvest date	3, 12	1.17	0.3614
	Plant	12, 16	1.12	0.4107
	Block	16, 311	4.26	0.0001*

[a] number of hyphal pelotons/ number of cortical cells
[b] number of amorphous pelotons/ number of cortical cells
[c] hyphal + amorphous pelotons/ number of cortical cells

pelotons, which may last only a couple of weeks. An ANOCOV of the effect of life history (vegetative or flowering) was performed. Harvest date was chosen as the covariate to account for the different time periods the plant was in the soil and therefore exposed to additional fungal infection. Neither life history nor harvest date were significant (Table 6). The function of mycorrhizae in adult orchids is still poorly understood. This analysis suggests that flowering has no lasting effect on mycorrhizal infection. However, small sample size may have contributed to a lack of significance.

Plant nutrient concentrations--Total carbon, nitrogen, and phosphorus were determined for the roots, leaves, peduncle, axis, and flowers and fruits. Nutrient levels are summarized in Table 7. On average root and shoot C were nearly equal (root / shoot C: 0.93 ± 0.026) while nitrogen concentrations were higher in leaves (root / shoot N: 0.65 ± 0.075). The C:N ratio for roots was almost double that of leaf C:N because root N levels were much lower than leaf N levels (C:N root: 17.15 ± 1.47, $N = 18$; C:N leaf: $9.38 + 0.36$, $N = 8$). It is possible that leaves have higher nitrogen levels in response to the physiological demands of photosynthesis.

Seasonal variation in root nutrient levels--Through the growing season, the root system of S. cernua must respond to a variety of resource demands. Like other

Table 6. ANOCOV of the effect of life history status in 1996 (vegetative or flowered) on amorphous infection observed in 1997 with harvest date as the covariate.

Source	DF	F	P
Life history	1, 3	0.95	0.3486
Covariate	3, 13	0.00	0.9578

Table 7. Nutrient concentrations in S. cernua. Sample size is given in parentheses. Units are as follows: total C and total N: % per g dry weight; and total P: mg P per g dry weight.

Structure	Total Carbon	Total Nitrogen	Total Phosphorus
Leaf	41.64 ± 0.55 (8)	4.48 ± 0.18 (8)	---
Root	39.43 ± 0.59 (18)	2.58 ± 0.22 (18)	2.57 ± 0.24 (8)
Axis	42.10 ± 1.20 (3)	3.59 ± 1.30 (3)	---
Peduncle	43.85 ± 0.46 (2)	1.65 ± 0.20 (2)	1.26 (1)
Flowers & fruit	44.63 ± 0.36 (3)	4.24 ± 0.59 (3)	4.96 ± 0.6 (2)

perennials, growth of new leaves and roots probably depends on root system reserves. Later, when the leaves of the basal rosette are actively photosynthesizing, the roots function in storage. Root tissue nutrients were analyzed with a one-factor ANOVA to detect differences in nutrient levels related to harvest date. Root total nitrogen varied among harvest dates ($F = 7.78$, $P = 0.0027$, $DF = 3, 14$) as did the carbon to nitrogen ratio ($F = 4.96$, $P = 0.0150$, $DF = 3, 14$). The greatest difference in root nitrogen was between the first and second harvest dates on 19 May and 30 June (Fig. 11). There was no difference among dates for total carbon ($F = 1.08$, $P = 0.3908$, $DF = 3, 14$) or phosphorus ($F = 1.24$, $P = 0.3540$, $DF = 2, 6$). There was also no difference in nutrient levels between the vegetative and reproductive phases. Because most of the plants in the sample did not flower, a difference between the phases was not expected. Changes in the root system of non-flowering plants likely occurred during the vegetative season.

Soil nutrient concentrations--The soil nutrient status at Nine-Mile Prairie is described in Table 8. The S. cernua population is located on a gentle north-facing slope and the orchids in this study were enclosed in six of the seven plots, labeled A through G. Soil moisture and nutrient concentration variation among plots were analyzed with a one-factor ANOVA. Spatial variation in soil nutrient and moisture levels could confound

Figure 11. Seasonal variation in mean root total nitrogen. Bars indicate one SE of the mean. Means with the same letter are not significantly different. Sample size was N = 3 for each date.

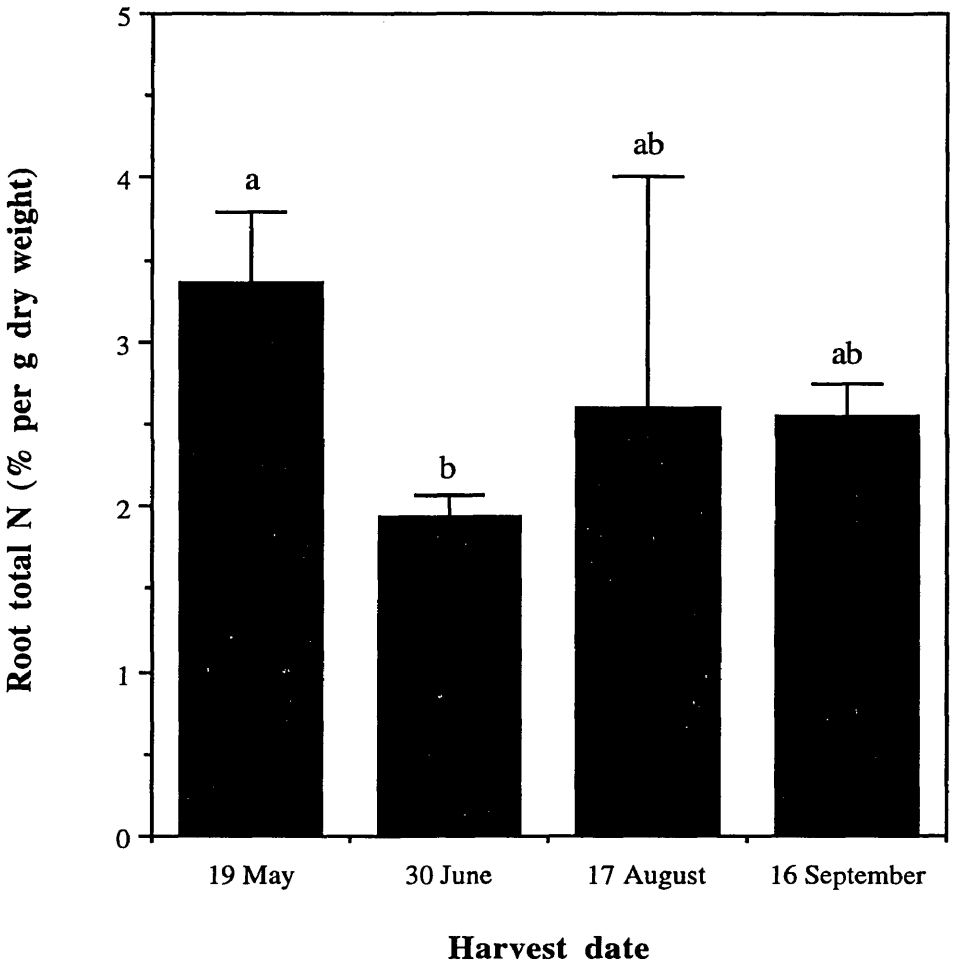

Root total N (% per g dry weight)
5
4
3
2
1
0
a
b
ab
ab
19 May
30 June
17 August
16 September
Harvest date

Table 8. Soil nutrient and moisture concentrations. Units are as follows: ammonium-N, nitrate-N, and phosphorus: μg/gds; total carbon and soil moisture: %.

Nutrient	N	Mean ± SE
Ammonium-N	49	4.54 ± 0.61
Nitrate-N	49	1.23 ± 0.11
Phosphorus (Bray)	49	5.62 ± 0.13
Total carbon	49	3.38 ± 0.07
Soil moisture	49	24.99 ± 0.89

interpretation of the role of mycorrhizae. No significant differences among plots were found for any of the soil nutrients or soil moisture (Table 9).

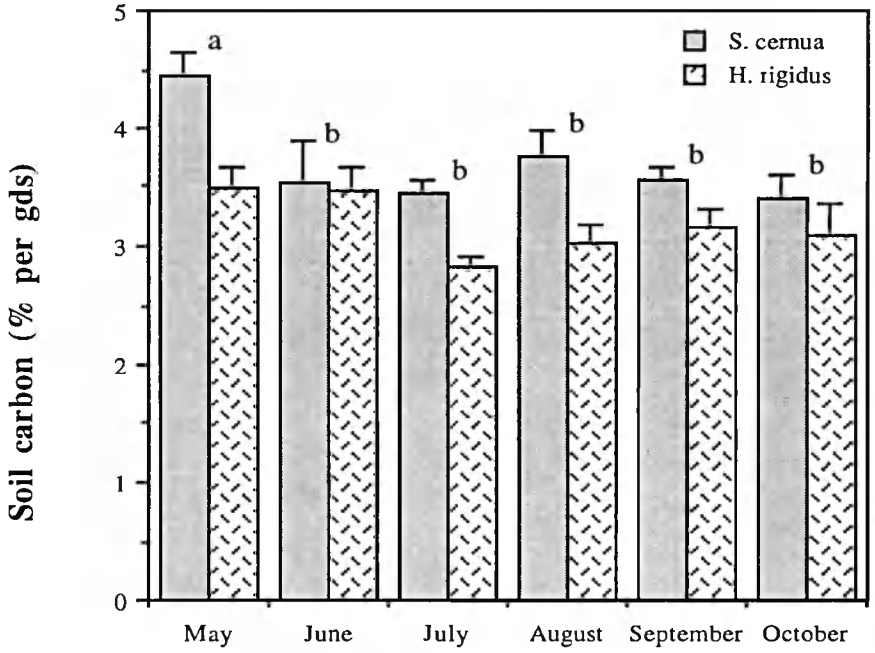
Comparison of soil nutrients near S. cernua and H. rigidus through the growing season--Soils vary spatially and temporally in nutrients and moisture. Mycorrhizae help their host plants with the acquisition of water and minerals and are able to search larger volumes of soil. Helianthus rigidus is not known to be mycorrhizal and provided a control for the mycorrhizal soil surrounding S. cernua roots. Both H. rigidus and S. cernua have non-fibrous root systems. Differences in nutrient levels between S. cernua and H. rigidus soil through the season were analyzed with a two-factor ANOVA with month and species as the main effects. There was a significant difference in soil carbon between the two species ($F = 25.26$, $P = 0.0001$, $DF = 1, 37$) but not for other nutrients (Figs. 12 – 16). Spiranthes cernua had a greater amount of carbon in the soil around the roots. Though not a significant difference ($F = 3.08$, $P = 0.0873$, $DF = 5, 37$), H. rigidus consistently had higher mean ammonium-N in every month except June. Monthly differences in soil moisture ($F = 12.09$, $P = 0.0001$, $DF = 5, 37$), total carbon ($F = 5.03$, $P = 0.0013$, $DF = 5, 37$), ammonium-N ($F = 10.11$, $P = 0.0001$, $DF = 5, 37$) and nitrate-N ($F = 6.12$, $P = 0.0003$, $DF = 5, 37$) were significant, but differences in phosphorus were not (Figs. 12 – 16). May, July, and October were the wetter months and September was

Table 9. One-factor ANOVA of the effect of location on soil nutrient concentration and moisture. Soil was taken from within six 10 ×10 plots (A through F).

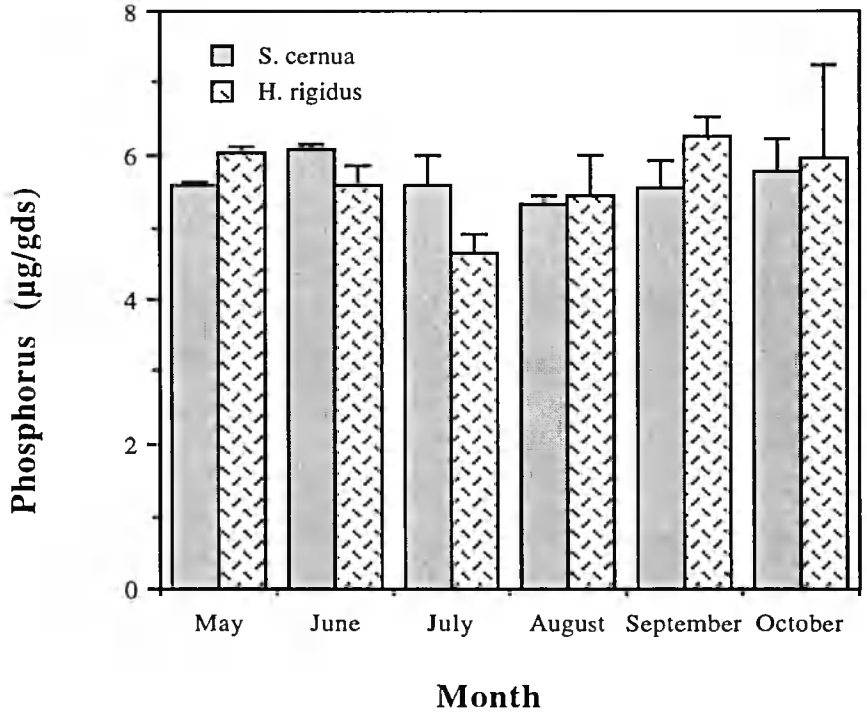
Nutrient	DF	F	P
Ammonium-N	5, 43	0.97	0.4487
Nitrate-N	5, 43	1.39	0.2472
Total carbon	5, 43	0.96	0.4524
Bray phosphorus	5, 43	1.76	0.1416
Soil moisture	5, 43	0.52	0.7593

Figure 12 – 16. Comparison of soil nutrients and moisture near the roots of Spiranthes cernua and Helianthus rigidus. Means ± SE are given. Monthly means with the same letter are not significantly different. Sample sizes were: May, N = 3 for both species; June, N = 3 for both species; July, N = 6 for both species; August, N = 3 for both species; September, N = 6 for both species; and October, N = 4 for S. cernua and N = 3 for H. rigidus. (12) Total carbon. Across all dates, total carbon was significantly greater in S. cernua soil. (13) Phosphorus (Bray). (14) Ammonium-N. (15) Nitrate-N. (16) Percent moisture.

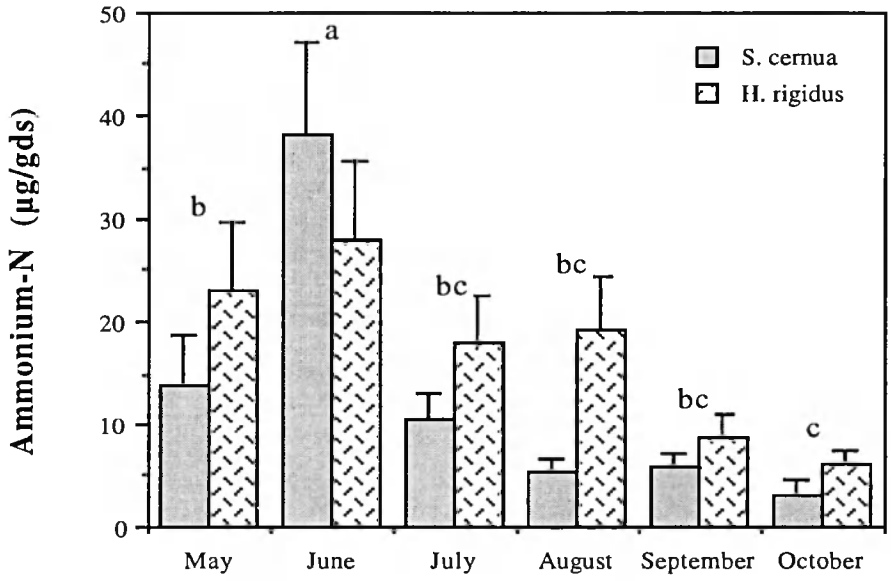
12


Soil carbon (% per gds)
5
4
3
2
1
0
a
b
b
b
b
b
S. cernua
H. rigidus
May
June
July
August
September
October

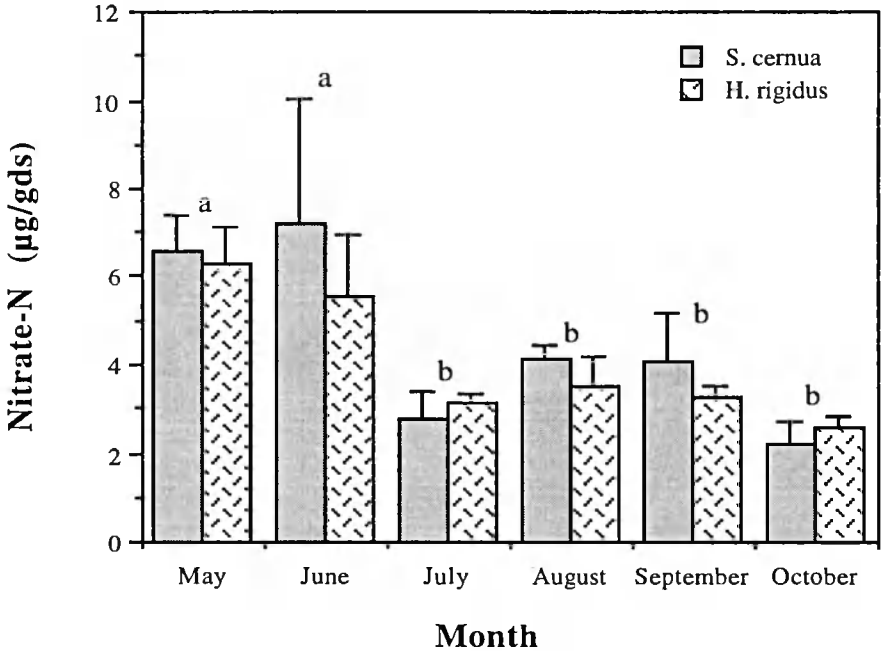
13


Phosphorus (µg/gds)
8
6
4
2
0
S. cernua
H. rigidus
May
June
July
August
September
October
Month

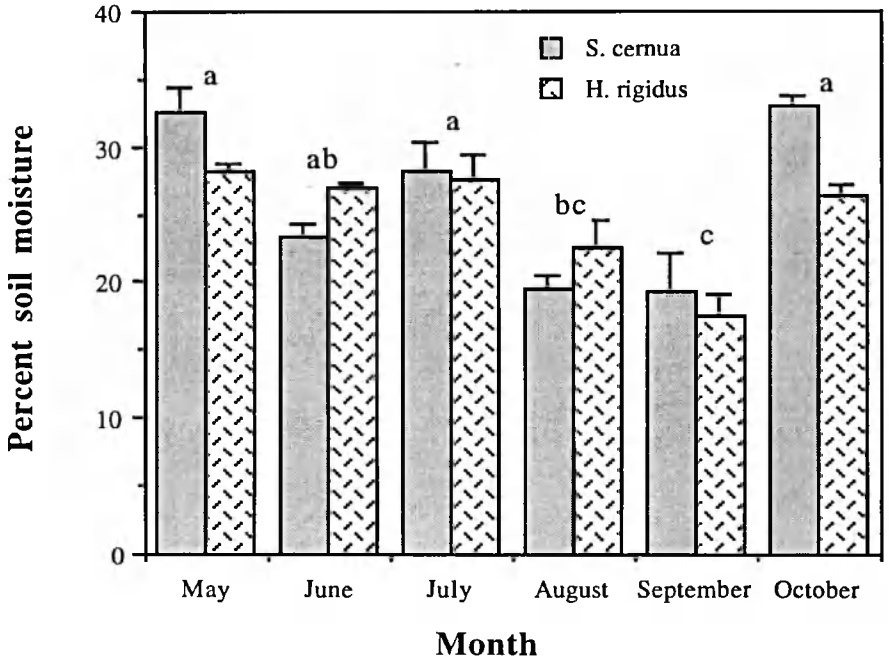
14


Ammonium-N (µg/gds)
50
40
30
20
10
0
S. cernua
H. rigidus
a
b
bc
bc
bc
c
May
June
July
August
September
October

15


Nitrate-N (µg/gds)
12
10
8
6
4
2
0
S. cernua
H. rigidus
a
a
b
b
b
b
May
June
July
August
September
October
Month

16


40
30
20
10
0
Percent soil moisture
S. cernua
H. rigidus
a
ab
a
bc
c
a
May
June
July
August
September
October
Month

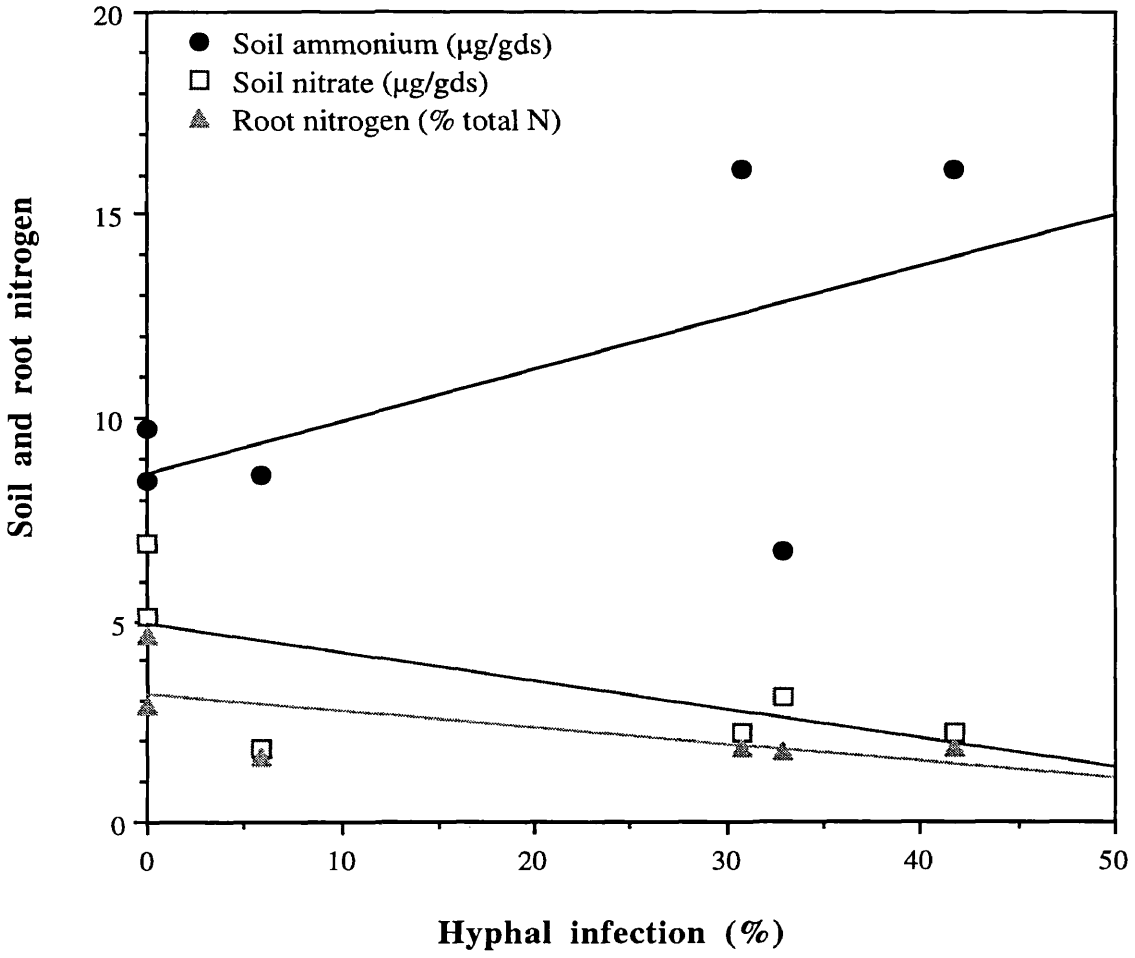
the driest. Consistent with results in other grasslands (DeLuca and Keeney, 1994; Turner et al., 1997), inorganic nitrogen levels decreased through the growing season. It is important to note that nitrogen mineralization was not measured. It is possible that soil inorganic nitrogen does not represent the amount of total available nitrogen. Soil carbon was highest in May and then decreased. Month by species interactions were not significant for any of the soil nutrients or moisture.

Nutrients and mycorrhizal infection--Plant and soil nutrients may be related to mycorrhizal infection if the fungus serves as a conduit between the soil and the plant. At Nine-Mile Prairie, S. cernua is photosynthetically active for only four to five months of the year and may depend on mycorrhizal fungi when belowground. Further, mycorrhizal infection of protocorms is followed by rapid growth and the same may be true for adult plants. Therefore, S. cernua may also be dependent on mycorrhizae during periods of aboveground growth such as the breaking of seasonal dormancy and flowering. Because so little is known about the mycorrhizal relationship in adult plants, and the possibility that different orchid species are dependent on mycorrhizal fungi to differing degrees, several hypotheses about the relationship between mycorrhizal infection and tissue and soil nutrient levels can be investigated.

First we hypothesized that the orchid would have increased mycorrhizal infection in soil with lower nutrient levels (Wetzel and van der Valk, 1996). Hyphal infection was analyzed in bud and mature roots separately. Of the six bud roots sampled, four had hyphal infection present. Three of the roots had over 30% of the cortical cells infected (the fourth root had about 5% infection). There was little difference among the soil samples from around the roots of these six plants in carbon or phosphorus and no correlation with hyphal infection was observed (Table 3, Appendix C). Bud roots were only present through early summer when soil ammonium and nitrate were at their highest levels. There were no significant correlations between hyphal infection in bud roots and soil ammonium-N ($r = 0.26471$, $P = 0.6122$, $N = 6$) or nitrate-N ($r = -0.52941$, $P = 0.2801$, $N = 6$). However, scatterplots of the data suggest an association between hyphal infection and inorganic soil nitrogen (Fig. 17). Plants with the highest soil ammonium-N levels also had the highest infection levels. In contrast, the two plants without hyphal infection occurred in soil with the highest nitrate-N levels: 5.085 and 6.925 μg/gds. The nitrate-N level of soil surrounding plants with infection ranged from 1.811 to 3.107 μg/gds. No correlation was found between soil nutrient levels and hyphal infection in mature roots (Table 3, Appendix C).

Next we hypothesized that roots with lower root nutrient levels would have higher levels of mycorrhizal infection. Bud and mature roots were not separated when tissue nutrient concentrations were determined. Therefore, root nutrient values are assumed to be representative of the entire root system of an individual plant. As above, hyphal infection was examined and a greater response was expected in buds. Root phosphorus was determined in only 8 plants due to the mass requirements of the analysis. There was no significant correlation observed between root phosphorus or root carbon and hyphal infection in either bud or mature roots (Table 3, Appendix C). As with soil nitrate-N, plants with greater root nitrogen appear to have less hyphal infection (Fig. 17). This probably reflects the plant's ability to meet its own nitrate requirements in soil with higher nitrate-N levels. However, no significant correlation was observed between hyphal infection in bud roots and total root nitrogen ($r = -0.52941$, $P = 0.2801$, $N = 6$). The lack of any detectable relationship between tissue nutrients and fungal infection may be explained by the fact that the mycorrhizae is tolypophagous (characterized by waves of infection, fungal lysis and reinfection) and probably necrotrophic (Rasmussen, 1995). The orchid receives mineral nutrients upon fungal digestion (when pelotons become amorphous). If pelotons are digested quickly, it would be difficult to observe the plant-fungal response to nutrient levels. Unfortunately, the persistence time of amorphous

Figure 17. Relationship of hyphal infection in bud roots to soil ammonium-N and nitrate-N and root total nitrogen. N = 6.


Soil ammonium (µg/gds)
Soil nitrate (µg/gds)
Root nitrogen (% total N)
Soil and root nitrogen
Hyphal infection (%)
0
5
10
15
20
0
10
20
30
40
50

pelotons is unknown and amorphous pelotons from multiple infection episodes are indistinguishable.

DISCUSSION

Root systems of terrestrial orchids have a variety of underground structures. In Spiranthes the root system is composed of several tuberoid roots which are thought to function in both storage and nutrient and water acquisition (Stern et al., 1993). Masuhara and Katsuya (1992) found two types of roots in S. sinensis: genuine roots which were primarily mycorrhizal and therefore involved in mineral and water acquisition, and tuberous roots which were primarily involved in storage. These roots differed in time of development and infection level but were morphologically indistinguishable. A similar situation may exist in S. cernua with the bud roots functioning as the mycorrhizal roots and the mature roots functioning primarily in storage. Bud roots developed and senesced within the vegetative season. Old mature roots senesced during the vegetative season, but development of storage roots was not observed. Excavation of S. cernua during dormancy (October through March) is needed to determine when tuberous roots develop.

In studying the root system of replanted plants, volume was a better predictor of size than root number due to root senescence. To estimate growth of the root system,

changes in the presence and absence of individual roots should be documented. This could be achieved if roots were individually marked. Also, the mini-rhizotron technique might be useful in following root development and senescence, especially when the prairie sod is frozen.

Spiranthes cernua tolerated excavation well and, with the exception of one plant (thought to have been destroyed by a small mammal), all plants were alive in 1998. Plants were able to survive the loss of a root either by accidental breakage or collection. This may be related to their ability to reproduce asexually through root splitting (Brickell and Zuk, 1996). These results indicate that S. cernua would be a good candidate for reestablishment through transplantation. In this study, plants were better able to survive transplanting during the vegetative or early reproductive phase.

Bud roots had a significantly larger proportion of infected cortical cells and higher proportions of active infection as compared to mature roots. Though no seasonal change in mycorrhizal infection was detected when all roots were analyzed, there was seasonality to mycorrhizal infection in S. cernua. The bud roots represent the majority of the infected tissue and the bud roots were only observed during the vegetative phase (i.e., the seasonality of the mycorrhizal infection is linked to the phenology of bud roots). In orchid protocorms, mycorrhizal infection is followed by rapid growth (Rasmussen,

1990), and it is likely that mycorrhizae are important for nutrient acquisition during vegetative growth of adult orchids as well.

Rasmussen (1995) notes that different orchid species rely on mycorrhizal fungi to different degrees in the adult stage. For example a non-chlorophyllous orchid, such as Corallorhiza sp., is entirely dependent on its fungal associate while a chlorophyllous orchid may not be. Levels of active mycorrhizal infection in S. cernua do not appear to be related to current nutrient levels in the root system. There are several possible explanations. First mycorrhizae may not respond to root nutrient levels but to some other factor such as temperature or soil nutrient concentrations. A second possibility is that because the relationship is necrotrophic, amorphous peloton numbers should be used for analyses instead of hyphal pelotons. Unfortunately, the persistence time of amorphous pelotons is unknown and different infection events would be indistinguishable. Finally, available carbon and nitrogen, rather than total values, might correlate better with mycorrhizal infection. It is possible that total carbon and nitrogen estimates masked changes in available carbon and nitrogen. Saarinen (1998) suggests that biochemically available carbon (non-structural carbohydrates) and nitrogen (as free amino acids) better represent plant carbon and nitrogen status than do total carbon and nitrogen.

Zimmerman (1990) found that total non-structural (TNC) carbon decreased in pseudobulbs of the epiphytic orchid Catasetum viridiflavum with the development of the new shoot. In another epiphytic orchid, Oncidium 'Goldiana', Hew and Ng (1996) found that TNC in pseudobulbs not on the current shoot decreased with inflorescence development. In S. cernua no change was found in root total carbon during the growing season. Because only one plant that flowered in 1997 was harvested, a decrease in carbon due to inflorescence production was not tested. An increase in root carbon was expected in the spring and early summer due to storage of photosynthates. It is possible that no change was observed in S. cernua through the growing season because total and not available carbon was measured.

Total nitrogen in roots of S. cernua was greater than total nitrogen found in the pseudobulbs of the epiphyte C. viridiflavum (Zimmerman, 1990) and the wintergreen terrestrial orchid Tipularia discolor (Whigham, 1984). Total nitrogen decreased during shoot development and growth in C. viridiflavum (Zimmerman, 1990). A similar trend was observed in S. cernua with a significant decrease in root nitrogen from May to June. This decrease is probably related to the development of new tissues and the production of photosynthates. Root nitrogen increased after leaf senescence in T. discolor (Tissue et

al., 1995) and in the epiphyte O. 'Goldiana' (Hew and Ng, 1996). A slight, but not significant, increase in root nitrogen was observed after leaf senescence in S. cernua.

In C. viridiflavum, pseudobulb total phosphorus increased when the plant was dormant and subsequently decreased during development and growth of the new shoot (Zimmerman, 1990). Hew and Ng (1996) found that phosphorus of the back pseudobulbs decreased in proportion to the increase in the newly developing pseudobulb in O. 'Goldiana'. No significant change in phosphorus during the growing season was observed in S. cernua roots. Whigham (1984) found that phosphorus decreased in the first year corm with inflorescence development and new corm initiation in T. discolor, however differences were not analyzed statistically. Whigham (1984) also found that nutrient uptake from the litter and soil was necessary for inflorescence development. It is possible that different strategies of mineral nutrient translocation and storage are utilized by terrestrial and epiphytic orchids. The epiphytic environment is marked by highly variable nutrient and water levels compared to a terrestrial environment. Terrestrial orchids may depend more on direct acquisition from the soil than nutrient translocation.

The soil at Nine-Mile Prairie was more acidic (range of pH = 5.4 – 5.95 vs. pH = 6.1) and had higher amounts of ammonium-N, nitrate-N and phosphorus than Konza Prairie Research Natural Area, a native tallgrass prairie in Riley Co., KS (Bentivenga and

Hetrick, 1992). Differences between Konza and Nine-Mile Prairies may be explained by differences in parent material and species composition. When soil ammonium-N and nitrate-N concentrations from Nine-Nile Prairie were compared with Allwine Prairie, a restored prairie in Douglas Co., NE, the Nine-Mile Prairie values were considerably higher (4.54 ± 0.61 NH_4-N μg/gds vs. 0.53 NH_4-N μg/gds and 1.23 ± 0.11 NO_3-N μg/gds vs. 0.273 NO_3-N μg/gds; Vinton, unpublished data). Allwine Prairie may have poorer quality soil due to prior cultivation. Since the Nine-Mile Prairie soil samples were taken from the rhizosphere of S. cernua, they may have higher nutrient concentrations.

Similar to other grassland studies (DeLuca and Keeney, 1994; Turner et al., 1997), soil ammonium-N and nitrate-N decreased through the growing season at Nine-Mile Prairie. Not only does inorganic nitrogen tend to decline through the growing season, it is often a limiting nutrient in prairies (Schimel et al., 1991). Spiranthes cernua has bud roots with high mycorrhizal infection at the time that soil inorganic nitrogen is highest. It is possible that S. cernua is able to meet its nitrogen requirements with a lower overall number of pelotons and, therefore, at a lower cost. Plants that did not have hyphal infection in the bud roots had the highest soil nitrate-N concentrations (Fig. 17), and it is likely these plants were able to acquire enough nitrogen without mycorrhizae.

Compared to the microbial flora, plants are better able to acquire nitrate-N than ammonium-N from the soil (Jackson, Schimel, and Firestone, 1989). No hyphal infection was found in bud roots of the plants in soil with the highest soil nitrate-N. Interestingly, plants with higher hyphal infection were associated with soils lower in nitrate-N and higher in ammonium-N. Stephen and Fung (1971) tested the nitrogen requirements of two mycorrhizal fungal isolates from the orchid Arundina chinensis and found the fungi were unable to use nitrate as a nitrogen source. Perhaps the fungus in S. cernua has similar preferences and is more abundant in soil with higher ammonium, which may result in higher mycorrhizal infection.

VAM are thought to be most important in phosphorus acquisition (Carlile and Watkinson, 1994; Smith et al., 1994) and movement of phosporus from the fungus to seedlings has been demonstrated in orchids (Alexander, Alexander, and Hadley, 1984). Though no change in soil phosphorus was observed over the growing season, changes in plant resource demands were expected. No correlation between hyphal and soil phosphorus was detected. It is possible that phosphorus is not limiting S. cernua at Nine-Mile Prairie.

Carbon transfer to protocorms and developing seedlings from mycorhizae is well documented in orchids (Smith, 1966, 1967; Alexander and Hadley, 1985), however, the

movement of carbon to adult chlorophyllous orchid plants has not been confirmed. Soil from around the roots of adult orchids was tested for total carbon, and soil from near the roots of the forb H. rigidus was used as a non-mycorrhizal control. The rhizosphere soil was expected to have high carbon values because root exudates are a major source of carbon addition in grassland soils (Anderson and Coleman, 1985). Though carbon concentrations showed no significant seasonal variation, S. cernua had significantly higher carbon values than H. rigidus. It is unlikely that the observed difference between carbon levels in soil from S. cernua and H. rigidus was due only to differences in soil sampling (i.e., rhizosphere vs. not) because significant differences were not observed in the other nutrients as well. Further research is necessary to determine why S. cernua had higher soil carbon than H. rigidus. No relationship between mycorrhizal infection levels and soil carbon was detected during the growing season.

Few plants flowered in 1997 and the effect of flowering on mycorrhizal infection and nutrient allocation was not examined. Information from herbarium specimens provided information on the root system morphology of flowering plants. It appears that plants must reach a critical size both above (Antlfinger and Wendel, 1997) and below ground before flowering can occur. Because S. cernua does not have bud roots at the

time of flowering, nutrient acquisition and storage is accomplished during the vegetative phase. No flower primordia were observed.

The terrestrial orchid S. cernua appears to have two types of tuberoid roots like its congener S. sinensis. Mycorrhizal infection was highest in bud roots, which are present during the vegetative phase of the growing season. Hyphal infection did not significantly correlated with root or soil nutrient levels. However, mycorrhizal infection did correspond to inorganic soil nitrogen and was highest when ammonium-N concentrations were highest, thus allowing the orchid to acquire nitrogen with a lower numbers of pelotons and, therefore, at a lower cost. This study suggests that orchid mycorrhizae may be important for nitrogen acquisition.

Further research is needed to completely understand the phenology of the root system and the importance of mycorrhizae during dormancy. The development of storage roots was not observed during the growing season and is expected to occur during the dormant phase. Although no trend was observed between highly mycorrhizal bud roots and carbon levels in the soil, it is possible that mycorrhizae are important in carbon acquisition during prolonged periods of dormancy.

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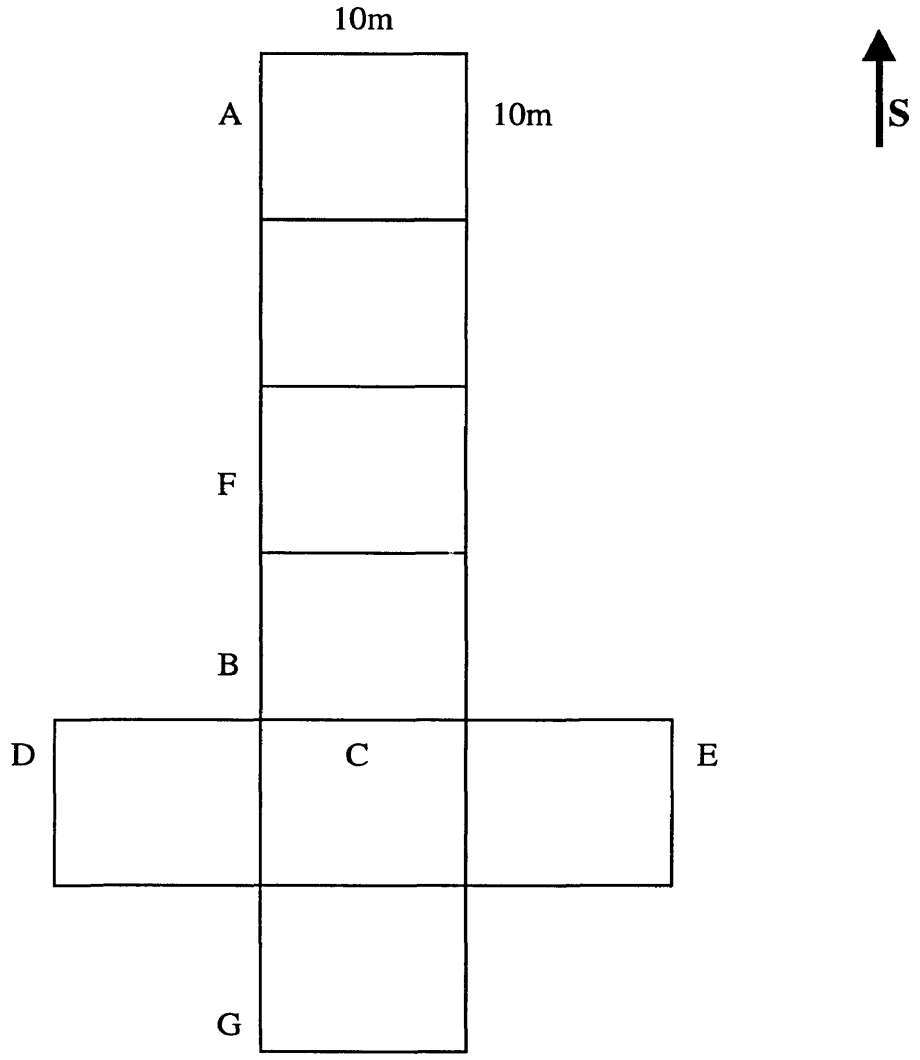
APPENDIX A

MAP OF PLOTS A-G



APPENDIX B

ROOT CLEARING AND STAINING TECHNIQUES

Protocol development--The roots of three seedlings (grown in symbiotic culture) and two plants harvested from Nine-Mile Prairie on 29 April 1997 (N = 6) were used to investigate root clearing and staining techniques. Four stains were tested: 0.05% trypan blue in lactophenol, cotton blue in modified lactophenol, 1% safranin in 50% ethanol, and 0.05% toluidine in dH_2O. Stains were applied with and without clearing the root. Due to the large amount of starch in the root, clearing was deemed necessary. Roots were cleared before sectioning. Two methods of clearing were tried:

1. Soaked in 10% KOH for 24 h at room temperature (Stoutamire, 1991).
2. Heated in 10% KOH for one hour at 90 °C. Washed with fresh 10% KOH and immersed in 10% H_2O_2 for 5 min at 20 °C. Rinsed once in deionized water. Immersed in 1% HCl for 3 min before staining (Alexander and Hadley, 1984; Reich and Barnard, 1984).

Roots subjected to the second clearing protocol did not maintain their structural integrity and therefore, was not used.

Stains were first tested on a pure culture of the fungus (strain TN29) used for symbiotic seed germination (Antlfinger, unpublished data). Hyphae were placed on microscope slides and exposed to the stain at room temperature and on a hot plate set on low (dial set at 2). Specimens were removed from the hot plate when most of the stain had evaporated. In general, heating was not effective. The results of hyphal staining at room temperature are summarized below:

1. Trypan blue: positive staining; showed distinct hyphae, agar not stained
2. Cotton blue: no staining.
3. Safranin: hyphae stained well, agar stained.
4. Toluidine blue: hyphae stained well, agar not stained.

Next uncleared roots of two seedlings were stained. The root tissue was exposed to stain for 10 min at room temperature. Results were as follows:

1. Trypan blue: light staining of both fungus and cellular contents of cells with broken cell walls.
2. Cotton blue: no fungal staining and appeared to collapse the plasmalemma.
3. Safranin: dark staining of both fungus and root.

4. Toluidine blue: dark staining of fungal strands and light staining of root cells.

Based on these experiments, we decided to discontinue cotton blue and safranin because they did not selectively stain the fungus. Also we decided to clear the root to achieve better staining with trypan blue. In addition, clearing the root would eliminate cellular contents that could be erroneously identified as pelotons.

Refinement of staining method--The staining procedure consisted of heating cleared tissue (hot plate set at 2) completely immersed in stain in hanging-drop slides for five and ten minutes. Only trypan blue and toliudine blue were used. As in Alexander and Hadley (1984), trypan blue-stained tissue was rinsed once with plain lactophenol to remove excess stain. Tissue was sectioned after staining. There was little difference between staining for 5 or 10 min. Trypan blue stained better with heating. There was little difference between stains. Root cells and stele were well defined.

Finally, we tried staining tissue that was already sectioned. Tissue was sectioned by hand under a dissecting microscope. Root cross-sections were exposed to toluidine blue at room temperature for 5 and 10 minutes. Trypan blue was prewarmed and cross-sections were exposed for ten minutes on or off of the hot plate. As above, tissue stained with trypan blue was rinsed with plain lactophenol. Sectioning the roots prior to staining

allowed better exposure to both stains. For this study, roots were cleared, sectioned and then stained with trypan blue on the hot plate for ten minutes.

APPENDIX C

ADDITIONAL TABLES

Appendix C, Table 1. One-factor ANOVA of the effect of location in the root (block) on mycorrhizal infection. ANOVAs performed separately for each plant root.

Plant ID	Infection type	DF	F	P
139	hyphal	6, 30	2.10	0.0832
	amorphous	6, 30	0.81	0.5711
	total	6, 30	1.22	0.3227
139 bud	hyphal	2, 15	1.49	0.2568
	amorphous	2, 15	0.17	0.8423
	total	2, 15	2.31	0.1333
140	hyphal	14, 84	1.20	0.2889
	amorphous	14, 84	3.56	0.0001*
	total	14, 84	3.04	0.0008*
143	hyphal	2, 16	15.05	0.0002*
	amorphous	no amorphous infection		
	total	2, 16	15.05	0.0002*
145	hyphal	no hyphal infection		
	amorphous	7, 29	8.26	0.0001*

Appendix C, Table 1. (cont.)

Plant ID	Infection type	DF	F	P
	total	7, 29	8.26	0.0001*
150	hyphal	no hyphal infection		
	amorphous	4, 15	1.95	0.1541
	total	4, 15	1.95	0.1541
150 bud	hyphal	1, 7	1.31	0.2897
	amorphous	no amorphous infection		
	total	1, 7	1.31	0.2897
16	hyphal	1, 6	0.30	0.6036
	amorphous	1, 6	1.34	0.2912
	total	1, 6	2.52	0.1636
167	hyphal	6, 40	19.77	0.0001*
	amorphous	6, 40	0.93	0.4843
	total	6, 40	9.21	0.0001*
178	hyphal	10, 57	2.01	0.0486*
	amorphous	10, 57	3.98	0.0004*

Appendix C, Table 1. (cont.)

Plant ID	Infection type	DF	F	P
	total	10, 57	4.68	0.0001*
181	hyphal	8, 52	36.63	0.0001*
	amorphous	8, 52	7.53	0.0001*
	total	8, 52	7.39	0.0001*
21	hyphal	no hyphal infection		
	amorphous	5, 14	3.43	0.0314
	total	5, 14	3.43	0.0314
2a bud	hyphal	1, 12	12.49	0.0041*
	amorphous	1, 12	21.38	0.0006*
	total	1, 12	0.16	0.06984
2b	hyphal	7, 27	1.52	0.2019
	amorphous	7, 27	1.69	0.1534
	total	7, 27	3.32	0.0112*
2b bud	hyphal	2, 5	0.43	0.6753
	amorphous	2, 5	0.42	0.6802

Appendix C, Table 1. (cont.)

Plant ID	Infection type	DF	F	P
	total	2, 5	0.45	0.6600
45	hyphal	5, 22	3.88	0.0114*
	amorphous	5, 22	4.34	0.0068*
	total	5, 22	13.69	0.0001*
72	hyphal	no hyphal infection		
	amorphous	5, 30	2.91	0.0295*
	total	5, 30	2.91	0.0295*
95	hyphal	3, 13	0.87	0.4798
	amorphous	3, 13	3.65	0.0417*
	total	3, 13	2.67	0.0912
npc	hyphal	8, 50	9.80	0.0001*
	amorphous	8, 50	2.02	0.0625
	total	8, 50	2.15	0.0480*

Appendix C, Table 2. Nested ANOVA of the effect of growing season phase (reproductive or vegetative) on cortical cell infection.

Infection type	Effect	DF	F	P
Hyphal[a]	Phase	1, 18	1.57	0.2260
	Plant	18, 20	1.58	0.1623
	Block	20, 609	6.11	0.0001*
Amorphous[b]	Phase	1, 18	1.33	0.2643
	Plant	18, 20	0.46	0.9500
	Block	20, 609	6.35	0.0001*
Total[c]	Phase	1, 18	0.25	0.6255
	Plant	18, 20	0.84	0.6473
	Block	20, 609	6.30	0.0001*
Hyphal/Total	Phase	1, 14	1.20	0.2915
	Plant	14, 16	1.35	0.2814
	Block	16, 311	4.26	0.0001*

[a] number of hyphal pelotons/ number of cortical cells
[b] number of amorphous pelotons/ number of cortical cells
[c] hyphal + amorphous pelotons/ number of cortical cells

Appendix C, Table 3. Correlation analysis of hyphal infection and soil or plant tissue nutrient concentrations. Correlation was performed on bud and mature roots separately.

Root type	Nutrient source	Nutrient	N	R	P
Mature	Soil	C	16	-0.13604	0.6154
		P	16	-0.12697	0.6394
		NH_4	16	-0.37494	0.1524
		NO_3	16	-0.10606	0.6958
		H_2O	16	-0.27635	0.3002
	Root	N	13	-0.28825	0.3393
		C	13	0.10174	0.7409
		P	7	-0.18531	0.6908
	Leaf	N	7	0.21622	0.6414
		C	7	0.01802	0.9694
Bud	Soil	C	6	-0.52941	0.2801
		P	6	-0.70588	0.1170
		NH_4	6	0.26471	0.6122
		NO_3	6	-0.52941	0.2801
		H_2O	6	0.26471	0.6122
	Root	N	6	-0.52941	0.2801
		C	6	0.50000	0.3125
		P	3	0.86603	0.3333

Appendix C, Table 3. (cont.)

Root type	Nutrient source	Nutrient	N	R	P
	Leaf	N	3	-0.86603	0.3333
		C	3	-0.86603	0.3333