

PRE-INOCULATION BY AN ARBUSCULAR MYCORRHIZAL FUNGUS ENHANCES MALE REPRODUCTIVE OUTPUT OF *CUCURBITA FOETIDISSIMA*

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Male and female reproductive output of *Cucurbita foetidissima*, a gynodioecious native perennial, was examined in a 2-yr greenhouse/outplanting study. Plants were divided into three treatment groups: (1) a low-phosphorus (P) soil mix control; (2) a low-P soil mix with the addition of mycorrhizal inoculum (*Glomus intraradices*); and (3) a high-P soil mix. Plants were outplanted after one summer of greenhouse growth and harvested in the fall of the second year. High-P treatment plants grew best during the first year, having significantly longer vines than either low-P treatment. By the end of the second year, however, treatment had no significant effect on either aboveground biomass or weight of the tuberous storage root. Tissue concentrations of N and P also did not differ significantly with treatment. Male reproductive output was significantly enhanced by the addition of mycorrhizal inoculum, resulting in a threefold increase over control plants in the production of male flowers. In contrast, treatment had no significant effect on aspects of female reproductive output, including number of female flowers, percent fruit set, total fruit biomass produced by the plant, or mean fruit weight. Fruit production was correlated with vegetative aboveground biomass and is likely reflective of carbon status. These results suggest that mycorrhizal colonization may differentially influence male and female components of plant reproduction.

Keywords: buffalo gourd, *Cucurbita foetidissima*, *Glomus intraradices*, male reproductive output, gynodioecy, sex allocation.

The majority of terrestrial vascular plant species form some type of mycorrhizal association, those with arbuscular mycorrhizal fungi (AMF) being the most common (Gerdemann 1975). The association of mycorrhizal fungi with a host plant root is known to aid in the uptake of phosphorus and certain other elements from the soil, often resulting in an increase in plant growth and tissue nutrient content (Mosse 1973; Rhodes and Gerdemann 1980; Stribley 1987). Additional benefits include reduced susceptibility to pathogens and improved resistance to salinity and drought (Hirrell and Gerdemann 1980; Rosendahl and Rosendahl 1990, 1991; Ruiz-Lozano et al. 1995; Harrison 1997; Stahl et al. 1998). While studies demonstrating increased growth due to mycorrhizal colonization abound, the role of mycorrhizae in plant reproduction has received far less attention. Mycorrhizal studies in which male and female reproductive components were examined separately are rare.

Certain plants, including cucurbits, are known to alter their floral sex expression in response to environmental conditions, including light intensity, photoperiod, temperature extremes, soil moisture and nutrient availability, and trauma to leaves, buds, or storage tissue (Galun 1961; Freeman et al. 1980; Condon and Gilbert 1988; Delesalle 1989, 1992 and references therein). Soil phosphorus and nitrogen levels have been shown to affect both male and female reproductive success in *Cucurbita pepo* (Lau and Stephenson 1993, 1994). These findings suggest that colonization by arbuscular mycorrhizal fungi, which is known to affect uptake of both of these nutrients,

may play a significant role in reproductive success of both sexual functions. The effect of the soil microbial environment on floral sex expression remains largely unexplored. However, recent publications report a significant increase in pollen grain number, size, and vigor of mycorrhizal zucchini (Stephenson et al. 1994, 1998; Lau et al. 1995).

The objective of this study was to determine the effect of inoculation with an arbuscular mycorrhizal fungus on male and female components of reproduction, including floral sex expression, using a native perennial, *Cucurbita foetidissima*. This species was chosen for two reasons. As a native, *C. foetidissima* has not been subject to intense horticultural selection for response to added nutrients and should be reflective of other native field species. Also, the use of a monoecious perennial allowed for easy assessment of male and female reproductive output.

Material and Methods

Cucurbita foetidissima HBK, or buffalo gourd, is a long-lived perennial common to wash bottoms and roadsides in arid regions of the western United States and northern Mexico (Bemis and Whitaker 1969). Populations are gynodioecious, composed of both monoecious and gynodioecious individuals, presumably controlled by a single locus (Dossey et al. 1981; Kohn 1989). Plants are robust, having a large fleshy storage root of up to 40 kg and numerous stems that range from 6 to 12 m in length (Hogan and Bemis 1983; Kohn 1989). Reproduction is both sexual, by means of seed, and asexual, through adventitious rooting at the nodes. Buffalo gourd has been in-

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vestigated as a potential dryland food and fuel crop (Hogan and Bemis 1983; DeVeaux and Shultz 1985).

Seeds of *C. foetidissima* were collected from plants growing near the Snowfield exit of I-15 in Washington County, Utah. Seeds were surface-sterilized by shaking in 70% ethanol for 1 min and 2% bleach for 15 min, followed by rinsing in sterilized deionized water. Seeds were transferred to sterile blotter papers in plastic petri dishes and stratified at 2°C for 2 wk before being transferred to a 30°C germinator. All seeds germinated within 3 d.

Pregerminated seeds were planted in 15-cm pots containing one of three soil treatments: (1) a low-P potting mixture (low-P); (2) a low-P potting mixture to which spores of *Glomus intraradices* in an expanded clay carrier (Nutri-link H1000, Native Plants) had been added (low-P+AMF); and (3) a high-P potting mixture (high-P). A total of 20 pots per soil treatment were planted with two to three seeds each, then thinned to one plant per pot on emergence of the germinating seeds. The base potting mixture for all treatments was composed of peat moss, surface, vermiculite, and sand in a 4 : 2 : 3 : 2 ratio, amended with fertilizer at a rate of 700 g dolomite, 350 g limestone, 95 g calcium nitrate, 294 g gypsum, 126 g Osmocote 18-6-12 (manufactured by Grace Sierra), 65 g STEM trace minerals, and 4 g Fe-138 per 0.0832 m³ of potting mixture. Superphosphate was added to the high-phosphorus mixture at a rate of 64 g per 83.25 L of potting soil. The mixtures were steamed at 71°C for 1 h. Following steaming, water extracts (Warncke and Krauskopf 1983) of the mixture had a pH of 6.75, with 123 ppm NO₃-N and 126 ppm plant-available K. Plant-available P was 0.6 ppm for the low-P mixture and 10.6 ppm for the high-P mixture. Soil analyses were carried out at the Brigham Young University Plant and Soil Testing Laboratory, Provo, Utah.

Plants were grown in the greenhouse for 8 wk, after which 20 plants of each treatment were transplanted into 26-L containers filled with the appropriate low- or high-P potting mixture described above. A spot check of root samples taken at the time of transplanting confirmed that mycorrhizal colonization had taken place in plants of the low-P+AMF treatment (Koske and Gemma 1989). None of the root samples taken from the other two treatments were colonized.

Plants were grown in the greenhouse for another 3 mo, after which all vine tops were measured, harvested, dried, and weighed. Pots were overwintered in the ground under a bed of leaves. The following spring, plants of all three treatments were randomized at a field site in a 21 × 33-m grid, with 3

m between plants. Pots were placed in the ground and the root systems allowed to extend into the surrounding soil. Soil at the site had 2.2% organic matter, with a pH of 7.5, 23 ppm available N, 17 ppm available P, 171 ppm available K, and an electrical conductivity of 0.93 dS/m (bicarbonate extraction; Page et al. 1982). Watering was accomplished by means of a drip irrigation system, and the area surrounding each plant was covered with weed cloth and gravel to prevent adventitious rooting at the nodes.

Flowering was monitored on a regular basis throughout the second growing season. The presence of male and female flowers was marked at each node using colored tape. At the end of the growing season, stem lengths were measured and a map of flowering nodes constructed for each plant. Plants that produced only female flowers were classified as gynoecious. Plants having at least one male flower were classified as monoecious. Root samples were collected from soil layers directly below each pot and examined for mycorrhizal colonization. Systematic sampling of the entire root system was not possible, however, because of the great depth to which these roots grow.

Vines, gourds, and storage tubers were harvested, dried, and weighed. Vine leaf and stem tissue from 10 randomly chosen plants per treatment were ground and analyzed for content of N, P, K, Zn, Fe, Mn, Cu, Ca, and Mg by the Plant and Soil Testing Laboratory at Brigham Young University. Tissue nitrogen was determined using the Kjeldahl procedure (Horwitz 1980). Content of other bioessential elements was determined using atomic absorption procedures on tissue samples digested in a 1 : 5 solution of concentrated sulfuric and nitric acid (Horwitz 1980).

Biomass and colonization data were statistically analyzed using the GLM and CORR procedures on SAS, version 6.1, for the personal computer (SAS 1989). Mean separations were accomplished using the Student-Newman-Keuls multiple-range test. Differences in plant tissue concentrations were analyzed using MANOVA. Percentage data were arcsine transformed before analysis. Differences in the numbers of male and female flowers per plant were examined using the nonparametric Kruskal-Wallis test, followed by the Dunn Q-test for nonparametric multiple comparisons.

Results

At the end of the first season, plants in the high-P treatment averaged the greatest growth, having a significantly longer

Table 1
Proportion of Gynoecious, Monoecious, and Nonflowering Plants Occurring in Each Treatment

	Low-P			Low-P+AMF			High-P		
	<i>n</i>	Male flowers	Female flowers	<i>n</i>	Male flowers	Female flowers	<i>n</i>	Male flowers	Female flowers
Nonflowering	4	5	6
Gynoecious	6	...	2.3 ± 0.3	6	...	2.5 ± 1.1	3	...	3.0 ± 1.2
Monoecious	10	7.5 ± 2.8	3.5 ± 1.3	8	28.3 ± 8.3	3.4 ± 2.2	11	9.4 ± 3.2	2.8 ± 0.7

Note. Mean numbers of male and female flowers occurring on monoecious and gynoecious plants is also given.

Table 2

Results from General Linear Models Analysis of Growth Parameters of Buffalo Gourd Grown under Three Soil Treatments

	<i>n</i>	Stem and leaf dry weight (g)	Storage root dry weight (g)	Total dry weight (g)	Number of primary stems	Ratio of 2° to 1° stems
High-P	20	702.5 ± 56.3	279.9 ± 21.5	982.4 ± 71.5	5.00 ± 0.40 ^A	0.66 ± 0.12 ^A
Low-P	20	713.7 ± 63.8	308.8 ± 20.8	1022.5 ± 80.0	4.85 ± 0.45 ^A	1.08 ± 0.27 ^{AB}
Low-P+AMF	19	786.8 ± 49.3	319.9 ± 22.0	1106.7 ± 63.6	3.53 ± 0.25 ^B	1.41 ± 0.21 ^B
<i>P</i> value		0.5348	0.4009	0.4725	0.0166*	0.0456*

Note. Data were taken at the end of the second growing season. Treatment means and standard errors are given for each variable. Asterisks denote significant differences at $P = 0.05$. Means followed by different letters are significantly different.

mean stem length of 446 ± 88 cm compared with 375 ± 80 cm and 348 ± 90 cm for the low-P and low-P+AMF treatments, respectively ($n = 60$, $F = 7.01$, $df = 2$, $P = 0.0019$). Stem lengths for the two low-P treatments were not significantly different. The high-P treatment also had the greatest above-ground dry weight (106.6 ± 24.1 g vs. 94.2 ± 14.5 g for the low-P and 94.3 ± 15.2 g for the low-P+AMF treatments), although these differences were not statistically significant ($n = 60$, $F = 3.02$, $df = 2$, $P = 0.0569$). Fewer than one-third of the plants (five to six plants per treatment) flowered during the first season, too few for quantitative analysis.

Forty-four of 59 surviving plants flowered during the second growing season (one plant died from squash beetles). Of these, 15, or 34%, were gynoecious, a percentage very close to the 32% average reported for native populations (Kohn 1989). The numbers of gynoecious, monoecious, and nonflowering plants occurring in each treatment are given in table 1. A χ^2 analysis of these numbers (Zar 1984) determined that the percentage of gynoecious plants was independent of treatment ($\chi^2 = 1.53309$, $v = 2$, $0.25 < P < 0.50$). Consequently, biomass and reproductive data of the two morphs were combined in the following analyses.

At the end of the second growing season, following out-planting into field soil, no significant treatment differences were observed in either aboveground vegetative biomass or weight of the tuberous storage root (table 2). High-P plants, which had been larger at the end of the first year, averaged the smallest biomass figures at the end of the experiment. By this time, the one-time application of additional phosphorus in the high-P soil mix had been reduced from the original 10.6 ppm plant-available phosphorus to 1.9 ppm. Phosphorus concentrations in the two low-P soil mixes at the end of the experiment were even less, averaging 0.8 and 0.9 ppm for the low-P and mycorrhizal treatments, respectively. Phosphorus levels in the field soil, however, were more than adequate for normal plant growth. Leaf tissue concentrations of bioessential elements (table 3) did not differ significantly with treatment (Wilks's $\lambda = 0.503823$, $df = 18$, $P = 0.6212$). Univariate GLM results for individual elements, including N and P, also did not differ, with the exception of copper.

Roots of all plants were colonized with mycorrhizal fungi by the end of the second growing season. Root samples collected from below the pots were colonized an average of $46.0\% \pm 17.3\%$ for low-P treatment plants and $48.3\% \pm 11.1\%$ for high-P plants. Pre-inoculated plants had a signifi-

cantly higher percentage of their roots colonized, with a mean of $62.8\% \pm 12.5\%$ ($n = 59$, $F = 8.22$, $df = 2$, $P = 0.0007$).

Flowering of monoecious plants followed a typical cucurbit pattern (Scheerens et al. 1987; Delesalle 1992). Only male flowers were produced on proximal nodes, while on the more distal portions of the stem, male and female flowers were interspersed. Flowering pattern (node number at which male and female flowers were produced) did not differ with treatment (data not shown). Treatment did, however, have a significant effect on some measures of branch architecture, reflecting a tendency for the pre-inoculated mycorrhizal plants to have fewer, longer, and more branched primary stems. Inoculated plants had significantly fewer primary stems per plant and significantly more branches per primary stem than did plants from the other two treatments (table 2). Inoculated plants also tended to have longer primary and secondary stem lengths, although these differences were not significant ($F = 0.70$, $df = 2$, $P = 0.4988$, and $F = 0.38$, $df = 2$, $P = 0.6877$, respectively). The mean length of major primary stems (stems >1.5 m) on inoculated plants was 25.0 ± 2.7 m, compared with 24.9 ± 2.9 m for low-P plants and 24.0 ± 3.1 m for high-P plants. The mean length of secondary stems was 20.7 ± 2.0 m for inoculated plants, 20.6 ± 2.2 m for low-P plants, and 20.2 ± 2.0 m for high-P plants.

The most notable treatment effect occurred in plant sexual expression. Treatment had a profound effect on the number of male flowers produced by monoecious plants during the second growing season ($H_c = 7.8289$, $df = 2$, $0.01 < P < 0.025$). Pre-inoculated plants produced an average of 28.3 male flowers per plant compared with averages of 7.5 for the low-P treatment and 9.4 for high-P plants, a threefold difference (fig. 1). In contrast, no significant treatment differences occurred in either the number of female flowers ($H_c = 0.9350$, $df = 2$, $P > 0.50$), mean gourd dry weight, total gourd dry weight per plant, or percentage fruit set (fig. 1; table 4). This was true for both sexual morphs (statistical results not shown). Total gourd dry weight per plant was significantly correlated with aboveground biomass ($r = 0.61884$, $n = 32$, $P = 0.0002$), suggesting that acquisition of photosynthate was a limiting factor.

Discussion

Although a number of studies have documented mycorrhizal enhancement of seed production and resulting fitness gains

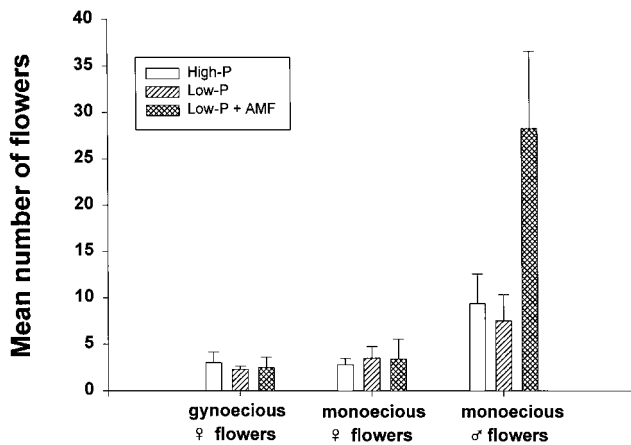


Fig. 1 Mean numbers of male and female flowers produced by gynoeious and monoecious plants grown in three soil treatments. Error bars represent standard error of the mean.

through the female function, very little is known about the effect of mycorrhizal colonization on reproduction through the male function. This study is the first to examine the effect of mycorrhizal colonization on male reproductive output in a native plant species. Male reproductive success, as measured by number of male flowers, increased threefold in monoecious plants of the low-P+AMF treatment. The large increase in male flower number was independent of either plant size or female reproductive output. No trade-off between male and female reproduction was observed. Results from this study lend support to the proposition that male and female reproductive functions are limited by different resources (Freeman et al. 1976; Ashman and Baker 1992) and indicate that mycorrhizal colonization may differentially affect reproductive success through male and female sexual functions.

Previous studies have also reported mycorrhizal enhancement of the male reproductive function. Lau et al. (1995) documented an increase in pollen grain number and size for mycorrhizal zucchini (*Cucurbita pepo*). Pollen from mycorrhizal plants also produced significantly longer pollen tubes *in vitro* (Stephenson et al. 1998). In contrast to the present study, Lau et al. (1995) observed no differences in number of male flowers in *C. pepo*. Stephenson et al. (1998) reported a significant trade-off between male and female reproductive success of my-

corrhizal tomato plants. Plants that were not permitted to set fruit produced significantly more flowers and had significantly faster pollen tube growth. Response differences among species may be attributed to a number of factors, including unique physiological factors, growth form and life cycle, and selection history. Further research on multiple species is needed.

Pollen production, whether measured by number of male flowers as in *Cucurbita foetidissima* or by number of pollen grains per flower as in *C. pepo*, depends completely on the sporophyte for nutrient acquisition. Ashman and Baker (1992) found that pollen of hermaphroditic *Sidalcea oregana* had the highest phosphorus requirement of all reproductive tissues and suggested that male and female reproductive functions may be limited by different resources. Mycorrhizae are known to improve phosphorus uptake from the soil (Harley and Smith 1983). Under conditions of high phosphorus availability, *C. pepo* plants demonstrated an increase in pollen number and size similar to that produced by mycorrhizal plants (Lau and Stephenson 1994; Lau et al. 1995). Phosphorus acquisition, whether through the plant's own root system or through a mutualistic association with mycorrhizal fungi, appears to be particularly important to pollen production.

Previous reports regarding the effect of arbuscular mycorrhizal fungi on plant reproduction have focused primarily on the female reproductive function, particularly seed size and yield of grain and legume crops (e.g., Jensen 1982; Schenck and Smith 1982; Vejsadova et al. 1993; Bethlenfalvai et al. 1994, 1997; Subramanian and Charest 1997). Most report enhanced reproductive output in the presence of mycorrhizal fungi, especially under low-nutrient conditions. Various factors affect the magnitude and direction of the mycorrhizal effect, including species or isolate of the fungus (Jensen 1982; Schenck and Smith 1982; Bethlenfalvai et al. 1997), plant species or cultivar (Ganade and Brown 1997), soil type and nutrient status (Dodd et al. 1983; Bethlenfalvai and Barea 1994), and environmental effects, such as soil temperature and moisture stress (Schenck and Smith 1982; Subramanian and Charest 1997).

In the most comprehensive set of studies to date, Koide et al. have documented increased seed production of mycorrhizal individuals through changes in plant phenology, shoot architecture, inflorescence production, fruit set, number of fruits, and number of seeds per fruit (Koide et al. 1988, 1994; Bryla and Koide 1990a, 1990b; Lewis and Koide 1990; Koide and

Table 3

Mineral Concentrations (\pm SE) in Leaf and Stem Tissue of Buffalo Gourd Plants Grown under Three Soil Treatments

	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	Zn (ppm)	Fe (ppm)	Mn (ppm)	Cu (ppm)
High-P	3.54 \pm 0.10	0.28 \pm 0.01	3.52 \pm 0.10	6.83 \pm 0.27	1.31 \pm 0.04	27.6 \pm 1.6	134.9 \pm 6.2	33.1 \pm 3.2	6.0 \pm 0.26 ^{AB}
Low-P	3.74 \pm 0.17	0.30 \pm 0.02	3.77 \pm 0.11	6.28 \pm 0.41	1.19 \pm 0.07	26.7 \pm 1.5	129.4 \pm 5.2	35.2 \pm 4.3	6.6 \pm 0.16 ^A
Low-P+AMF	3.56 \pm 0.11	0.26 \pm 0.01	3.68 \pm 0.13	6.66 \pm 0.28	1.31 \pm 0.06	26.2 \pm 1.7	138.3 \pm 3.7	27.2 \pm 2.1	5.7 \pm 0.30 ^B
P value	0.5392	0.2494	0.3064	0.4458	0.2790	0.8100	0.4558	0.2163	0.0466*

Note. Ten plants per treatment were analyzed. Attained significance values from univariate General Linear Model analyses are given below. Asterisks denote significant differences at $P = 0.05$. Means followed by different letters are significantly different.

Table 4
Results from General Linear Models Analysis of Fruiting Parameters of Buffalo Gourd Grown under Three Soil Treatments

	<i>n</i>	Mean gourd dry weight (g)	Total gourd dry weight (g)	Percent fruit set
High-P	11	13.8 ± 1.3	50.8 ± 8.7	93.2 ± 4.9
Low-P	12	10.3 ± 1.1	44.0 ± 11.8	81.9 ± 8.1
Low-P+AMF	9	12.1 ± 1.2	61.9 ± 24.6	88.4 ± 5.6
<i>P</i> value		0.1199	0.7133	0.5070

Note. Data were taken at the end of the second growing season. Treatment means and standard errors are given for each variable.

Lu 1992; Stanley et al. 1993; Lu and Koide 1994; Shumway and Koide 1994a, 1994b, 1995). Phosphorus content of the seed produced was consistently higher for mycorrhizal plants of all species studied, resulting in enhanced growth, reproduction, and competitive ability of subsequent generations (Lewis and Koide 1990; Koide and Lu 1992; Lu and Koide 1994; Shumway and Koide 1994a, 1994b; Heppel et al. 1998).

Mycorrhizal effects on flowering parameters have also been examined in a limited number of perfect-flowered species, including *Lycopersicon esculentum* (Bryla and Koide 1990a; Stephenson et al. 1998), *Capsicum annuum* (Dodd et al. 1983), *Glycine max* (Schenck and Smith 1982), *Avena sativa* (Koide et al. 1988), *Vicia sativa* and *Vicia hirsuta* (Ganade and Brown 1997), and *Abutilon theophrasti* (Lu and Koide 1994). Reproductive responses include earlier flowering, prolonged flowering, and an increase in the number of flower buds, inflorescences, and fruits.

In my study, no significant treatment differences in female function was observed in either sexual morph. However, treatment application was largely confined to the first year of growth. During the second season, P availability at the field site was adequate for plant growth, and the soil contained indigenous mycorrhizae, allowing plants of all treatments to become colonized. Although high-P plants produced larger storage tubers during the first season, first-year tuber size differences did not result in significant differences in plant size, flowering, or tuber weight by the end of the second season. There was, however, a consistent trend for the AMF-treatment plants to be larger in size and to have greater fruit production.

This trend suggests that, under conditions of continuous phosphorus limitation, mycorrhizae could enhance growth and female reproductive output, as has been found in other plant species.

Further study of the effect of mycorrhizal colonization on male and female reproductive success is clearly warranted. Reproductive output in this study was monitored for only 1 yr. It is expected that, under field conditions, the initial differences due to pre-inoculation would become less pronounced with time. Nevertheless, under the experimental conditions reported here, mycorrhizal colonization resulted in significant differential enhancement of the male reproductive function. Numerous studies document shifts in plant sex expression as a result of resource limitation (Freeman et al. 1976, 1980, 1997). Because hermaphroditic plant populations gain half of their fitness through the male function, an understanding of male reproductive success through pollen is equally important to that of female reproductive success through seed production. Male reproductive output may be particularly important to the reproductive fitness of monoecious plants of gynodioecious species such as *C. foetidissima*.

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