Evolutionary Ecology Research, 2010, **12**: 477–490

Root fungi in wild strawberry: root colonization depends on host inbreeding

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ABSTRACT

Question: What is the effect of level of host inbreeding on fungal root colonization?

Hypothesis: Selfed plants are poorer hosts than outcrossed ones and should be less intensively colonized.

Organism: Hermaphroditic individuals of the self-compatible wild strawberry *Fragaria virginiana* (Rosaceae).

Methods: Selfed and outcrossed progeny were grown at three resource levels. Plants were scored for vegetative size, leaf disease, and root fungi in semi-natural conditions.

Results: Hyphal colonization was greater in outcrossed than in selfed plants, but the effect was marginal for vesicular colonization. Neither resource level nor maternal genotype influenced the effect of plant inbreeding on colonization. But fungi associated with plants in high resources produced more vesicles. Inbreeding depression in plant vegetative size was positively correlated with cross differential root colonization.

Keywords: *Fragaria virginiana*, inbreeding depression, root fungi, Rosaceae, vesicular arbuscular mycorrhizal (VAM) fungi.

INTRODUCTION

Recent studies have emphasized the links between above- and belowground communities (Huhta, 2007), and the fact that the outcome of species interactions is likely influenced by the ecological context of interacting partners. The type of an interaction may indeed change depending on ecological conditions [e.g. a mutualism may become an antagonism (Bronstein, 1994)], and species interactions are better described as a continuum. Associations with root fungi fall into this continuum for the plant host. Under some conditions, well-characterized root pathogens can be of no effect or even become beneficial to their plant host [which may change the evolutionary outcomes of these interactions (Salvaudon *et al.*, 2007)], whereas well-known root mutualists such as mycorrhizal fungi, which contribute to plant mineral

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nutrition and receive carbon in return (Smith and Read, 1997), may be occasionally detrimental to their hosts (Egger and Hibbett, 2004; Jones and Smith, 2004). From the fungus's point of view, the association may also have different outcomes, depending on factors that affect the plant's ability to fight or sustain the fungus, which may derive from environmental or genetic features (Graham and Eissenstat, 1994).

There may be considerable variation in the outcome of the interaction depending on the host–fungus pair, as well as the host genetic variation (Smith and Goodman, 1999). However, to our knowledge, no study has examined the effect of level of host inbreeding on root fungi. This oversight is surprising given that the mating system is arguably one of the most important determinants of genetic structure in populations (Loveless and Hamrick, 1984). Selfed individuals often exhibit a reduction in fitness and fitness-related traits compared with outcrossed individuals [i.e. inbreeding depression (Charlesworth and Charlesworth, 1987)]. Such an effect may, in turn, translate into an altered interaction with mutualists or antagonists. This effect has been studied for pollinator mutualists [inbreeding reduces attractivity and visitation (e.g. Carr and Dudash, 1996; Ouborg *et al.*, 2000; Ivey and Carr, 2005)] and antagonists [inbreeding reduces resistance/tolerance to attacks (e.g. Carr and Eubanks, 2002; Carr *et al.*, 2003; Ivey *et al.*, 2004; Stephenson *et al.*, 2004)], and might occur with root fungi as well. Specifically, inbreeding level could affect the plant's relationship with root fungi by directly interfering with the steps involved in the initiation of the interaction (e.g. signalling and recognition), or by indirectly reducing the plant's ability to sustain mutualist fungi (e.g. carbon provisioning to mycorrhizal fungi via plant size; efficiency of the interaction) or to counter colonization by antagonist fungi (i.e. resistance or tolerance). Whatever the underlying mechanism, host inbreeding level is expected to modify the frequency and the intensity of the interaction with root fungi, whether they are mutualists or antagonists, and this may feed back on the intensity of inbreeding depression (Botham *et al.*, 2008).

In the present study, we tested the hypothesis that, overall, selfed and outcrossed plants would show different relative root colonization. Depending on the functional role of root fungi that colonize strawberry roots, we can contrast two main situations. Specifically, if mycorrhizal fungi predominate, we expect selfed plants to be less colonized than outcrossed ones because they are expected to be poorer hosts and bring fewer benefits to mutualist fungi. In contrast, if pathogens predominate, selfed plants will be more colonized than outcrossed ones because of a lower resistance to attack (see above). Although the opportunity for cohabitation between different fungal types exists, it is reduced because mycorrhizal fungi compete with and can affect the incidence of other root-inhabiting fungi (Saikkonen *et al.*, 1998; Borowicz, 2001), through a variety of mechanisms (Azcón-Aguilar and Barea, 1996). This was confirmed by two studies showing that most colonization was mycorrhizal when roots were colonized by both mycorrhizal and other fungi (Gange *et al.*, 1999; Schroeder and Janos, 2004), and the use of mycorrhizal fungi as biocontrols against root pathogens (Harrier and Watson, 2004; Whipps, 2004).

We used the Virginian wild strawberry, *Fragaria virginiana*, a species that displays a mixed mating system and is known to be naturally colonized by root fungi, in particular mycorrhizal fungi. We performed controlled pollinations to obtain selfed and outcrossed progeny and compared the relative fungal root colonization between the two inbreeding levels. As plants were installed in a semi-natural environment, they were passively inoculated and our results thus reflected the net effect of colonization by a range of naturally occurring fungal root species. In addition, both selfed and outcrossed plants were grown under contrasting levels of resources to investigate the effect of the host plant

resource availability on the relative colonization of the two inbreeding levels. Indeed, environmental conditions may modify the traits of selfed relative to outcrossed individuals, especially in stressful conditions (Armbruster and Reed, 2005; Cheptou, 2006), and this may change the relative colonization between selfed and outcrossed hosts.

We asked the following specific questions: (1) Under semi-natural conditions, are the roots of selfed wild strawberry plants less often and/or less heavily colonized by fungi than those of outcrossed plants (i.e. mutualist hypothesis) or is the opposite the case (antagonist hypothesis)? (2) Does the relative difference in colonization between crosses vary with host plant resource availability? (3) Is inbreeding depression in plant vegetative size correlated with cross differential in root colonization? The sign of the relationship would be indicative of which hypothesis is more likely (i.e. mutualist vs. antagonist) and allow a discussion of the importance of plants' inbreeding level in determining their interaction with root fungi and the potential role of root fungi on plant mating and sexual system evolution, whether they are mutualists or antagonists. We found that outcrossed plants are more colonized than selfed ones and that this difference in colonization correlates positively with the intensity of inbreeding depression, although the effect of inbreeding on root colonization does not vary with the host's resource availability.

MATERIALS AND METHODS

Study system

The Virginian wild strawberry *Fragaria virginiana* Mill. (Rosaceae), a perennial herbaceous plant, occurs naturally in North America (Staudt, 1989). This species is octoploid (Rousseau-Gueutin *et al.*, 2009), although most of its genome now behaves as a diploid [i.e. disomic segregation of markers (Ashley *et al.*, 2003)]. In northwestern Pennsylvania, natural populations are sexually dimorphic, and self-compatible hermaphrodites co-exist with females and the sex ratio varies dramatically among populations (Ashman, 1999). Hermaphrodites exhibit a broad range of fruit setting ability (Ashman, 1999) and selfing rates (L. Penet *et al.*, unpublished manuscript). Moreover, selfed progeny show a decrease in vegetative size compared with outcrossed progeny [i.e. inbreeding depression (C.L. Collin *et al.*, unpublished manuscript)], and the expression of inbreeding depression is affected by mycorrhizal fungi, though differently among life stages (Botham *et al.*, 2008).

Most Rosaceae species form mycorrhizal associations (84%) that mainly involve vesicular arbuscular mycorrhizal (VAM) fungi from the Glomeromycota phylum, and only a few species interact with ectomycorrhizae or both (Wang and Qiu, 2006). Vesicular arbuscular mycorrhizal fungi are characterized by the presence of arbuscules in host roots (i.e. structures involved in nutrient exchange between host and fungus). Hyphae serve to transport nutrients from the soil to the roots and carbon from the host to the fungus and its storage organs (i.e. vesicles). Among wild strawberries, mycorrhizal associations have been described in *Fragaria chiloensis* (Fontenla *et al.*, 1998), *F. vesca* (Harley and Harley, 1987; Mark and Cassells, 1996), and *F. virginiana* (Botham *et al.*, 2008; J. Taylor *et al.*, unpublished data), although detailed studies mostly focused on the cultivated hybrid *Fragaria* × *ananassa* (e.g. Sharma and Adholeya, 2004, and references therein). Strawberry roots also host a variety of root fungi that can cause more or less severe root diseases (Nemec, 1975), and the potential use of mycorrhizal fungi to control fungal root diseases seems a promising way to protect cultivated species (Norman *et al.*, 1996; Whipps, 2004).

Genotypes, cross types, and resource treatments

We used eight hermaphrodite maternal genotypes originating from a natural population [PR population in Ashman (1999)], near the Pymatuning Laboratory of Ecology (Crawford County, PA). For each maternal genotype, flowers were hand-pollinated in an insect-free greenhouse at the University of Pittsburgh, and selfed and outcrossed seeds were produced. Seedlings of both cross types were transplanted into three different resource levels, achieved by manipulating pot size, and nutrient and fertilizer supply. In the Extra-Low treatment, 130-ml pots were filled with one part of Fafard® #4 potting soil (Conrad Fafard) and two parts of sand; in the Low treatment, 130-ml pots with 2:1 Fafard/sand; and in the High treatment, 200-ml pots with 2:1 Fafard/sand. Seedlings grown in the Extra-Low, Low, and High treatments received 2, 4, and 8 pellets of Osmocote® fertilizer (nitrogen/phosphorus/potassium 14:14:14), respectively. In the fall, potted plants were transferred to the Pymatuning Laboratory of Ecology where they were randomized to overwinter among the natural vegetation.

Root sampling and plant traits

In the spring of 2006, plants were transferred into larger pots (901 ml), topped up with the same proportions of potting soil (nutrient-free) and sand as above. We did not add fertilizer and prevented transplanted plants from obtaining additional resources from the soil. At this stage, plants expressed vegetative differences due to the resource treatments, especially for plant size, which was positively influenced by resource level [i.e. total plant biomass (see Ashman *et al.*, 2001)]. Mean (±S.E.) estimated vegetative size (see Ashman, 1999) for the Extra-Low, Low, and High resource treatments was 86.5 (±4.6), 150.4 (±8.6), and 212.0 (±13.8), respectively, and differed significantly among treatments (Welch ANOVA allowing unequal variances, $F_{2,73.2} = 51.3$, $P < 0.0001$). Fungal colonization of potted plants occurred naturally at the Pymatuning Laboratory of Ecology, where wild strawberry populations show substantial colonization by VAM fungi (J. Taylor *et al.*, unpublished data). In July, we used apple corers to extract cores of soil and collect root fragments from 2–4 plants per cross type per genotype within each resource level (total of 128 plants). Because both plant size and amount of leaf damage are likely to influence fungal root colonization [e.g. mycorrhizal fungi (Gehring and Whitham, 1994)], we measured these at the sampling time to use as covariates in our statistical analyses. We scored disease status as the proportion of leaves showing fungal damage [leaf scorch or leaf spot (Cole and Ashman, 2005)].

Root colonization scoring

Root samples were preserved in 70% ethanol until processing. We prepared about ten 1-cm root segments and stained them using the ink and vinegar method of Vierheilig and colleagues (1998), with the following modifications. At each step we added 0.5 ml of chemicals to root segments in microcentrifuge tubes: clearing in 10% KOH (10 min at 95°C), rinsing with tap water, staining in a 5% Cross® black ink-vinegar solution (3 min at 95°C), destaining with acidified tap water (20 min). Root segments were mounted on slides and examined individually under a compound microscope to score fungal colonization (magnification: 100× to 400×). The proportion of root segments colonized with hyphae

constituted the incidence of hyphal colonization, and the proportion of these colonized segments containing vesicles constituted the incidence of vesicular colonization. The percentage of root length that was colonized was also estimated and converted into a non-linear scale from 0 to 5 as the intensity of hyphal colonization (0 = no hyphae; 1, 2, 3, 4, and 5 = 1–5%, 6–25%, 26–50%, 51–75%, and 76–100% of the root length with hyphae present, respectively), following Graham *et al.* (1995). This allowed us to distinguish between poorly colonized roots (1–5%), which may indicate either a poor or an early association, and un-colonized roots (0%). A total of 870 root pieces from 128 plants were individually scored for fungal colonization.

Fungal hyphae and vesicles of different size, density, and aggregation patterns were encountered throughout the samples. These could belong to endomycorrhizal fungi as well as to other root-inhabiting fungi. We observed no root necrosis, suggesting that if pathogenic root fungi were present, these were likely biotrophic (Mendgen and Hahn, 2002). Nor did we observe hyphae networks on root surface or colonization of the vascular system. The presence/abundance of hyphae in roots reflects the existence of an interaction between host plants and root fungi. The presence of vesicles indicates that the fungi received carbon from their host, although an analysis of the lipid content would precisely determine the storage status of the fungus (van Aarle and Olsson, 2003). Arbuscules were found in preliminary samples stained with Trypan Blue (J. Taylor, personal communication) but they were not observed in all root samples with the staining process we used here. While our staining technique may have prevented the observation of arbuscules (Gange *et al.*, 1999), it is also possible that these structures had disappeared from the old root samples we scored, as they are transient structures with a rapid turnover (Smith and Read, 1997). In general, the presence of arbuscules is the definite indication of an active association with VAM fungi because of their role in nutrient exchange (Smith and Read, 1997). Thus, although the structures we scored could not be unambiguously attributed to mycorrhizal fungi, exposing our results to our expectations should give a quite reliable indication of the fungal type colonizing wild strawberry roots in semi-natural conditions.

Statistical analyses

To determine whether outcrossed and selfed plants were colonized differently across maternal genotypes and environments, we employed full factorial mixed-model analyses of covariance, with maternal genotype as a random factor, cross type and resource level as fixed factors, and plant size and plant disease status as covariates. Analyses were performed in JMP v.5 (SAS Institute, Inc., 2002) using the REML method. Maternal genotype was included to account for genetic differences among families, and it was considered a random factor because we did not choose the genotypes specifically within the natural population (we present the percentage of the total variance explained by maternal genotype). An overall effect of cross type is expected if selfed and outcrossed plants differ in their ability to establish or fight or sustain interactions with root fungi, whereas a significant interaction between cross type and resource level would indicate that plant resource availability influences the effect of inbreeding on root colonization. A significant plant size effect would indicate that root colonization depends on host size independently of, or in addition to, factors inducing variation in size – that is, cross type, maternal genotype, and resource level in our design. Incidence and intensity of hyphal colonization were highly correlated ($r = 0.91$, $P < 0.0001$), and yielded qualitatively similar results (not shown), and therefore we

present analyses only for the intensity of colonization (i.e. a more precise measure of root colonization by fungal hyphae) and the incidence of vesicular colonization.

Based on the hypothesis that host inbreeding alters the relationship with root fungi, we predicted that inbreeding depression in plant vegetative size would be correlated with cross differential fungal root colonization, across genotypes. The sign of this correlation would depend on the type of fungus present in wild strawberry roots: positive if selfed plants are poorer hosts than outcrossed ones and their roots are less colonized by mutualist fungi; and negative if selfed plants are less able to defend against antagonists and are more colonized than outcrossed plants. We estimated inbreeding depression in vegetative size following Ågren and Schemske (1993); that is, for each maternal genotype, we calculated $1 - s/o$ when selfed progeny were smaller or of equal size as outcrossed progeny and $o/s - 1$ otherwise, with s and o the mean vegetative size of selfed and outcrossed progeny, respectively. To determine whether inbreeding depression in vegetative size was correlated with cross differential root colonization, we used the same formulae to calculate the relative difference in colonization between crosses for the two colonization traits ($n = 8$ maternal genotypes for each correlation).

RESULTS

Selfed plants showed lower root colonization

In hermaphrodite plants of *Fragaria virginiana*, root colonization rate was high with ~70% of the root pieces colonized with fungal hyphae. We found that outcrossed plants were significantly more colonized by hyphae than selfed plants (intensity of hyphal colonization) (see Table 1; Fig. 1, top panel), suggesting that wild strawberry roots were colonized in the main by mycorrhizal fungi (mutualist hypothesis). A similar and close to significant effect of cross type was also found for the incidence of vesicular colonization among the root

Table 1. Restricted maximum likelihood ANCOVA of total fungal colonization in roots of the wild strawberry *Fragaria virginiana*

		Intensity of hyphal colonization[a]		Incidence of vesicular colonization[b]	
Source	d.f.	*F*	*P*	*F*	*P*
Cross type	1	4.96	**0.029**	3.54	*0.064*
Genotype (random)[#]	7	—	—	—	—
Cross × Genotype	7	1.80	*0.099*	0.90	0.514
Resource level	2	0.72	0.489	4.35	**0.016**
Cross × Resource	2	0.75	0.475	0.72	0.490
Genotype × Resource	14	0.68	0.788	0.76	0.712
Cross × Genotype × Resource	14	1.10	0.367	1.24	0.266
Vegetative plant size	1	0.23	0.630	2.59	0.112
Disease status	1	1.82	0.182	0.13	0.717

Bold values, $P < 0.05$; italic values, $P < 0.1$.

Number of degrees of freedom for error: [a] 78 and [b] 74.

[#] Shrunken; percent of the total variance explained: [a] 1.9% and [b] 7.5%.

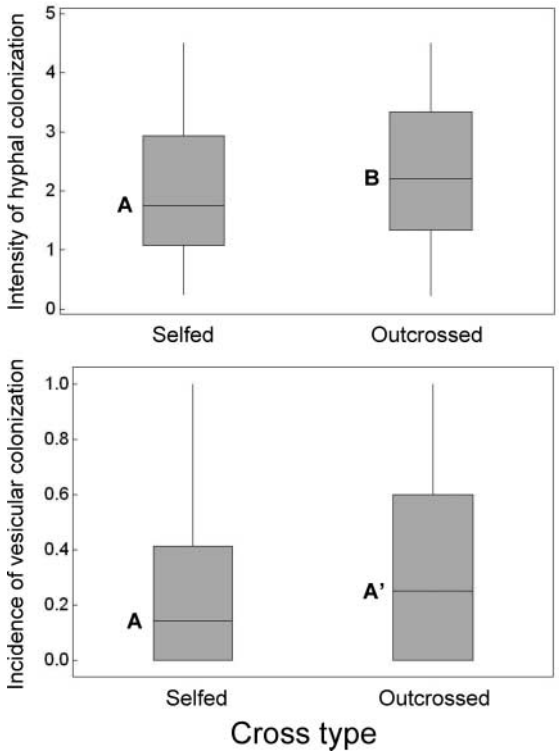

Fig. 1. Box-and-whisker plots of the fungal colonization in roots of selfed and outcrossed plants of the wild strawberry, *Fragaria virginiana*. The intensity of hyphal colonization (top panel) and the incidence of vesicular colonization (bottom panel) were quantified (see text for details). The figure shows median fungal colonization (lines), 25% to 75% quartiles (boxes), and ranges (whiskers). Different letters denote significant differences ($P < 0.05$); the same letter with a prime indicates that the difference was marginally significant ($P = 0.06$).

pieces that were colonized by hyphae. Roots from outcrossed plants contained vesicles 1.5 times more often than those from selfed plants (mean incidence ± S.E.: 0.32 ± 0.04 and 0.22 ± 0.04, respectively, $P = 0.064$) (see Table 1; Fig. 1, bottom panel).

Resource level and root colonization

Host plants were submitted to contrasting resource levels, but this did not influence the relative difference in root colonization between outcrossed and selfed plants (cross × resource interactions, $P > 0.5$ for both analyses; Table 1). Resource availability as a main factor did not have an effect on hyphal colonization ($P = 0.489$) but the incidence of vesicular colonization differed significantly among resource levels ($P = 0.016$; Table 1). Plants grown under harsh (Extra-Low) conditions showed significantly less vesicular colonization than the plants in the rich (High) environment, whereas plants in the Low condition were colonized by vesicles at an intermediate rate between the two extreme treatments (see Fig. 2 for *a posteriori* contrasts with an adjusted critical level of 0.017).

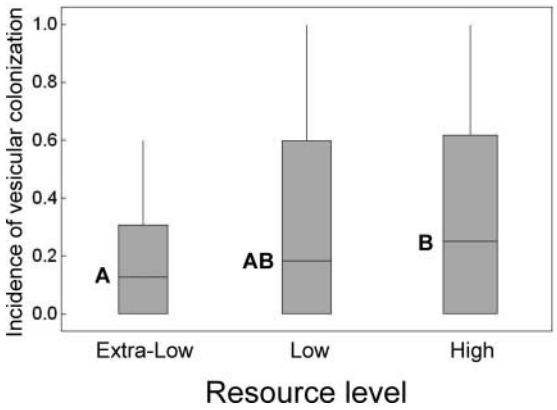

Fig. 2. Box-and-whisker plots of the vesicular colonization in roots of the wild strawberry, *Fragaria virginiana* grown under three resource levels. The figure shows median fungal colonization (lines), 25% to 75% quartiles (boxes), and ranges (whiskers). Different letters denote significant differences at the adjusted alpha value of 0.017.

Positive relationship between cross differential plant size and root colonization

The marginally significant cross type × genotype interaction suggests that host genetic background may play a role in the intensity of hyphal colonization, although not the incidence of vesicular colonization ($P = 0.099$ and $P = 0.514$, respectively; Table 1). Among the maternal genotypes we sampled, inbreeding depression in plant vegetative size ranged from slightly negative to 26% (i.e. selfed plants varied from slightly larger to much smaller than outcrossed plants). We nevertheless found a positive correlation between inbreeding depression in vegetative size and difference in root colonization between crosses within maternal genotypes (Fig. 3), which was significant for the intensity of hyphal colonization ($r = 0.88$, $P = 0.004$) but not for the incidence of vesicular colonization ($r = 0.51$, $P = 0.20$; Fig. 3).

Vegetative plant size is known to vary not only between crosses but also among maternal genotypes and resource levels; however, in the present experiment, plant size did not significantly influence root colonization beyond these effects ($P > 0.1$ for both analyses; Table 1). Contrary to what might have been expected, plant disease status did not affect any of the colonization traits we scored ($P > 0.2$ for both analyses), perhaps because the damage was not severe enough to impact the plants' relationship with root fungi (on average less than half of the plant leaves showed signs of infection).

DISCUSSION

We found that roots of outcrossed plants of the wild strawberry *Fragaria virginiana* were more colonized by root fungi than those of selfed individuals. This is in line with the hypothesis that semi-natural fungal root colonization in this species and under our experimental conditions was by fungal mutualists, probably mycorrhizae, and shows a novel aspect of how host genetics might impact biotic interactions. In contrast, relative differences in root colonization between cross types were not affected by host resource availability. The overall difference in root colonization between selfed and outcrossed plants, however, may

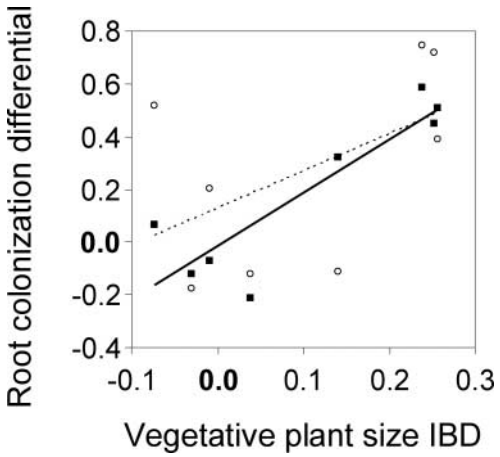

Fig. 3. Correlation between inbreeding depression (IBD) in vegetative plant size and cross differential fungal colonization in roots of the wild strawberry, *Fragaria virginiana*. Solid squares represent the intensity of hyphal colonization (solid line; $r = 0.88$, $P = 0.004$) and open circles represent the incidence of vesicular colonization (dashed line; $r = 0.51$, $P = 0.20$); $n = 8$ maternal genotypes.

still feed back on the expression of inbreeding depression (e.g. Botham *et al.*, 2008). In the following paragraphs, we discuss these findings in more detail and suggest the potential impact of plant–root fungi interactions on the evolution of plant mating and sexual system.

Host inbreeding alters fungal root colonization

Host inbreeding represents an important source of variation among host plants to a wide range of mutualist and antagonist partners (Carr and Dudash, 1996; Smith and Goodman, 1999; Ouborg *et al.*, 2000; Carr and Eubanks, 2002; Carr *et al.*, 2003; Ivey *et al.*, 2004; Stephenson *et al.*, 2004; Ivey and Carr, 2005). In the present study, we found that inbreeding level altered the relationship between root fungi and wild strawberry hosts, with selfed plants displaying less colonization than outcrossed plants. In particular, inbreeding level had the greatest impact on hyphal colonization of strawberry roots (Fig. 1, top panel), which could indicate that selfed plants were less prone to interact with root fungi, possibly because of altered mechanisms of signalling or recognition (e.g. Gadkar *et al.*, 2001). The fact that selfed plants tended to contain fewer vesicles than outcrossed plants after the establishment of the interaction indicates that carbon transfer from plant to fungi may have been less efficient in selfed hosts, or that root fungi colonizing selfed plants could sequester fewer resources from them (but see van Aarle and Olsson, 2003). Carbon allocation within plants is a delicate balance, especially when host plants support symbionts (see Matyssek *et al.*, 2005), and, based on the hypothesis that inbreeding level negatively affects plants' ability to control their carbon allocation, we would have observed greater root colonization in selfed than in outcrossed plants instead. Our results thus suggest that host plant inbreeding level may mostly alter the plant's ability to interact with root fungi and, to a lesser extent, the efficiency of this interaction once it is initiated. Yet, the underlying mechanisms remain to be determined. In our design, we controlled for modifications in vegetative plant size and indirect plant-size mediated effects, which suggests that host plant inbreeding level is more likely to have direct effects on fungal root colonization. To our knowledge, this is the first study to directly quantify fungal root colonization in selfed and outcrossed plants. As

F. virginiana is an octoploid species, it might retain higher heterozygosity levels than true diploids after selfing. Therefore, we should expect more dramatic inbreeding effects on root colonization in diploid species. As our results are based on a range of naturally occurring species, experimental studies controlling the inoculation by single or paired fungal species would help us to understand the effect of inbreeding level on root colonization in greater detail, such as species-specific effects.

In our experimental design, root colonization occurred in semi-natural conditions and fungi may have ranged from mutualist to pathogenic. However, we suspected that colonization by mycorrhizal fungi could predominate, as *F. virginiana* forms strong associations with VAM fungi in surrounding natural populations (J. Taylor *et al.*, unpublished data), and a preliminary sampling demonstrated the presence of arbuscules in both selfed and outcrossed plants. This is in line with our main result showing less fungal root colonization in selfed plants, as expected if the interaction is mostly with mutualist fungi. There was also a positive correlation between inbreeding depression in plant size and cross differential root colonization. Indeed, if outcrossed hosts were more colonized by root pathogens instead of mutualist mycorrhizal fungi, the opposite correlation would have been found. First, outcrossed plants, being more heavily colonized, would have suffered more from the infection relative to selfed plants. As a consequence, this would have resulted in a negative correlation between inbreeding depression in plant size and cross differential root colonization. Alternatively, since selfed individuals are usually less able to defend against or tolerate attacks by antagonists, we would have expected the roots of selfed plants to be more colonized than those of outcrossed plants and for this relative difference in colonization to have led to an increase rather than a decrease in inbreeding depression in plant size. Given that our results conflict with both of these expectations, we propose that we observed solely, or mainly, mutualist mycorrhizal fungi rather than antagonists. Our results, based on a range of naturally occurring mycorrhizal species, nonetheless point to experimental studies involving single or combined inoculation of known species (Botham *et al.*, 2008) to determine the impact of host inbreeding level on mycorrhizal colonization.

Mycorrhizal colonization, inbreeding depression, and mating and sexual system evolution

Host genetic composition affects the occurrence and the outcome of interactions between beneficial partners and their host plants (Smith and Goodman, 1999), and this may feed back on the host genetic variation and mating system. Our results suggest that, in *F. virginiana*, the feedback will differ between selfed and outcrossed plants and this may affect the magnitude of inbreeding depression. In another study, we showed that mycorrhizae indeed alter the expression of inbreeding depression, although the mycorrhizal effect strongly depends on the life-stage and reproduction mode (sexual vs. asexual) and conclusions on the net effect of mycorrhizal symbiosis on lifetime inbreeding depression in this clonal perennial species cannot yet be made (Botham *et al.*, 2008). However, based on our results and what we know of the effects of mycorrhizal fungi on plants, we can speculate that the lower ability of selfed plants to interact with mycorrhizal fungi should translate into fewer benefits to the plant, which would thus increase inbreeding depression in most cases. On the other hand, the interaction with mycorrhizal fungi could decrease the magnitude of inbreeding depression if outcrossed plants suffer from being heavily colonized – that is, if supporting great loads of mycorrhizae is detrimental to the plant, which commonly occurs under rich environments

Table 2. Expected effects of a greater root colonization by mycorrhizal fungi in outcrossed plant hosts than in selfed ones on the magnitude of inbreeding depression under contrasted environments

	Outcrossed host (more colonized)	Selfed host (less colonized)	Inbreeding depression
High resources	–[a]	+	decrease
Low resources	+	+	increase

[a] Mycorrhizal associations may be parasitic at high resource availability and outcrossed plants may then suffer from being more colonized.

(Johnson *et al.*, 1997). In summary, although the conditionality of mycorrhizal associations (Jones and Smith, 2004) may render it difficult to predict their net effect on host plants, we hypothesize that, overall, mycorrhizal fungi could decrease inbreeding depression under benign conditions, whereas an increase in inbreeding depression may be predicted in harsher environments (summarized in Table 2).

Our experimental design included a resource level dimension and we expected to find a greater relative difference in root colonization between selfed and outcrossed plants in the Extra-Low than in the High resource treatment. This would have suggested an interactive effect between host resource availability and inbreeding level on root fungal colonization. However, we did not find such an effect of host resource availability on root colonization (Table 1), possibly because the low resource treatments we imposed did not constitute stressful enough conditions, or because the resources that varied among treatments did not limit mycorrhizae (Sharma and Adholeya, 2004). Therefore, the joint manipulation of mycorrhizal inoculation and resource level is necessary to estimate inbreeding depression in the presence and absence of mycorrhizal fungi along a range of resource levels, and thus to test formally the potential for mycorrhizal fungi to contribute to the evolution of dioecy (separate sexes) from gynodioecy in harsh environments (Ashman, 2006).

In addition to direct effects on host resources and indirect effects on the expression of inbreeding depression, mycorrhizal fungi also indirectly affect the host's reproductive system through alterations of plant–pollinator (Gange and Smith, 2005) or plant–enemy interactions (Bennett *et al.*, 2006). Such interactive effects may be further complicated in sexually polymorphic species, if mycorrhizal fungi differentially benefit sex morphs, such as females and hermaphrodites of the gynodioecious *F. virginiana*. Sex morphs of gender dimorphic plant species allocate energy differently (Case and Ashman, 2005) and this could alter their interactions with root fungi [e.g. mycorrhizae in juniper (Gehring and Whitham, 1992)], and contribute to sex-ratio dynamics in natural populations provided that root fungi affect relative seed production between sexes. Interactive effects are well studied in factorial experiments (Morris *et al.*, 2007) and such designs would provide a more comprehensive understanding of the potential for plant–root fungi interactions to impact on plant mating and sexual system evolution.

CONCLUSIONS

This study highlights inbreeding level of host plants as an important component determining their interaction with root fungi. Our results in *F. virginiana*, together with those of

Botham and colleagues (2008), suggest that we need to be aware of the consequences of associations with root fungi, and particularly mycorrhizal fungi, in selfed and outcrossed hosts, as well as on abiotic conditions, to assess the effect of environment on the evolution of the plant reproductive system (Ashman, 2006). Although we did not find an effect of resources on the relationship between inbreeding level and fungal root colonization, we nonetheless advocate that mutualistic and parasitic interactions deserve to be investigated as host genotype × partner genotype × environment effects [G × G × E (see Salvaudon *et al.*, 2007)] because abiotic factors add another dimension to the conditionality of interactions.

ACKNOWLEDGEMENTS

This project benefited from the assistance of D. Cole, L. Hefferman, E. Korns, A. Krueger, K. Kurtz, V. N'Guyen, J. Robinson, L. Wright, and especially P. Roszczenko. E. York helped with plant care and J. Taylor identified VAM structures in a preliminary sampling. We thank L. Penet and an anonymous reviewer for constructive comments on the manuscript. This work was supported by NSF (DEB 0449488 and REU supplements). This is contribution 263 to the Pymatuning Laboratory of Ecology (PLE).

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