BRIEF COMMUNICATION

SEX-SPECIFIC EFFECT OF Microbotryum violaceum (Uredinales) Spores on Healthy Plants of the Gynodioecious Gypsophila repens (Caryophyllaceae)

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Females of gynodioecious species need to have reproductive advantages to compensate for their lack of male function and assure their maintenance in natural populations. Females may be more fecund than hermaphrodites because they reallocate resources from male to female function, avoid selfing and inbreeding depression, or produce higher quality offspring by screening arriving pollen. A novel type of female advantage is proposed that may be important for several species of gynodioecious Caryophyllaceae. The anther smut fungus Microbotryum violaceum generally sterilizes its infected hosts but even without infection, spore deposition can negatively affect the reproduction of healthy individuals. In Gypsophila repens, flowers of hermaphrodite plants experimentally pollinated with both fungal spores and pollen produced significantly fewer fruits and seeds than those that received pollen alone, whereas female reproduction was unaffected by spore deposition. This unexplored reproductive advantage of females is probably due to the larger stigmatic surface in pistillate flowers, that allows pollen germination despite the presence of spores. Because longer stigmas may also lead to increased spore deposition, these results raise questions regarding the infection probability for each sex, possible sex-specific defense mechanisms, and the general role of pathogens in the maintenance of females in natural gynodioecious populations.

Key words: female maintenance; fungus; gynodioecy; Gypsophila; Microbotryum; pathogen; Uredinales.

Gynodioecy is a reproductive system in which females and hermaphrodites co-occur in the same populations. Because of their lack of male function, females can only spread and persist in populations if they incur compensatory reproductive advantages over hermaphrodites (Lewis, 1941). Females may produce more fruits, more and heavier seeds with better germination, and better seedling survival (reviewed in Shykoff et al., 2003). Furthermore, in self-compatible species, females can avoid inbreeding depression that can affect hermaphrodites (Charlesworth and Charlesworth, 1978). Females may be less susceptible to herbivore attack and may escape herbivore damage more easily (Mutikainen and Delph, 1996; Ashman, 2002; Collin et al., 2002). For example, female frequency in some natural populations of Sidalcea hendersonii can only be explained by differential predation of seeds from different sexes (Marshall and Ganders, 2001). In Dianthus sylvestris, Eritrichium arctioideis, and Fragaria virginiana, herbivores prefer floral structures and seeds from hermaphrodites and may act as an important selective force permitting female maintenance (Ashman, 1992; Puterbaugh, 1998; Collin et al., 2002). In contrast, sex-specific effects of pathogens in gynodioecious plants remain largely unexplored.

In this study, we experimentally investigated whether the fungus Microbotryum violaceum (Pers.) Deml. & Oberw. (= Ustilago violacea Pers.) differentially affects the reproduction of healthy females and hermaphrodites of Gypsophila repens L. (Caryophyllaceae). This fungus is an important parasite of the Caryophyllaceae. It is a sexually transmitted pathogen that causes anther smut disease in almost 100 species of the family (Thrall et al., 1993). In infected individuals, the fungus induces the production of anthers that contain spores and further destroys the ovary in pistillate flowers that normally do not produce anthers, thereby sterilizing the plant (Baker, 1947; Uchida et al., 2003). Infection is transmitted by pollinators that visit diseased plants and transport spores to healthy plants. In populations with high disease prevalence, the fungus may also affect reproduction of healthy plants in two ways. First, because infection prevents pollen production, the presence of infected individuals reduces the number of pollen donors in the population, thus enhancing pollen limitation (Alexander, 1987). Second, the presence of fungal spores on healthy stigmas may reduce reproductive success (Alexander, 1987; Elmqvist et al., 1993; Marr, 1997; Carlsson-Granér et al., 1998), by physically and/or chemically preventing pollen germination (Marr, 1998).

The effect of M. violaceum on healthy individuals has been investigated in natural populations and controlled conditions in Silene latifolia (Alexander, 1987), S. dioica (Elmqvist et al., 1993; Carlsson-Granér et al., 1998), and S. acaulis (Marr, 1997, 1998). However, sex-specific effects of pathogens in gynodioecious plants remain largely unexplored and to our
knowledge only one study has investigated the sex specific effect of *M. violaceum* on healthy plants (Marr, 1998). Sex-specific effects might occur, because the smaller stigma surface of hermaphrodites observed in several gynodioecious Caryophyllaceae (Dulberger and Horovitz, 1984; Shykoff, 1992) could render this sex more sensitive to the effect of fungal spores. In *S. acaulis*, the inhibition of pollen germination by fungal spores was suggested to be chemically mediated because heterospecific pollen grains did not show the same impact as spores (Marr, 1997, 1998). Nevertheless, even small pollen grains are larger than fungal spores and may therefore have different physical effects.

We addressed the following questions: (1) Is the reproduction of healthy individuals of *G. repens* affected by the presence of fungal spores on their stigmas? (2) Is the effect of the fungus similar for female and hermaphrodite individuals? (3) Is pollen inhibition by the fungus chemically or physically mediated?

**MATERIALS AND METHODS**

*Study species and plant material*—Gypsophila repens is a perennial geophyte distributed in the mountains of south and central Europe. It typically occurs in sunny and periodically dry places in rocky and grassy habitats (Alégro et al., 2000). This species is gynodioecious-gynodioecious, with populations composed of female and hermaphrodite individuals (having pistillate and perfect flowers, respectively) and gynodioecious individuals (bearing both types of flowers). Perfect flowers are protandrous, self-compatible, and larger than pistillate ones (López-Villavicencio et al., 2003). In natural conditions, plants flower from early June until late October. The most common pollinators are small solitary bees and syrphid flies (J. A. Shykoff, personal observation). Pollinators visit many flowers on the same plant and neighboring plants before moving over longer distances.

This species, like many other members of the Caryophyllaceae, is infected by the fungus *Microbotryum violaceum*; however, the anther smut disease of *G. repens* appears to be quite particular. In the majority of species infected by this fungus, the disease leads to complete female sterility (Baker, 1947). This is not the case in *G. repens* (López-Villavicencio et al., 2003), for which infected plants may be either totally or partially infected, the latter producing a mixture of healthy and diseased flowers. Whereas most infected plants bear flowers with greatly reduced stigmas, as observed in other infected species, some have well-formed functional stigmas and are able to produce seeds (López-Villavicencio et al., 2003).

Bulk seed of *G. repens* was collected from a wild population found in the Italian Alps (Grosio, 46°17′24″ N and 10°15′11″ E). Between June and July 2002, 33 female and 36 hermaphrodite plants grown in greenhouse conditions were used for the experiments.

**Effect of *M. violaceum* on the reproduction of healthy plants**—Plants were hand-pollinated using *G. repens* outcrossed pollen alone or in combination with *M. violaceum* spores. For each of 69 plants, the following treatments applied on two flowers: (1) pollen (P), (2) pollen and living spores (SG), (3) pollen and dead autoclaved spores (DSG), and (4) pollen and spores collected from infected individuals of the sympatric Caryophyllaceae *Dianthus sylvestris* Wulf. (SD). The spores collected from *D. sylvestris* did not differ in size from those of the fungus on *G. repens* (M. Le Gac, Université Paris-Sud, France, personal communication). Killed spores from the same species were used to distinguish between chemical and mechanical mediated effects. If the effect of fungal spores on the reproduction of healthy plants is only mediated by physical obstruction, the treatments with live and dead spores will have a similar impact on seed set. In contrast, if spores inhibit pollen growth primarily by chemical means, the dead spores will have no effect. Specificity of the potential chemical effect was investigated using spores from *D. sylvestris*. Because host races of *M. violaceum* are known to be genetically highly differentiated and specialized (Bucheli et al., 2000), they may have evolved specific chemical interactions with their host plants. If the treatment with live spores from *G. repens* has a more dramatic effect on seed set than the two treatments without live spores, this will indicate some specific chemically mediated inhibition of pollen growth. For the first treatment, we pollinated each mature flower by brushing two or three anthers presenting ripe pollen across the stigma. The anthers were taken from at least two different individuals. For the other treatments, spores were first spread over the stigmatic surface using a toothpick, followed by the procedure used for first treatment.

Once the fruits were mature (15–20 d after pollination), they were collected and their seeds counted. Mean seed mass was assessed by individually weighing all the seeds contained in a fruit to the nearest 0.01 mg.

**Stigma measures**—To study differences in the surface available for pollen deposition and germination between females and hermaphrodites, samples of pistillate and perfect flowers were collected in vials filled with 95% ethanol. Stigma length was then measured for one flower from each of 12 female and 13 hermaphrodite plants. The stigmas were mounted individually on slides and measured under a dissecting microscope.

**Statistical analyses**—We performed all statistical analyses using the SAS statistical package (SAS-Institute, 2002). Normality of the residuals and homoscedasticity of data were tested using Shapiro-Wilk’s test.

Because fruit set followed a binomial distribution, differences among treatments (P, SG, DSG, and SD) were studied using generalized linear mixed models (PROC GLIMMIX; SAS, 2002). This type of model, contrary to conventional generalized linear model for binomial error distribution, permits analysis of randomized block designs, in our case, treating plant as a random block effect (Littell et al., 1996). Plant sex, treatment, and the interaction were included in the models as fixed effects, while plant individual, nested within plant sex, was considered as a random block effect.

Differences between treatments in seed number, seed mass, and stigma size were tested using MIXED models (PROC MIXED; SAS, 2002). The distributions of seed number and seed mass were square root transformed, while stigma size was natural logarithm transformed to improve normality and homoscedasticity of residuals. The degree to which seed mass was affected by seed number and treatment, was determined using a minimal factor ANCOVA (PROC MIXED in SAS, 2002) with seed number as the covariate and step-wise elimination of nonsignificant interactions. Again, in all the previous models, we considered plant sex, treatment and the interaction as fixed effects and plant individual within plant sex as a random effect.

For all the cases with a significant interaction between main effects (plant sex and treatment), we used the SLICE option in the LSMEANS statement to determine which groups differed significantly. To test if the action of fungal spores from *M. violaceum* infecting *G. repens* was chemically or mechanically mediated, we compared differences between flowers receiving live or killed spores (SG vs. DSG). The species-specific effect was tested by comparing flowers receiving live spores from *G. repens* and from the sympatric *D. sylvestris* (SG vs. SD). All the pairwise comparisons were made with Bonferroni adjustment using GLIMMIX or MIXED depending on the variable.

**RESULTS**

Fruit set was significantly affected by treatment but not by plant sex (*F*~3,68~ = 6.66, *P* = 0.0002). Flowers that received only pollen produced more fruits than those receiving pollen and spores. The interaction between plant sex and treatment was, however, marginally significant, suggesting that the effect of spore deposition was different between females and hermaphrodites (*F*~3,68~ = 2.41, *P* = 0.066). Testing the sexes separately showed that the addition of fungal spores reduced fruit set in hermaphrodite plants (*F*~3,47~ = 7.37, *P* < 0.0001) but not in females (*F*~3,47~ = 1.41, *P* = 0.2385) (Fig. 1a). For hermaphrodites, the addition of live vs. autoclaved spores from *G. repens* had no differential effect on fruit set, but the addi-
Perfect flowers ± SE = 0.33 ± 0.01, N = 13; $F_{1,25} = 48.17$, $P < 0.0001$).

**DISCUSSION**

Fruit set and mean seed number for *G. repens* were significantly decreased after the experimental application of fungal spores of *M. violaceum*, as already found for *Silene latifolia* (Alexander, 1987), *S. dioica* (Elmqvist et al., 1993; Carlsson-Granér et al., 1998), and *S. acaulis* (Marr, 1997, 1998). Although our results were obtained using hand pollinations only in the greenhouse, they suggest that the effects of *M. violaceum* in natural populations are probably not limited to sterilization of diseased individuals. The fungus could also have an impact on fitness of plants that remain healthy, reducing their fecundity when spores are deposited along with pollen, and possibly contributing to pollen limitation in natural populations with high disease prevalence (Alexander, 1987).

Sex-specific effects on the impact of spore deposition on seed set in gynodioecious species have not been previously found (Marr, 1998). Here we found that reduction in fertility of *G. repens* was significant only for hermaphrodite plants, with no detectable difference for females between flowers pollinated with only pollen and those also receiving spores.

Spores may decrease seed set by physical interference, reducing the available surface for pollen germination, or by chemical interference, producing metabolites that inhibit the germination of the pollen tube (Marr, 1998). In *S. acaulis*, the inhibition of pollen germination by fungal spores was suggested to be chemically mediated because heterospecific pollen grains did not cause the same reduction as did spores (Marr, 1997, 1998). However, even the tiny (mean diameter 11 ± 0.16 μm) pollen grains of *Mertensia viridis* used by Marr (1998) are larger than fungal spores (mean diameter 6–9 μm) (Zogg, 1985) and may therefore have different physical effects. In hermaphrodite plants of *G. repens*, addition of spores similarly affected fruit set and mean seed number, regardless of whether the spores were alive or autoclaved. This suggests that the effect of *M. violaceum* spores on fruit set and mean seed number in this species mainly resulted from mechanical

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**Table 1.** Differences in mean seed number and seed mass between females and hermaphrodites after four pollination treatments: outcrossed pollen (P), pollen and spores of *Gypsophila repens* (SG), pollen and dead spores of *Microbotryum violaceum* (DSG), and pollen and *M. violaceum* spores from *Dianthus sylvestris* (SD). Differences in mean seed number were tested using MIXED models (PROC MIXED in SAS, 2002). Seed mass was analyzed with a minimal factor ANCOVA with seed number as a covariate and stepwise elimination of non significant interactions (PROC MIXED in SAS, 2002). Plant sex, treatment, and the interaction were considered as fixed effects, while plant individual within plant sex (not shown) was treated as a random effect. Significant values are in bold.

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**Fig. 1.** Mean (a) fruit set and (b) mean seed number per fruit for females and hermaphrodites under four pollination treatments: pollen (P), pollen and live spores of *Gypsophila repens* (SG), pollen and dead autoclaved spores of *Dianthus sylvestris* (SD). Differences among treatments within each sex were estimated with pairwise comparisons using Bonferroni adjustment in SAS GLIMMIX (for fruit set) or SAS MIXED (for mean seed number). Treatments within sex types sharing the same lowercase letter did not differ significantly. Bars indicate ±1 SE. Note that neither fruit set nor mean seed number differed significantly for females and hermaphrodites treated with pollen alone (P).
rather than chemical interference, possibly because the spores clog the stigma and prevent efficient pollen success. The diminution in fertility in hermaphrodites may result from a mere reduction of stigmatic surface available for pollen adherence and germination. Indeed, pistillate flowers with their significantly longer stigmas than perfect ones suffered no disadvantage from spore deposition. For the hermaphrodites, live spores from the sympatric D. sylvestris reduced fruit set even more than live spores from G. repens, but seed number was not similarly affected. Although we do not yet understand the nature of this species-specific effect, a chemical interaction, with foreign spores being more damaging than those from the same host species, seems likely. In gynodioecious species, pistillate flowers generally present longer stigmas with larger papillae (Dulberger and Horovitz, 1984; Shykoff, 1992; Puterbaugh et al., 1997; Caruso et al., 2003), making them superior in their ability to capture pollen (Dulberger and Horovitz, 1984). Pistillate flowers are usually smaller than perfect ones and less attractive to pollinators (Delph, 1996). They receive less pollen than do hermaphrodites in natural populations and are expected to be more pollen-limited than their hermaphro-
dite counterparts (Lloyd, 1974; Philipp, 1980; Uno, 1982; Maurice and Fleming, 1995; Williams et al., 2000), even though increased pollen limitation in female plants is restricted to local patches of high female frequency (McCauley and Brock, 1998; Graff, 1999). Nevertheless, the higher stigmatic surface may compensate for lower insect visitation and may help females to catch pollen even under low pollen availability. The difference in stigma size between females and hermaphrodites has been proposed as one of the mechanisms involved in the maintenance of females in natural populations. Pollen grains have been found to germinate better on stigmas of female flowers (Marr, 1998). Longer stigmas in female flowers may also increase pollen competition leading to higher quality progeny (Shykoff, 1988, 1992) although this hypothesis remains unconfirmed (Delph and Mutikainen, 2003). Indeed, in natural populations females usually have a higher seed set than hermaphrodites, and no evidence exists for a pattern of higher pollen limitation in females (Shykoff et al., 2003). The general pattern of longer female stigmas in gynodioecious species may also account for the sex-specific impact of fungal spores on seed set. Our results showed that in G. repens, females have a reproductive advantage over hermaphrodites in the presence of fungal spores. The longer stigmas in females likely account for this advantage, by allowing adequate pollen germination despite the presence of spores. On the other hand, fruit set and mean seed number were similar for females and hermaphrodites when pollen was applied alone. This is somewhat surprising since females are expected to have reproductive advantages that compensate for their lack of male function (reviewed in Shykoff et al., 2003), though female advantage may arise through the better pollen trapping ability of pistillate flowers discussed previously. In addition, the presence of this pathogen may be an important factor for maintenance of females in populations of this species if there is no strong female fecundity advantage (Marshall and Ganders, 2001) and if contamination with fungal spores differently impedes seed production of female and hermaphrodite plants.

This greenhouse study suggests that contamination with fungal spores has sex-specific effects on healthy plants of G. repens. Although we have not measured spore deposition in natural populations of this species, about one quarter of flowering plants are infected in the population that we have been monitoring in the Italian Alps (López-Villavicencio et al., 2003). At such high disease frequency, contamination of healthy plants with spores is extremely likely (Bucheli and Shykoff, 1999). Because we found that fecundity of hermaphrodites is reduced by this contamination, females are expected to have a reproductive advantage in the presence of this fungus.

On the other hand, females and hermaphrodites may differ in their risk of infection. In natural populations, spore and pollen deposition are correlated (Elmqvist et al., 1993, Collin et al., 2002), as are flower size and spore deposition (Elmqvist et al., 1993; Shykoff et al., 1997). In S. latifolia, flowers of females are larger than those of males and they can receive more spores per flower (Bucheli and Shykoff, 1999). Because greater spore deposition may lead to higher disease risk in gynodioecious species (Alexander and Antonovics, 1988; Antonovics and Alexander, 1992), females and hermaphrodites could perhaps differ in their infection probability, if the larger stigmas of females are more efficient at trapping spores as well as pollen (Collin et al., 2002). Indeed, it would be interesting to look for sex-specific defense mechanisms. The behavior of females and hermaphrodites in relation to such pathogens could, therefore, provide new insights regarding the maintenance and spread of females in gynodioecious populations and the evolution of sexual systems.

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