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PLANT–MYCORRHIZAL FUNGUS INTERACTIONS AFFECT THE EXPRESSION OF INBREEDING DEPRESSION IN WILD STRAWBERRY

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The influence of biotic interactions on the expression of inbreeding depression has received only modest investigation; however, these interactions may be important in determining the magnitude and/or variation of inbreeding depression seen in the wild and invoked in models of mating and sexual system evolution. We present the first experimental test of the effects of plant–mycorrhizal fungus interactions on the expression of inbreeding depression. We inoculated selfed and outcrossed seedlings from eight hermaphrodite genotypes of wild strawberry (*Fragaria virginiana*) with mycorrhizal fungal spores and determined the effect on vegetative growth and sexual and asexual reproduction. We found that inoculated plants grew at rates similar to those of control plants but produced fewer flowers and more plantlets than controls. However, some of these effects varied with maternal genotype and cross type. As a consequence, mycorrhizal fungal inoculation had variable and trait-dependent effects on the expression of inbreeding depression. We discuss the results in light of the conditional nature of plant-mycorrhiza associations and in light of their potential to influence mating and sexual system evolution.

Keywords: *Entrophospora*, *Fragaria*, *Glomus*, mating system, mutualism, plant-fungus interactions.

Introduction

Inbreeding depression, i.e., the reduction in fitness of selfed relative to outcrossed progeny, is the main genetic cost of selfing, and thus the magnitude and variability of inbreeding depression are thought to play a central role in the evolution of mating and sexual systems (Charlesworth 1999 and Goodwillie et al. 2005 for reviews). Inbreeding depression is caused, in part, by recessive deleterious or partially deleterious alleles (Carr and Dudash 2003) that, when expressed in homozygous form, can be purged by selection; thus, the inbreeding history of individuals and populations has long been a central focus of studies (Husband and Schemske 1996). More recently, however, the importance of environmental context in mediating the severity of inbreeding depression has come to the forefront, and the necessity of investigating inbreeding × environment interactions has been emphasized (Armbruster and Reed 2005). While numerous studies in plants have focused on abiotic aspects of the environment, such as drought or nutrient stress (Sakai et al. 1997; Cheptou et al. 2000; Heschel et al. 2005), and a fair number of studies have addressed plant-plant competitive interactions (Carr and Dudash 1995; Cheptou et al. 2001; Pujol and McKey 2006), far fewer have paid attention to how plant-animal or plant-fungus interactions may affect inbreeding depression (Hayes et al. 2004; Ivey et al. 2004; Ivey and Carr 2005).

Biotic interactions can affect inbreeding depression if the biotic agent interacts differently with selfed and ouctrossed individuals. In fact, the effect of this type of interaction on the expression of inbreeding depression may be especially strong because there is the potential for feedback between interactors (Lively and Howard 1994) and complex dynamics between multiple interactors (Gange and Smith 2005; Ivey and Carr 2005). For instance, when considering plant-enemy interactions, workers have found that selfed plants can be less resistant to or less tolerant of attack by enemies (reviewed in Steets et al. 2007), which could lead to greater inbreeding depression in the presence of enemies. Likewise, although this phenomenon is studied much less frequently, selfed and outcrossed plants may interact differently with mutualists such as insect pollinators (Ivey and Carr 2005), which influence plant mating patterns (Vogler and Kalisz 2001), or mycorrhizal fungi (Nuortila et al. 2004), which influence plant nutrient uptake (Smith and Read 1997). To our knowledge, the only published study to address the effects of plant inbreeding level (hereafter "cross type") on pollinator behavior found that fewer pollinators were attracted to selfed plants than to outcrossed plants but that these foraged longer on selfed plants than on outcrossed plants (Ivey and Carr 2005). The only published study to address plant cross type and mutualistic root fungi found that the mycorrhizal status of the maternal plant increased the early growth rate of their outcrossed seedlings more than that of selfed seedlings (Nuortila et al. 2004). While these two studies imply that the strength of inbreeding depression could be mutualist dependent, neither quantified the effect of the mutualist on inbreeding depression. Thus, we still lack a full understanding of whether the expression of inbreeding depression can be contingent on the plant-mutualist interaction. This type of information, however, is crucial if we are to have a full appreciation of whether biotic interactions can mediate inbreeding depression in a way that may maintain mixed mating and/or contribute to the evolution of sexual systems (see Ashman 2006 and Steets et al. 2007 for review).

Here we address this gap by studying the effect of mycorrhizal fungi on the expression of inbreeding depression in a wild strawberry, *Fragaria virginiana*. Specifically, we inoculated selfed and

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outcrossed plants from several maternal genotypes with mycorrhizal fungal spores and asked the following questions: (1) Does experimental inoculation with mycorrhizal fungi affect the vegetative growth and the sexual or asexual reproduction of wild strawberry plants? (2) Does the effect of mycorrhizal fungal inoculation depend on the cross type that produced the plant; i.e., does it differ between selfed and outcrossed plants? (3) Does inoculation with mycorrhizal fungi influence the magnitude of inbreeding depression?

Material and Methods

Study System

Plant. *Fragaria virginiana* (Rosaceae), the Virginia wild strawberry, is a creeping stoloniferous perennial herb that is native to North America (Staudt 1989). It has a gynodioecious/subdioecious breeding system, in which females coexist with hermaphrodites but some hermaphrodites never produce fruit and thus may be considered "functional males" (Staudt 1989; Stahler et al. 1995; Ashman 1999).

Fragaria virginiana reproduces sexually via flowers produced on inflorescences and asexually via plantlets produced along stolons. Both inflorescences and stolons develop from axillary buds. In the fall and early spring, axillary buds develop into inflorescences, whereas in the late spring/summer, they develop into stolons (Hancock 1999). Female plants require cross-pollination to set seed, but hermaphrodites can self-pollinate, and in northwestern Pennsylvania populations have a mixed mating system (L. Penet, C. L. Collin, C. Evenovski, A. Rhode, and T.-L. Ashman, unpublished data).

Mycorrhizal fungi. Like other members of the Rosaceae (Wang and Qiu 2006), *F. virginiana* associates with soilborne vesicular-arbuscular mycorrhizal fungi both in the wild (Taylor et al. 2007; J. Taylor, C. L. Collin, and T.-L. Ashman, unpublished data) and under cultivation (Stewart et al. 2005). Vesicular-arbuscular mycorrhizal fungi produce arbuscles (short-lived structures thought to be involved in nutrient transfer between plant and fungus), hyphae (structures involved in nutrient transport and vegetative fungal growth), and vesicles (lipid-filled terminal swellings of hyphae with a storage/perennating function) within the roots of their host plant (Smith and Read 1997). Vesicles are formed after arbuscles and thus are encountered once the plant-fungus interaction has been established; large numbers of vesicles are formed at the end of the growing season (Peterson et al. 2004). In northwestern Pennsylvania, wild strawberry shows extensive colonization, as measured by the density of hyphae and the abundance of vesicles within their roots (Taylor et al. 2007). However, there is also substantial variability in colonization, which is accounted for by several factors, including plant genotype, population, and time in the growing season (Taylor et al. 2007).

Plant-fungus interaction. Association with mycorrhizal fungi has been shown to increase plant nutrient acquisition, leading to increased plant vigor, especially when soils are nutrient deficient (Smith and Read 1997). But under some conditions, such as abundant resources, this relationship can turn parasitic, with the fungus obtaining photosynthates and reducing plant growth (Johnson 1993; Dunham et al. 2003). In cultivated strawberry, colonization by vesicular-arbuscular mycorrhizal fungi has been shown to increase plant growth, yield, and nutrition and to help in the prevention of soilborne diseases (Niemi and Vestberg 1992; Khanizadeh et al. 1995; Norman et al. 1996; Norman and Hooker 2000), but the level to which this occurs depends on the specific cultivar as well as the mycorrhizal inoculate used (Stewart et al. 2005).

Experimental Design

Plant cultivation and fungal treatment. To produce plants for this experiment, we hand-pollinated several hermaphrodite genotypes in the greenhouse. These plants were originally collected from a wild northwestern Pennsylvania population of *F. virginiana* (population "PR" in Ashman 1999) and were known to be capable of producing fruit. We conducted hand-pollinations with either self pollen or pollen from another hermaphrodite plant. All flowers were emasculated before anthesis and were pollinated with the aid of toothpicks. We chose eight maternal families of seeds for this study, using two conditions: (1) they presented signs consistent with inbreeding depression at the seedling stage in a previous study, and (2) they had enough seeds to conduct the study. While this may result in an overestimation of inbreeding depression for the population of origin, the purpose of this study was not to estimate the magnitude of inbreeding depression per se but to determine whether association with mycorrhizal fungi could alter its expression.

We planted 100 seeds from each maternal genotype–cross type combination in Sunshine germination soil and allowed them to germinate and grow for ~2 mo under a 14L : 10D cycle and temperatures of 24°/12°C (day/night). For this experiment, we randomly selected 20 seedlings per cross type–maternal genotype combination and assigned each to either the mycorrhizal treatment or a control treatment; the remaining seedlings were used in a separate experiment. Seedlings were transplanted into pots filled with 200 mL of soil (a 2 : 1 mix of Sunshine germination soil and sand). Soil of plants in the mycorrhizal treatment was inoculated with the fungus spores in a carrier medium, whereas soil of plants in the control treatment was amended with the carrier medium alone. The inoculum used ("Mexican" formulation of the MiniPlug; Plant Health Care, Pittsburgh) was a commercially available mix of spores of two generalist vesicular-arbuscular mycorrhizal fungi, *Entrophospora columbiana* and *Glomus intraradices*. These fungi are known to readily form associations with strawberry (Taylor et al. 2007) and are native to the regions where the plants grow (Klironomos 2000). We applied the inoculum at the recommended level, which corresponds to ~127 spores per pot in our experiment. To minimize accidental contamination of the control plants, we prepared the soil mix with the mycorrhizal fungus spores in a separate room. We placed the plants ~15–20 cm apart on a greenhouse bench in a randomized design. They were exposed to natural day length and day/night temperatures of 24°/18°C for ~2 mo before we cold-stratified them in a darkened growth chamber at 4°C (i.e., "overwintering"). On May 2, 2007, we moved the plants back to the greenhouse. Because some plants did not survive transplanting/overwintering, the final number of plants was 287.

Measurement of the plant-fungus interaction. The extent of root colonization was characterized just before the overwintering period. We collected approximately four to eight root samples from each plant and stored these in 70% ethanol until quantification. To assess the efficacy of our treatments and the level of the plant-fungus interaction, we stained root samples

from a subsample of the plants and scored two mycorrhizal structures: vesicles and hyphae. We prepared and stained roots from 32 plants (one from each maternal genotype–cross type–treatment combination), using a black-ink–vinegar staining protocol adapted by C. L. Collin from Vierheilig et al. (1998). To assess the level of hyphal colonization, we cut and stained 10 1-cm segments of young, thin roots from each plant. We scored the percentage of the root length colonized by hyphae and calculated the mean percentage colonization per plant. Because few of these young roots contained vesicles, we also sampled 10 additional 1-cm segments from older, thicker roots, and in these we scored vesicle number per root segment. The exact number of vesicles found in each 1-cm segment was counted up to 50 vesicles/segment, but beyond this number exact counts were not feasible because of heavy clumping and overlap of vesicles. We scored roots with this high level of vesicles as >50, but for analysis purposes we used the value of 50. This approach is expected to be quite conservative because these root pieces likely contained upwards of 150 vesicles.

Ambient contamination is often a problem in mycorrhizal fungus experiments (Sharma and Adholeya 2004). Despite our efforts to avoid contamination of the uninoculated control treatment, plants in this treatment were colonized naturally by ambient mycorrhizal fungi. Roots of plants in the control group, however, were significantly less colonized than those in the experimentally inoculated group (27.2% ± 5.4% vs. 50.4% ± 2.8% hyphal colonization; $t_{\text{paired}} = 1.72$, $P = 0.001$, $N = 28$). Moreover, vesicle number was three times as high in the experimentally inoculated group as in the control group (3.2 ± 1.1 vs. 9.9 ± 2.3; $t_{\text{paired}} = 1.75$, $P = 0.05$, $N = 32$), suggesting that the control group was colonized after the experimentally inoculated group. Thus, although we do not have a nonmycorrhizal treatment, we still have a significant difference between the two groups with respect to the intensity, and possibly the duration, of the mycorrhizal relationship. We acknowledge, however, that the mycorrhizal infection of the control group may not be the same species used in the experimentally inoculated plants. In light of these facts, we will continue to refer to the control as the control, but we acknowledge that it does not represent a noninoculated state.

Measurement of plant traits. We measured the vegetative size of plants just before overwintering (January 8, 2007), hereafter the "juvenile stage," and when flowering (June 11, 2007), hereafter the "adult stage." Plant size was estimated nondestructively as the product of the number of leaves and the diameter of the central leaflet on the largest trifoliate leaf. This estimate has been shown to closely predict aboveground biomass (Ashman 1999). We recorded the total number of flowers produced per plant as an estimate of sexual reproduction and the number of plantlets produced between May 2 and June 26, 2007, as an estimate of asexual reproduction. Since only 20% of plants did not produce flowers, plants with no flower production were included in the data set. All traits were normally distributed.

Statistical Analysis

We assessed the effects of mycorrhizal inoculation and cross type on plant vegetative size at the juvenile and adult stages using a repeated-measures fixed-effect ANOVA. Maternal genotype was considered a fixed effect because of the limited number of genotypes and the fact that it was not chosen randomly from a set of all possible genotypes (Gotelli and Ellison 2004). We included maternal genotype in the design to account for the potential effects of inbreeding history, and thus we were not specifically interested in estimating family-level inbreeding depression per se. Maternal genotype, cross type, and mycorrhizal treatment were between-subject effects, and stage was the within-subject (repeated) factor. In this ANOVA, a significant overall effect of mycorrhizal treatment would suggest that mycorrhizal fungi affect mean plant vegetative size, whereas a significant treatment × stage effect would indicate that the effect of mycorrhizal fungi on plant size depends on stage, i.e., affects vegetative growth rate. Likewise, an interaction with cross type or maternal genotype would suggest that the effect of mycorrhizal treatment varied with inbreeding level or maternal genetic background.

We determined whether maternal genotype, cross type, or mycorrhizal inoculation affected sexual or asexual reproduction (flowers or plantlets, respectively) by using three-way ANCOVAs. Maternal genotype, cross type, and treatment were fully crossed fixed effects, and adult plant size was the covariate. Plant size was included to determine whether there were significant effects of cross type or mycorrhizal treatment (or their interaction) independent of effects on plant size. In both ANCOVAs, the three-way interaction of maternal genotype × cross type × treatment was not significant and was removed from subsequent analyses. In these analyses, we were particularly interested in whether there was a significant mycorrhizal treatment × cross type interaction, because this would suggest that the effect of mycorrhizal fungi on plant reproduction was dependent on the level of inbreeding.

To further determine whether mycorrhizal fungi can affect the expression of inbreeding depression, we calculated the inbreeding-depression coefficient for each maternal genotype as $\delta_p = 1 - (\bar{x}_s/\bar{x}_o)$, where $\bar{x}_s$ is the mean of each plant performance trait for selfed progeny and $\bar{x}_o$ is the mean for outcrossed progeny (Johnston and Schoen 1994). We compared the mean inbreeding depression (across maternal genotypes) between mycorrhizal-treatment groups by using paired *t*-tests. All analyses were conducted with SAS (SAS Institute 2006).

Results

Effects of Mycorrhizal Fungi on Plant Vegetative Size and Growth Rate

The repeated-measures ANOVA revealed no overall effect of mycorrhizal fungi but significant cross type and maternal genotype effects on average plant size (table 1, top). However, there was a significant interaction between stage, mycorrhizal treatment, and maternal genotype as well as one between treatment, stage, cross type, and maternal genotype (table 1, bottom), suggesting that mycorrhizal inoculation affected vegetative size but in a stage-dependent manner and that this depended on both maternal genotype and cross type. This latter interaction occurred in complex ways (fig. 1).

Effects of Mycorrhizal Fungi on Plant Reproduction

There were significant effects of mycorrhizal treatment on sexual (flower number) and asexual (plantlet) reproduction (table 2; fig. 2). The effect depended on cross type for the former but not for the latter. Specifically, experimental inoculation had no effect

Table 1

Repeated-Measures ANOVA for *Fragaria virginiana* Vegetative Size

Source of variation	df	*F*	*P*
Between subjects:			
Mycorrhizal treatment	1	2.53	.11
Cross type	1	23.01	.0001
Maternal genotype	7	2.34	.03
Mycorrhizal treatment × cross type	1	.58	.45
Mycorrhizal treatment × maternal genotype	7	1.21	.30
Cross type × maternal genotype	7	1.23	.28
Mycorrhizal treatment × cross type × maternal genotype	7	1.65	.12
Within subjects:			
Stage	1	797.49	.0001
Stage × mycorrhizal treatment	1	2.78	.10
Stage × cross type	1	8.79	.003
Stage × maternal genotype	7	5.97	.0001
Stage × mycorrhizal treatment × cross type	1	.05	.82
Stage × mycorrhizal treatment × maternal genotype	7	2.04	.05
Stage × cross type × maternal genotype	7	.72	.65
Stage × cross type × mycorrhizal treatment × maternal genotype	7	2.04	.05
Error	252		

Note. Maternal genotype, cross type (outcross or self-pollination), and mycorrhizal treatment (inoculated or control) are fixed between-subjects effects, whereas stage (juvenile or adult) is the within-subject repeated factor.

on flower production of selfed plants but decreased that of outcrossed plants by 28% (fig. 2*a*). A different pattern was seen for asexual reproduction. There, experimental inoculation increased plantlet production by 30% for selfed and 23% for outcrossed progeny (fig. 2*b*). This analysis also revealed that the effects of cross type on plantlet production depended heavily on maternal genotype (interaction: $P < 0.0001$) but that cross type and maternal genotype had more or less independent effects on flower production (interaction: $P = 0.2$). Adult plant size had significant effects on both forms of reproduction (table 2).

Effects of Mycorrhizal Fungi on the Expression of Inbreeding Depression

Estimates of inbreeding depression ($\delta_p = 0.14$–0.22; fig. 3) paralleled the ANOVA results showing trait-dependent effects

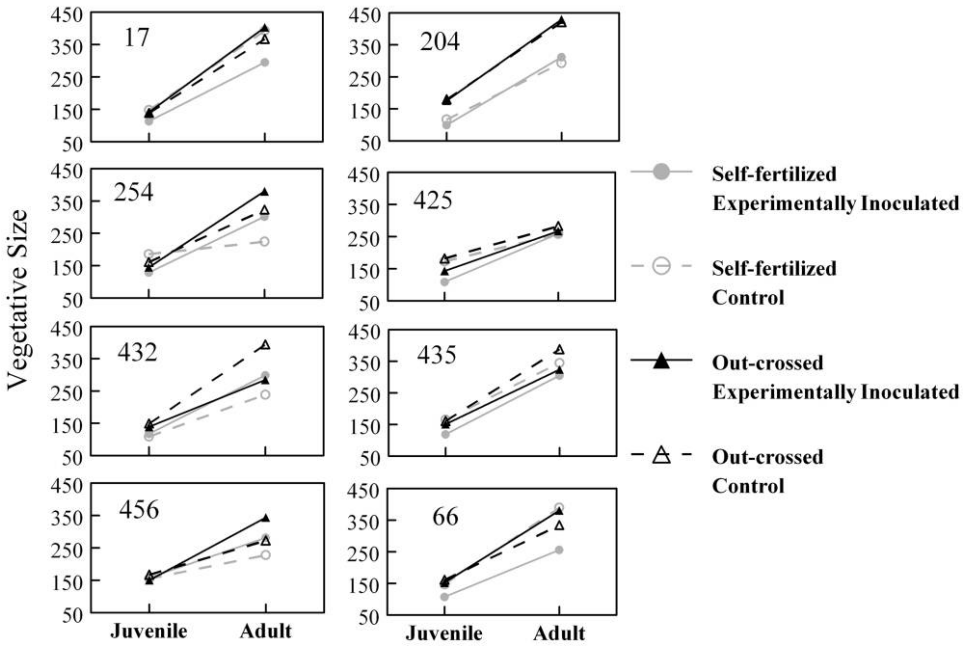

Fig. 1 Vegetative plant size for *Fragaria virginiana* at two stages (juvenile and adult) for plants produced by two cross types (outcrossed or selfed) and grown under two mycorrhizal treatments (experimentally inoculated and control). One graph is presented for progeny of each of the eight maternal genotypes (maternal identity noted in upper left corner).

Table 2

ANCOVAs for *Fragaria virginiana* Sexual (Flower Production) and Asexual (Plantlet Production) Reproduction

Source of variation	df	*F*	*P*
Flower production:			
Mycorrhizal treatment	1	6.93	.009
Cross type	1	3.22	.07
Maternal genotype	7	4.89	.0001
Mycorrhizal treatment × cross type	1	5.84	.02
Mycorrhizal treatment × maternal genotype	7	.64	.72
Cross type × maternal genotype	7	1.41	.20
Adult vegetative size	1	21.67	.0001
Error	262		
Plantlet production:			
Mycorrhizal treatment	1	17.26	.0001
Cross type	1	8.06	.005
Maternal genotype	7	8.82	.0001
Mycorrhizal treatment × cross type	1	.01	.94
Mycorrhizal treatment × maternal genotype	7	.91	.50
Cross type × maternal genotype	7	5.28	.0001
Adult vegetative size	1	10.70	.001
Error	262		

Note. Maternal genotype, cross type (outcross or self-pollination), and mycorrhizal treatment (inoculated or control) are fixed effects, and adult vegetative size is the covariate.

of cross type (tables 1, 2). In addition, the effects of mycorrhizal treatment on the expression of inbreeding depression varied among traits. Only inbreeding depression for flower production was significantly lower for the mycorrhizal treatment than for the control treatment ($t_{1,7} = 2.31$, $P = 0.05$; fig. 3). Inbreeding depression for this trait was significantly less than 0 in the control treatment ($t_{1,7} = 3.52$, $P = 0.005$) but was not different from 0 in the inoculated treatment ($t_{1,7} = 0.02$, $P = 0.98$). Inbreeding depression in juvenile size was slightly higher in the inoculated treatment than in the control treatment ($t_{1,7} = 1.93$, $P = 0.09$; fig. 3).

Discussion

We found that experimentally inoculated *Fregaria virginiana* attained similar vegetative size but produced fewer flowers and more plantlets, relative to control plants. However, some of these effects varied with cross type and maternal genotype. We also found that both the magnitude and the direction of inbreeding depression changed with experimental mycorrhizal inoculation, but the effects were life-stage or trait dependent. We discuss these results in more detail and in light of their potential to influence mating and sexual system evolution.

Effects of Experimental Inoculation with Mycorrhizal Fungi on Wild Strawberry Plants

Given that inoculation with mycorrhizal fungi can sometimes (Gange and Smith 2005) but not always result in increased vegetative growth (Pendleton 2000; Dunham et al. 2003; Nuortila et al. 2004; Perner et al. 2007), the lack of an effect of mycorrhizal treatment on aboveground vegetative growth rate may not be surprising. The absence of a positive effect of mycorrhizal infection on vegetative growth has often been attributed to light limitation of photosynthesis (Dunham et al. 2003) or abundance of soil nutrients (Johnson 1993). However, this is not likely the case here because there were significant interactions between mycorrhizal treatment and maternal genotype (and between maternal genotype and cross type) on growth rate (fig. 1). This suggests that the overall lack of an effect was due more to heterogeneity in response to mycorrhizal inoculation among different maternal genetic lineages than to resource availability during the experiment. For example, for some maternal genotypes average growth rate was two to three times as high in the inoculated group as in the control group (fig. 1, maternal genotypes 254 and 456), whereas in other maternal genotypes there was no difference between treatments (fig. 1, maternal genotype 204). Such genotype-dependent effects of mycorrhizal inoculation are not uncommon (Mark and Cassells 1996; Khaosaad et al. 2006), but the cause of the variation is not well known. Here we consider two possibilities. First, variation in maternal genotypes in the response to inbreeding is expected for most additive traits (Schultz and Willis 1995), so if the mycorrhizal fungus–plant interaction is determined by genes with additive effects, then the presence of variation among maternal genotypes may reflect the variation in the distance families are from the population mean breeding value for the mycorrhizal fungus–plant interaction. Second, maternal genotype–dependent variation may reflect variation in the inoculation success between the treated and the control plants. In fact, differences in phenotypic variation between the treatments could contribute to differences in the magnitude of inbreeding depression because the environments that accentuate the phenotypic variation enhance the opportunity for selection and thus inbreeding depression (Waller et al. 2008). Further study is required to draw a definitive conclusion regarding the genesis of maternal genotype variation in response to experimental inoculation with mycorrhizal fungi.

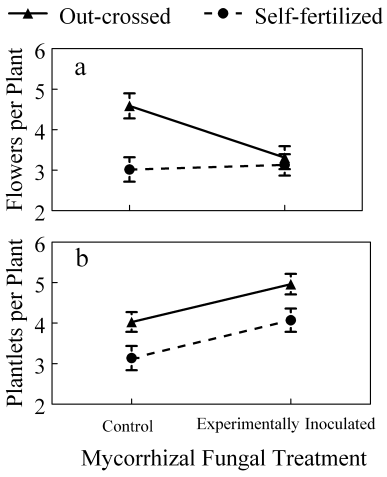

Fig. 2 Sexual (*a*, flowers/plant) and asexual (*b*, plantlets/plant) reproduction in *Fragaria virginiana* produced by two cross types (outcrossed or selfed) grown under two mycorrhizal treatments (experimentally inoculated and control). Means ± SE are presented. The interaction between cross type and mycorrhizal treatment was significant for flower production but not for plantlet production (see table 1 for details).

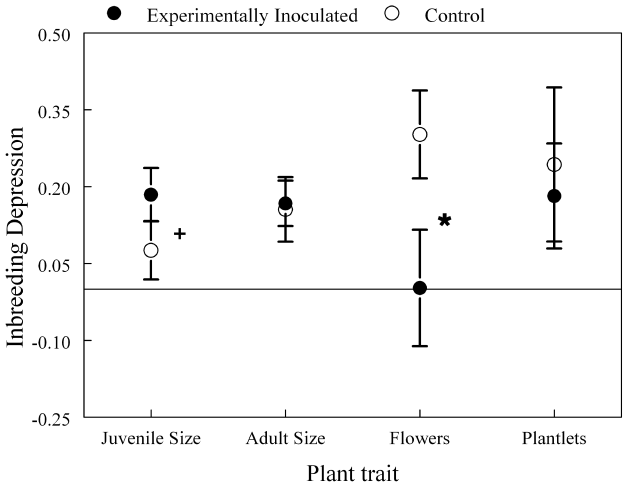

Fig. 3 Effects of mycorrhizal treatment on the expression of inbreeding depression in greenhouse-grown *Fragaria virginiana*. Estimates are mean (± SE) of inbreeding depression across eight maternal genotypes for progeny that were experimentally inoculated with mycorrhizal fungi or not inoculated (control). Inbreeding-depression coefficients that are significantly different at $P < 0.05$ are denoted with an asterisk, and those at $P < 0.10$ are denoted with a plus sign.

While flower or inflorescence production has been seen to increase in response to experimental inoculation with mycorrhizal fungi in several studies (Liu 1995; Pendleton 2000; Gange and Smith 2005; Perner et al. 2007), a study by Nuortila et al. (2004) and our study found that flower production was reduced by mycorrhizal fungal inoculation. Our study also revealed variation in the response of flower production to mycorrhizal inoculation based on cross type: outcrossed plants reduced flower production with experimental inoculation, whereas selfed plants showed no change in flower production (fig. 2*a*). This result may reflect competition for resources between developing flower buds and fungal storage structures, because both flower buds (Hancock 1999) and fungal storage structures, i.e., vesicles (Peterson et al. 2004), are largely formed at the end of the growing season. To explore this possibility, we revisited the older root samples taken at the end of the growing season. We found that roots of outcrossed plants in the inoculated group had the most vesicles; in fact, they had approximately four times as many vesicles per segment as those of the outcrossed plants in control group. In contrast, the roots of selfed plants had low vesicle densities regardless of treatment group, and these were as low as that of the control outcrossed group (cross type × treatment interaction: $F_{1,28} = 7.24$, $P = 0.012$). These findings perfectly mirror the pattern of differences in flower production (fig. 2*a*), suggesting that competition between the plant and the fungi for resources may well have occurred but in a cross type–dependent manner. These results also suggest that the timing of the formation of the plant-fungus relationship was delayed in the inoculated selfed plants and that the relationship perhaps occurred as slowly as the natural colonization by ambient fungi that occurred in the control groups. In *F. virginiana* plants grown under seminatural conditions and naturally colonized by mycorrhizal fungi, C. L. Collin and T.-L. Ashman (unpublished manuscript) also found that roots of outcrossed plants tended to have more vesicles than those of selfed plants just after reproduction. Another possibility is that resource competition occurred between flower buds and allocation to arbuscular cells. Specifically, recent work has shown that arbusculated cells represent greater sucrose sinks than corticular cells; this, combined with an associated activation of defense genes (see García-Garrido and Ocampo 2002 for review), may have represented a competing resource sink within the plant. Regardless of the mechanism, our results suggest that the plant–mycorrhizal fungus relationship differs, depending on cross type, and that the dynamics of this relationship should be studied in greater detail.

We found that inoculation with mycorrhizal fungi increased plant investment in asexual reproduction, as measured by the number of plantlets produced per plant. This was similar for both outcrossed and selfed plants, although the latter had fewer plantlets on average. A similar enhancement of clonal growth with experimental inoculation has been found in other strawberry species. Mark and Cassells (1996) found that runner number in *Fragaria vesca* was increased by inoculation with *Glomus fistulosum*. Likewise, Varma and Schuepp (1994) found increased production of stouter runners in field-transplanted *Fragaria* × *ananassa* inoculated with *Glomus intraradices*, although this varied with commercial variety, possibly because of differences in infection rate. Several recent studies have commented on the potential population implications of mycorrhizal fungus–mediated changes in asexual reproduction (Streitwolf-Engel et al. 2001; Cheplick 2004). Our results add to these and suggest that mycorrhizal colonization could promote clonal spread in wild strawberry populations and thus ultimately influence population genetic diversity and structure.

Stage- and Trait-Dependent Effects of Mycorrhizal Fungi on Inbreeding Depression

The effect of experimental inoculation with mycorrhizal fungi on inbreeding depression depended on the plant trait measured. This result may not be surprising, given that outcomes of potentially beneficial interactions often depend on the size or age of participants (Bronstein 1994), and changes in these characteristics can cause mutualisms to shift to parasitism, i.e., as the cost-benefit ratio tips toward costs for the plant (Bronstein 1994). We found that the effect of experimental inoculation on vegetative size first leads to increased inbreeding depression (owing to the relatively smaller size of selfed vs. outcrossed plants under experimental inoculation) but that this effect was lessened by the time plants reached the adult stage (fig. 3). At flowering, experimental inoculation instead decreased inbreeding depression (because of the reduced flower production by outcrossed plants under experimental inoculation; fig. 2*a*), but when plants began to reproduce asexually after flowering, the effect of experimental inoculation had again diminished (fig. 3). With these data, it is not possible to determine whether these variable results are due to trait dependence or stage dependence or whether they simply reflect feedback in the plant-fungus relationship. However, these results, while complex, add to the growing number of studies that demonstrate that biotic interactions can affect inbreeding depression and that the effects can be stage or trait dependent (Hull-Sanders and Eubanks 2005). Variable effects of biotic interactions on the expression of inbreeding depression are quite common and may depend on many factors, including the time at which the interaction develops. This has been seen to occur in plant-plant competitive

interactions in which the effect of competition on inbreeding depression increases in magnitude with increasing exposure. For example, inbreeding depression in *Hydrophyllum appendiculatum* increased with plant age in the presence of competitors but was more or less constant with age in the absence of competitors (Wolfe 1993). Interactions that can shift between beneficial and costly (i.e., mutualisms and parasitism) or that involve a third interactor (Bronstein 1994) might be more likely to produce variable results like those presented here. More studies of inbreeding depression that allow for and quantify biotic interactions are needed to test these ideas.

The power of this study is that it involved experimental addition of fungus spores, making the opportunity for colonization similar in selfed and outcrossed plants; however, surveys of mycorrhizal fungus–plant association in the field will also be important for assessing the likelihood that biotic interactions can mediate inbreeding depression. In one such study, C. L. Collin and T.-L. Ashman (unpublished manuscript) found that selfed wild strawberry plants were less likely than outcrossed ones to be colonized by root fungi under seminatural conditions. Moreover, studies in the wild allow for the additional possibility of three-way interactions between plants, mycorrhizal fungi, and other mutualists (e.g., pollinators) or antagonists (e.g., competitors, pathogens). These multiway interactions may be important for mediating inbreeding depression in the field and could explain why inbreeding depression is not always stronger when assessed in the field than in the greenhouse (Keller and Waller 2002).

Implications for Mating and Sexual System Evolution

The magnitude of inbreeding depression is an important predictor of mating and sexual system evolution (see Ashman 2006 and Steets et al. 2007 for review), so knowledge of how biotic interactions mediate its expression is paramount. Our results suggest that mycorrhizal fungi can affect the expression of inbreeding depression but that the effects are variable, and we do not yet know whether they are cumulative (i.e., have a lifetime effect) in this perennial plant. Thus, longer-term studies will be especially valuable. If mycorrhizal colonization affects cumulative inbreeding depression and the degree of mycorrhizal colonization varies among populations, then this biotic interaction could contribute to variation in sexual system expression in gynodioecious *F. virginiana* (see Ashman 2006 for review). However, knowledge of the effects of mycorrhizal fungi on the relative seed fitness of females and hermaphrodites would also be needed to draw definitive conclusions regarding their effect on sexual system evolution. While this study cannot definitively determine whether plant root–fungus interactions are important drivers of sexual system evolution, it has provided the first evidence of that possibility and should encourage other studies of this topic.

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